MARISLAINE ALVES DE FIGUEIREDO

BIOFORTIFICAÇÃO COM ZINCO, SELÊNIO E FERRO, E BIODISPONIBILIDADE DE FERRO EM CULTIVARES DE FEIJOEIRO-COMUM

LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para a obtenção do título de Doutor.

Dr. Messias José Bastos de Andrade
Orientador

Dr. Luiz Roberto Guimarães Guilherme
Dra. Li Li
Coorientadores

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APROVADA em 29 de abril de 2016.

Dr. Fabrício William Ávila	UNICENTRO
Dra. Joelma Pereira	UFLA
Dr. Guilherme Amaral de Souza	VANGUARDA AGRO S.A.
Dr. Luiz Roberto Guimarães Guilherme	UFLA

Dr. Messias José Bastos de Andrade
Orientador

LAVRAS – MG
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RESUMO

O feijão comum (*Phaseolus vulgaris* L.), alimento básico na dieta nutricional dos brasileiros e de populações de países em desenvolvimento, é uma leguminosa rica nutricionalmente com potencial à biofortificação. Aproximadamente um terço da população mundial sofre com deficiências nutricionais, sendo necessário o aumento do teor de nutrientes em vegetais, principalmente de ferro (Fe), selênio (Se) e zinco (Zn), que são micronutrientes importantes para as plantas e para a saúde humana. Nesse contexto, três estudos foram realizados objetivando-se avaliar o potencial de cultivares de feijoeiro-comum à biofortificação com Fe, Se e Zn, bem como verificar a interação entre esses minerais e a biodisponibilidade de Fe, de forma a contribuir para o aumento da qualidade nutricional dos grãos, diminuindo a deficiência desses micronutrientes e melhorando a saúde humana. No primeiro estudo, foram conduzidos experimentos em casa de vegetação, com dez cultivares de feijão em solução nutritiva, submetidas a diferentes tratamentos com Fe, Se e Zn. O crescimento da planta e o teor de minerais nos grãos de feijão foram avaliados, além de verificar a influência de níveis de polifenóis e de fitato na biodisponibilidade de Fe em grãos fortificados com Zn e Se. As cultivares de feijão avaliadas se mostraram promissoras à biofortificação simultânea com esses nutrientes sem afetar a biodisponibilidade de Fe. No segundo estudo, objetivou-se avaliar a interação entre o Fe, Se e Zn em cultivares consumidas no Brasil ou nos Estados Unidos. Expressão gênica e análise de microscopia de raiz foram realizadas com o intuito de entender o efeito positivo do suprimento de Zn na absorção de Fe pelas raízes. A expressão de genes relacionados ao transporte e absorção de Fe e Zn não explicou claramente a influência do Zn na nutrição de Fe. Já a microscopia de raízes e a avaliação das soluções nutritivas utilizadas mostraram que, na presença de Zn, houve acúmulo de Fe na epiderme das raízes e não no sistema vascular, tendendo a ser precipitado quando atravessa a membrana da raiz. No último estudo, um experimento de campo foi conduzido com o objetivo de avaliar o efeito da adubação de Zn, via solo e foliar, no teor e acúmulo de Fe e Zn nos grãos, e no rendimento de cultivares de feijoeiro-comum, bem como verificar a quantidade desses micronutrientes suprida pelo feijão biofortificado. A adubação com Zn não influenciou o rendimento, mas proporcionou alto teor desse micronutriente nos grãos das cultivares analisadas, representando 27% da ingestão diária recomendada de Zn. O maior teor de Fe nos grãos de feijão, obtido quando não houve aplicação de Zn via foliar, supre 56% da necessidade diária de Fe.

Palavras-chave: *Phaseolus vulgaris* L. Qualidade nutricional. Micronutrientes. Saúde humana.

ABSTRACT

The common bean (*Phaseolus vulgaris* L.), a staple food in nutritional diet of Brazilians and populations in developing countries, is a nutritionally rich legume with potential for biofortification. Approximately one third of the world population suffers from nutritional deficiencies, being necessary to increase the nutrient content in vegetables, especially iron (Fe), selenium (Se) and zinc (Zn), which are important micronutrients for plants and human health. In this context, three studies were carried out aiming to evaluate the potential of common bean cultivars to biofortification with Fe, Se and Zn, and verify the interaction between these minerals and iron bioavailability, in order to contribute to increased nutritional quality of grains, reducing the micronutrients deficiency and improving human health. In the first study, experiments were conducted in a greenhouse, with ten common bean cultivars in nutrient solution under different treatments with Fe, Se and Zn. The plant growth and the mineral content of the beans were evaluated in addition to verify the influence of polyphenol and phytate levels on Fe bioavailability in grains fortified with Zn and Se. The evaluated beans cultivars have proved promising for simultaneous biofortification with these nutrients without greatly affecting Fe bioavailability. In the second study, the aim was evaluate the interaction between Fe, Se and Zn in cultivars consumed in Brazil or in USA. Gene expression and root microscopy analysis were performed in order to understand the positive effect of Zn supply on the Fe uptake by roots. The expression of genes related to the transport and uptake of Fe and Zn did not clearly explain the influence of Zn in Fe nutrition. The roots microscopy and the evaluation of nutrient solutions used showed that, in the presence of Zn, there was Fe accumulation in epidermis of the roots and not in the vascular system, prone to be precipitated when it goes through the root membrane. In the latest study, a field experiment was conducted to evaluate the effect of Zn fertilization via soil and foliar, in the content and accumulation of Fe and Zn in grains and in the yield of common bean cultivars, in addition to verify the amount of these micronutrients supplied by biofortified beans. The fertilization with Zn did not affect the yield, but provided high levels of this nutrient in grains of the cultivars analyzed, representing 27% of the recommended daily intake of Zn. The higher Fe content in beans, obtained when there was no application of foliar Zn, supplies 56% of the daily requirement of Fe.

Keywords: *Phaseolus vulgaris* L. Nutritional quality. Micronutrients. Human health.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O feijão comum (*Phaseolus vulgaris* L.), alimento básico na dieta nutricional dos brasileiros (MORROW, 1991), possui alto teor de proteína, carboidratos, fibras, vitaminas e minerais (COSTA et al., 2006; THARANATHAN; MAHADEVAMMA, 2003). Meio bilhão de pessoas consomem feijão na América Latina e na África (CORTÉS et al., 2013), sendo o consumo no Brasil de, aproximadamente, 18 kg pessoa^{-1} ano^{-1}, fornecendo de 10 a 20% da quantidade de nutrientes requerida por um adulto (ANUÁRIO DA AGRICULTURA BRASILEIRA - AGRIANUAL, 2015; BROUGHTON et al., 2003; INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2011). Na África, esse consumo chega a 66 kg pessoa^{-1} ano^{-1} em áreas rurais do Kenya, representando 32% do suprimento de energia e 65% da proteína consumida nessa região (BLAIR et al., 2010; BROUGHTON et al., 2003).

O Brasil é um dos maiores produtores e consumidores mundiais de feijão comum, com uma produção estimada, no período 2015/2016, de 3,3 milhões de toneladas, correspondendo, aproximadamente, a 13% da produção mundial de feijão (FAOSTAT, 2016). A cultura possui ainda importância social e econômica evidenciada pela grande quantidade de pequenos produtores e trabalhadores rurais envolvidos na sua produção, e por representar importante fonte proteica na dieta alimentar da população, especialmente a de baixa renda (BROUGHTON et al., 2003; THARANATHAN; MAHADEVAMMA, 2003).

As deficiências nutricionais, principalmente as de ferro (Fe), selênio (Se) e zinco (Zn), afetam bilhões de pessoas no mundo, em especial, mulheres grávidas, adolescentes e crianças em países em desenvolvimento (ALLOWAY, 2009; BENOIST et al., 2008; COMBS JR., 2001; MÜLLER; KRAWINKEL,

2005). Os alimentos de origem vegetal, que geralmente possuem baixa disponibilidade de Fe, Se e Zn comparados aos alimentos de origem animal, são consumidos em grande quantidade por essas pessoas, aumentando assim o número de pessoas malnutridas (KABATA-PENDIAS; MUKHERJEE, 2007; WELCH et al., 2005).

O Fe, Se e Zn são essenciais para os organismos humanos, desenvolvendo importantes funções no metabolismo (ABBASPOUR; HURREL; KELISHADI, 2014; HATFIELD; BERRY; GLADYSHEV, 2012; HOTZ; BROWN, 2004). Para as plantas, somente o Fe e o Zn são essenciais (BROADLEY et al., 2012a; HÄNSCH; MENDEL, 2009), sendo o Se considerado benéfico para as mesmas (BROADLEY et al., 2012b).

Atualmente, estudos estão sendo desenvolvidos visando o enriquecimento das culturas agrícolas com micronutrientes, processo conhecido como biofortificação, seja agronômica ou genética, para que assim, haja o aumento no teor de nutrientes nas partes comestíveis das plantas de diferentes culturas (ÁVILA et al., 2014; PETRY et al., 2015; RAMOS et al., 2011, SOUZA et al., 2013; 2014; ZUO; ZHANG, 2009). Além da biofortificação, é necessário que estudos abordem também a biodisponibilidade de nutrientes para os consumidores, com foco em diminuir as substâncias inibidoras, como fatores antinutricionais.

Dessa forma, considerando a importância da cultura do feijoeiro-comum e do Fe, Se e Zn para a saúde humana, objetivou-se avaliar o potencial de cultivares de feijoeiro-comum à biofortificação com esses elementos, bem como verificar a interação entre eles e a biodisponibilidade de Fe, de forma a contribuir para o aumento da qualidade nutricional dos grãos, diminuindo a deficiência desses micronutrientes e melhorando a saúde humana.

2 REFERENCIAL TEÓRICO

2.1 Feijão-comum na alimentação humana

O feijão-comum (*Phaseolus vulgaris* L.) é considerado alimento básico de grande importância na dieta nutricional dos brasileiros, além de ser um produto muito consumido pela população de países da América Latina, África e Oriente Médio (COSTA et al., 2006; EL-NIELY, 2007; MORROW, 1991). Essa leguminosa possui alto teor de proteína, carboidratos e fibras, contribuindo também com quantidades significativas de vitaminas e minerais (THARANATHAN; MAHADEVAMMA, 2003). Dessa forma, deve-se considerar que os benefícios nutricionais obtidos com o consumo de feijão são de grande relevância para a nutrição humana, principalmente para as pessoas com carência na ingestão de alimentos de origem animal ou aquelas de baixa renda.

O Brasil destaca-se como um dos maiores produtores e consumidores mundiais de feijão, com consumo diário de feijão seco (cru) estimado em, aproximadamente, 50 g pessoa^{-1} (AGRIANUAL 2015; BROUGHTON et al., 2003; IBGE, 2011). Em 3 milhões de hectares cultivados com feijão nesse país, no período 2015/2016, foram produzidas, aproximadamente, 3,3 milhões de toneladas de grãos (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2016). Assim, a produtividade média para esta mesma safra foi de 1100 kg ha^{-1}. A dieta tradicional dos brasileiros é à base de arroz e feijão, alimentos que se complementam em relação aos aminoácidos essenciais. A proteína do arroz é pobre em lisina, mas é rica em aminoácidos sulfurados, como metionina e cistina, sendo o inverso observado no feijão (BRASIL, 2005).

Na África e na América Latina, estima-se que mais de 500 milhões de pessoas consomem feijão (CORTÉS et al., 2013). Em algumas regiões da

África, principalmente em regiões rurais, o consumo diário chega a 181 g pessoa^{-1} (BROUGHTON et al., 2003).

Conforme dados registrados pela Food and Agriculture Organization of the United Nations (FAO), em 2014, a produção mundial de feijão situou-se em torno de 26 milhões de toneladas. Os cinco principais países produtores, que juntos são responsáveis por mais de 60% da produção mundial, são: Índia, Mianmar, Brasil, Estados Unidos e México, sendo que ao todo, mais de 100 países cultivam algum tipo de feijão (FAOSTAT, 2016).

Benefícios à saúde humana devido ao consumo de feijão têm sido relatados por vários pesquisadores (CAMPOS-VEGA et al., 2013; HANGEN; BENNINK, 2002; JENKINS et al., 2012; MUDRYJ; YU; AUKEMA, 2014). Os grãos dessa leguminosa são benéficos na redução do risco de várias doenças, como a obesidade, doenças cardiovasculares, diabete e câncer.

De acordo com a Tabela Brasileira de Composição de Alimentos (TACO) e com o banco de dados do United States Department of Agriculture (USDA), 100 g de feijão cozido, possuem, em média, 2 mg de Fe e 1 mg de Zn (USDA, 2016; TACO, 2011). Em relação ao feijão seco, 100 g contém, em média, 6 mg de Fe e 3 mg de Zn (TACO, 2011). Esses valores variam de acordo com o tipo de feijão analisado. Na Tabela 1, é apresentada a composição nutricional dos grãos de feijão secos ou cozidos, de quatro tipos diferentes de feijão (Carioca, Preto, Navy e Pinto Beans).

O processamento desta leguminosa, ou seja, a utilização de feijão cozido, forma consumida pela população, apesar de diminuir os teores de nutrientes nos grãos devido à diluição pelo aumento do volume de água, aumenta a biodisponibilidade de nutrientes por meio da inativação de inibidores da tripsina e de hemaglutininas, e diminuição de fatores antinutricionais, como fitatos e taninos (OLIVEIRA et al., 2001; RAMÍREZ-CÁRDENAS; LEONEL; COSTA, 2008; THARANATHAN; MAHADEVAMMA, 2003).

Tabela 1 - Composição de nutrientes em 100 gramas de grãos de feijão.

Descrição	Proteína (g)	Carboidrato (g)	Lipídeo (g)	Fibra Alimentar (g)	Ferro (mg)	Zinco (mg)
Feijão carioca cru[1]	20,0	61,2	1,3	18,4	8,0	2,9
Feijão carioca cozido[1]	4,8	13,6	0,5	8,5	1,3	0,7
Feijão preto cru[1]	21,3	58,8	1,2	21,8	6,5	2,9
Feijão preto cozido[1]	4,5	14,0	0,5	8,4	1,5	0,7
Navy Beans cru[2]	22,3	60,8	1,5	15,3	5,5	3,7
Navy Beans cozido[2]	8,2	26,1	0,6	10,5	2,4	1,0
Pinto Beans cru[2]	21,4	62,6	1,2	15,5	5,1	2,3
Pinto Beans cozido[2]	9,0	26,2	0,7	9,0	2,1	1,0

Fonte: [1] Tabela Brasileira de Composição de Alimentos – TACO, 2011; [2] USDA, 2016.

Ribeiro et al. (2008), ao determinarem a composição de micronutrientes em grãos de 19 cultivares de feijão consumidas no Brasil, verificaram que os grãos foram constituídos em maior parte por Fe (7,15 mg 100 g^{-1}), seguido pelo Zn (3 mg 100 g^{-1}).

Em relação ao Se, Ferreira et al. (2002) encontraram em cultivares de feijoeiro-comum consumidas no Brasil um teor médio de selênio de 1,0 µg em 100 g de feijão cru . As concentrações desse nutriente no feijão preto, carioca e feijão cozido foram de 11,9, 0,1 e 1,7 µg 100 g^{-1} de grãos, respectivamente. No banco de dados do USDA, os valores de Se em grãos de feijão cozido, consumidos nos Estados Unidos, variaram de 1,2 a 5,7 µg 100 g^{-1} de grãos, dependendo do tipo de feijão (USDA, 2014).

2.2 Ferro

O Fe, segundo metal mais abundante na crosta terrestre com solubilidade extremamente baixa, é um elemento essencial para as plantas e para o homem (ABBASPOUR; HURREL; KELISHADI, 2014; BROADLEY et al., 2012a; MORRISSEY; GUERINOT, 2009). As formas predominantes de Fe solúvel nos solos e nas soluções nutritivas são os quelatos de Fe, sendo que os íons Fe^{+2} e Fe^{+3} também são absorvidos (BRIAT; CURIE; GAYMARD, 2007; MARSCHNER, 2012; MORRISEY; GUERINOT, 2009). Este micronutriente é constituinte de proteínas heme e não heme (Fe-S) que participam do sistema redox, o qual desintoxica as células pela redução do peróxido de hidrogênio (H_2O_2) em água e oxigênio (BROADLEY et al., 2012a). Além disso, o Fe faz parte da hemoglobina, proteína transportadora de oxigênio nos glóbulos vermelhos (GERMANO; CANNIATTI – BRAZACA, 2002).

A disponibilidade de Fe no solo pode ser afetada pelo pH, presença de metais catiônicos como, por exemplo, Cu, Mn e Zn, matéria orgânica, fósforo, aeração e umidade do solo (MALAVOLTA, 2006). A concentração crítica de Fe nas folhas para que não cause deficiências varia de 50 a 150 mg kg^{-1} de matéria seca, e concentrações acima de 500 mg Fe kg^{-1} de matéria seca de folhas podem causar toxidez (BROADLEY et al., 2012a).

A deficiência de Fe em humanos é considerada como a carência com maior distribuição no mundo (LYNCH, 2011; STOLTZFUS, 2011), afetando cerca de dois bilhões de pessoas e causando, aproximadamente, um milhão de mortes por ano (MAYER; PFEIFFER; BEYER, 2008; WORLD HEALTH ORGANIZATION - WHO, 2002). Crianças e mulheres grávidas são as mais afetadas devido à alta demanda de Fe durante o crescimento infantil e a gravidez (WHO, 2002).

Embora outros fatores como a malária, infecções parasitárias e deficiências nutricionais estejam envolvidos na ocorrência de anemia, um problema de saúde pública que afeta populações de países ricos e pobres, a

deficiência de Fe é a sua causa primária (BENOIST et al., 2008; WHO, 2007). Essa doença afeta negativamente o desenvolvimento cognitivo das crianças, a capacidade de trabalho dos adultos e o sistema imunológico das pessoas (FAO, 2013; WHO, 2001).

Assim, visando diminuir a carência com Fe e evitar os problemas causados pela sua deficiência, trabalhos estão sendo desenvolvidos para aumentar o teor desse micronutriente em cereais e leguminosas ou diminuir os fatores antinutricionais, melhorando a absorção de Fe (CAMPION et al., 2013; WELCH, 2002; WELCH; GRAHAM, 2004).

2.3 Selênio

O Se é essencial para o metabolismo humano e animal, mas é considerado como benéfico para as plantas (BROADLEY et al., 2012b; PILON-SMITS et al., 2009; RAYMAN, 2012). Esse elemento exerce atividade estrutural e enzimática, por meio de sua incorporação em proteínas, formando as selenoproteínas, que desempenham atividade antioxidante capaz de reduzir peróxidos do organismo, além de participar da síntese e da atividade do hormônio da tireoide (RAYMAN, 2000; 2002; 2012). Destaca-se ainda por suas propriedades anticancerígenas e de regulação hormonal (FAIRWEATHER-TAIT et al., 2011; STRATTON et al., 2003), e também por conter o desenvolvimento da virulência do HIV (RAYMAN, 2000; 2012). Por essas razões, a baixa ingestão dietética desse nutriente pode comprometer a saúde da população, causando redução na fertilidade e no sistema imunológico, e aumento no risco de câncer (RAYMAN, 2002; 2012).

Estima-se que entre meio e um bilhão de pessoas no mundo possuem ingestão inadequada de Se (COMBS JR., 2001). Uma das estratégias para diminuir a deficiência desse elemento tem sido aumentar seu teor nas culturas

agrícolas por meio de adubações, o que tem propiciado resultados promissores (ÁVILA et al., 2014; BOLDRIN et al., 2012; RAMOS et al., 2010; 2011; 2012, SOUZA et al., 2013, 2014). Contudo, fatores inerentes ao genótipo, solo, concentração e forma de aplicação do elemento, além de outros relacionados a interações com elementos, podem interferir na disponibilidade de Se nos solos e na resposta da planta que tem, nas doses próximas a 0,5 mg kg^{-1}, demonstrado maiores efeitos benéficos (SINGH; SINGH; BHANDARI, 1980).

Selenato (SeO_4^{2-}) e selenito (SeO_3^{2-}) são as formas inorgânicas solúveis em água, predominantemente absorvidas pelas raízes das plantas (WHITE et al., 2004). Os solos intemperizados, com elevadas concentrações de óxidos de Fe e alumínio (Al) na fração argila, podem adsorver Se na forma de selenito, o que reduz, consequentemente, sua disponibilidade para as plantas (ZHANG; SPARKS, 1990). O mesmo não ocorre com o Se na forma de selenato, que é estável em ambientes oxidados, móvel no solo e está prontamente disponível para as plantas. Além das diferenças de disponibilidade das formas de Se no solo, essas também diferem na absorção e na mobilidade do Se no interior das plantas, em que o SeO_4^{2-} (selenato), absorvido de forma ativa, é mais facilmente transportado para a parte aérea, enquanto o SeO_3^{2-} (selenito), absorvido de forma passiva, tende a acumular nas raízes das plantas (LI; MCGRATH; ZHAO, 2008; ZHANG et al., 2003).

Quanto às competições iônicas, o Se na forma de selenato compete com o sulfato e na forma de selenito compete com o fosfato (HOPPER; PARKER, 1999; LI; MCGRATH; ZHAO, 2008; SORS; ELLIS; SALT, 2005; WHITE et al., 2004). Alguns estudos têm demonstrado que o Se na forma de selenato pode aumentar a absorção de enxofre (S) quando disponibilizado em baixas concentrações para as plantas (BOLDRIN et al., 2013; RAMOS et al., 2011, SOUZA et al., 2013), além de utilizar os mesmos transportadores e rota de

assimilação desse macronutriente (PILON-SMITS; QUINN, 2010; ZHU et al., 2009).

Todo esse conhecimento acerca das relações existentes entre o Se e a produção vegetal e a saúde humana, sobretudo da suplementação na cultura do feijoeiro, alimento básico predominante na dieta brasileira, podem contribuir com os programas de biofortificação e contribuir para elevar a ingestão de Se pela população.

2.4 Zinco

O Zn é um elemento essencial em diversos processos bioquímicos, pois é componente de proteínas e enzimas envolvidas na produção de energia e manutenção da integridade estrutural de membranas (HÄNSCH; MENDEL, 2009), na síntese de ácidos nucleicos (DNA e RNA), no metabolismo da auxina e no crescimento e diferenciação celular (MAYER; PFEIFFER; BEYER, 2008). Esse micronutriente faz parte, aproximadamente, de 300 enzimas humanas, acarretando, quando deficiente, em sérios problemas de saúde (HOTZ; BROWN, 2004; STEIN et al., 2007). É o segundo elemento mais encontrado nos organismos e o único metal a participar dos seis grupos enzimáticos (BROADLEY et al., 2007). Atua também na redução da formação de radicais livres por atuar em inibidores como a NADPH oxidase e ser integrante da enzima superóxido dismutase (Cu-Zn-SOD) (CAKMAK, 2000; HÄNSCH; MENDEL, 2009; MAYER; PFEIFFER; BEYER, 2008).

O Zn é absorvido predominantemente como Zn^{2+} (BROADLEY et al., 2012a). A textura do solo, pH, matéria orgânica, atividade microbiana e concentrações de P e de elementos catiônicos afetam a disponibilidade de Zn para a absorção das plantas (ALLOWAY, 2008; 2009).

A adubação fosfatada é uma prática agrícola que pode diminuir a disponibilidade de Zn nos solos, acarretando assim, aumento da deficiência desse nutriente (ALLOWAY, 2009; MOUSAVI, 2011). Ao contrário, outras práticas como a adubação específica com fertilizantes contendo Zn, rotação de culturas e plantio direto, aumentam os teores de Zn no solo (GRAHAM et al., 2007).

As principais causas do antagonismo entre a adubação de P e a nutrição de Zn pelas plantas são o efeito de diluição de Zn nas plantas devido à maior produção de matéria seca provocada pelo acréscimo de P, a precipitação de compostos de P-Zn no solo, a inibição não competitiva no processo de absorção e o menor transporte do Zn das raízes para a parte área, induzindo ao sintoma de deficiência de Zn nas folhas (ALLOWAY, 2009; KABATA-PENDIAS, 2011; MALAVOLTA, 2006).

Em humanos, a ingestão inadequada de Zn na dieta, a absorção predominante de dietas à base de plantas, a amamentação abaixo do ideal, doenças que induzem perdas excessivas ou impedem a utilização do Zn, além dos estados fisiológicos que aumentam a necessidade de Zn, como períodos de rápido crescimento durante a infância e a gravidez, são os principais fatores responsáveis pela deficiência de Zn em países de baixa renda (GIBSON, 2006; HESS et al., 2009).

As principais consequências dessa deficiência no corpo humano são prejuízos no funcionamento do cérebro, no sistema imunológico e no crescimento físico. Estima-se que, no mínimo, um terço da população mundial seja afetada pela deficiência de Zn, principalmente crianças (HOTZ; BROWN, 2004; WELCH; GRAHAM, 2004). Além disso, segundo dados da WHO (2007), a deficiência de Zn é responsável por, respectivamente, 16, 18 e 10% das infecções respiratórias, índices de malária e diarreias. Em geral, 1,4% do total das mortes são atribuídas à carência de Zn: 1,4% nos homens e 1,5% nas

mulheres. Contudo, as informações a respeito da deficiência de Zn, em âmbito mundial, ainda são escassas (HESS et al., 2009).

Pesquisas sobre biofortificação com Zn em diferentes culturas estão sendo realizadas, mostrando que há uma variação na resposta à adubação com Zn de acordo com o genótipo (CORGUINHA et al., 2013; DUARTE et al., 2013; PROM-U-THAI, 2010; SOUZA et al., 2013;2014)

Para a cultura do feijoeiro-comum recomenda-se aplicar 1 mg de Zn kg^{-1} de solo, considerando-se 90% da produção relativa de matéria seca como parâmetro de determinação da dosagem adequada de Zn no solo e na planta (FAGERIA, 2000). Os níveis adequados desse micronutriente no solo variaram de acordo com o extrator utilizado, sendo que para o extrator Mehlich 1 foi de 0,7 mg de Zn kg^{-1} de solo e para o extrator DTPA foi de 0,3 mg de Zn kg^{-1} de solo. A toxidez de Zn no solo determinada pelos dois extratores foi de 25 mg de Zn kg^{-1} de solo. Os níveis adequado e tóxico na planta foram de 18 e 133 mg de Zn kg^{-1} de matéria seca, respectivamente (FAGERIA, 2000).

Dessa forma, a suplementação com esse micronutriente em feijão pode aumentar a qualidade nutricional dos grãos e causar uma diminuição dos problemas de saúde causados pela deficiência de Zn.

2.5 Biofortificação

A biofortificação é um processo que consiste no aumento dos teores de nutrientes nas partes comestíveis das plantas, seja pela adubação das culturas, via solo e/ou foliar (biofortificação agronômica), ou por meio do melhoramento genético (biofortificação genética) (CAKMAK, 2008; WHITE; BROADLEY, 2009). O combate às deficiências nutricionais é o principal objetivo da biofortificação, visto que milhares de pessoas morrem por causa da desnutrição (BOUIS; WELCH, 2010; KENNEDY et al., 2003; KHUSH et al., 2012).

Aproximadamente um terço da população está sob risco de deficiência de Fe e quase a mesma proporção deficiente em Zn (ALLOWAY, 2009; BENOIST et al., 2008; HOTZ; BROWN, 2004). Em relação ao Se, entre meio e um bilhão de pessoas no mundo são afetadas pela sua deficiência (COMBS JR., 2001).

Outras estratégias utilizadas atualmente para diminuir as deficiências de micronutrientes são, além da diversificação de alimentos consumidos, a fortificação industrial e a suplementação por meio de pílulas, cápsulas ou xaropes (ALLEN et al., 2006). Apesar de serem processos eficientes, eles são de alto custo para o consumidor e podem causar intoxicações caso haja o consumo de uma superdose (ZANCUL, 2004). Além disso, na fortificação industrial e na suplementação por meio de pílulas geralmente são introduzidas formas inorgânicas de Se (selenato e selenito) que possuem menor biodisponibilidade para o organismo humano (RAYMAN, 2008; RAYMAN; INFANTE; SARGENT, 2008). Nesse contexto, o incremento de micronutrientes via planta, ou seja, por meio da biofortificação, se torna mais recomendado, já que há maior facilidade de acesso dos consumidores aos vegetais, menor custo e menor risco de toxidez (BOUIS et al., 2011; MEENAKSHI et al., 2007; NESTEL et al., 2006). O Se, ao ser absorvido pelas plantas, é incorporado a compostos orgânicos que apresentam biodisponibilidade bem maior para os organismos humano e animal (RAYMAN, 2008; RAYMAN; INFANTE; SARGENT, 2008; ZHU et al., 2009).

Dentre os três nutrientes estudados nesse trabalho (Fe, Zn e Se), todos são considerados elementos essenciais para o homem, mas somente o Fe e Zn são essenciais para as plantas (BROADLEY et al., 2012a; 2012b). No metabolismo humano, os micronutrientes Fe e Zn atuam como cofatores de enzimas que participam de diversas vias metabólicas (ABBASPOUR; HURREL; KELISHADI, 2014; HOTZ; BROWN, 2004), e o Se participa de processos antioxidantes (RAYMAN, 2012).

Diversos autores verificaram aumento nos teores de Zn (CORGUINHA et al., 2013; DUARTE et al., 2013; PROM-U-THAI, 2010; SOUZA et al., 2013;2014), Fe (SOUZA et al., 2013) e Se (ÁVILA et al., 2014; BOLDRIN et al., 2013; RAMOS et al., 2010; 2011; 2012; REIS et al., 2014) nas plantas e/ou nos grãos de diferentes culturas quando submetidas à diferentes tratamentos envolvendo a suplementação desses nutrientes. Os resultados foram dependentes dos genótipos, das formas aplicadas do elemento e das doses e interações com outros elementos, possibilitando a identificação de cultivares promissoras para a biofortificação e consequente aumento da ingestão desses nutrientes na alimentação humana.

A estimativa da necessidade de ingestão de nutrientes por um indivíduo é feita pelo EAR (Estimated Average Requirement – necessidade média estimada), um dos parâmetros da ingestão dietética de referência (DRIs – Dietary Reference Intakes). O EAR é um valor de referência que corresponde ao nível de ingestão de nutrientes diário estimado para satisfazer os requisitos de metade dos indivíduos saudáveis em um grupo de mesmo gênero e estágio de vida. Ele é utilizado para avaliar as dietas de indivíduos ou grupos (INSTITUTE OF MEDICINE, 2006).

O RDA (Recommended Dietary Allowance - ingestão dietética recomendada), calculado a partir do EAR, é também outro parâmetro da DRIs, sendo o nível de ingestão dietética diária suficiente para atender as necessidades nutricionais de praticamente todos (97 a 98%) os indivíduos saudáveis de um determinado grupo de mesmo gênero e estágio de vida. O RDA não deve ser utilizado para avaliar a ingestão de nutrientes de um indivíduo (INSTITUTE OF MEDICINE, 2006).

Os valores de UL (Tolerable Upper Intake Level - limite superior tolerável de ingestão) correspondem aos limites de ingestão diária de um nutriente que aparentemente não oferecem nenhum efeito adverso à saúde

humana. O risco de toxidez aumenta à medida que a ingestão ultrapassa o UL (INSTITUTE OF MEDICINE, 2006).

Os valores de EAR, RDA e UL para Fe, Se e Zn são listados na Tabela 2.

Tabela 2 - Necessidade média estimada (EAR), ingestão dietética recomendada (RDA) e limite superior tolerável de ingestão (UL) dos nutrientes Fe, Se e Zn para homens e mulheres com idade entre 19 e 50 anos, e mulheres grávidas.

Nutrientes	**EAR**			**RDA**			**UL**
	Homens	Mulheres	Grávidas	Homens	Mulheres	Grávidas	
Ferro (mg)	6	8,1	22,5	8	18	27	45
Selênio (µg)	45	45	45	55	55	60	400
Zinco (mg)	9,4	6,8	10	11	8	11,5	40

Fonte: Institute of Medicine (2006).

O feijoeiro-comum é uma das culturas alvo de pesquisas e programas de biofortificação, como por exemplo, o programa HarvestPlus, que faz parte do programa de pesquisa do CGIAR (Consultative Group for International Agricultural Research) sobre agricultura para nutrição e saúde (Agriculture for Nutrition and Health - A4NH). Juntamente com seus parceiros, busca o desenvolvimento de novas variedades que forneçam produtos agrícolas mais nutritivos, com maiores quantidades de vitamina A, ferro ou zinco. As culturas biofortificadas por esse programa são feijão, mandioca, batata-doce, arroz, milho, milheto e trigo (BLAIR, 2013; BOUIS et al., 2011; PFEIFFER; MCCLAFFERTY, 2007).

A existência de variação genética em relação aos teores de micronutrientes torna essa leguminosa como promissora à biofortificação (BLAIR et al., 2010; RIBEIRO et al., 2008; 2013, TALUKDER et al., 2010).

O programa HarvestPlus estabeleceu níveis de Fe e Zn a serem alcançados em grãos de feijão biofortificados, que são 107 µg g^{-1} e 56 µg g^{-1},

respectivamente (BOUIS et al., 2011). Dessa forma, levando-se em consideração as necessidades diárias desses nutrientes, estudos sobre biofortificação de grãos de feijão se fazem necessários para suprimento dessa demanda.

2.6 Biodisponibilidade de minerais

Os riscos à saúde causados pelo fornecimento insuficiente ou excessivo de certos nutrientes não dependem apenas de sua concentração nos alimentos, mas também da proporção biodisponível desses elementos. Para a determinação dessa proporção é necessário a determinação da biodisponibilidade. Ela refere-se à quantidade ingerida de um nutriente que é absorvida e utilizada pelo organismo para o funcionamento normal do metabolismo (FAIRWEATHER-TAIT et al., 2005; FERNÁNDEZ-GARCÍA; CARVAJAL-LÉRIDA; PÉREZ-GÁLVEZ, 2009). É influenciada pela forma química do elemento no composto e pela presença de inibidores e/ou promotores de absorção desse elemento na digestão humana (GLAHN et al., 2002a; JIN et al., 2009; TAKO et al., 2014).

Muitas vezes o termo biodisponibilidade é confundido com a bioacessibilidade. Diferentemente, bioacessibilidade é definida como a quantidade de cada composto ingerido que fica disponível para ser absorvido no intestino após digestão (FERNÁNDEZ-GARCÍA; CARVAJAL-LÉRIDA; PÉREZ-GÁLVEZ, 2009; HEANEY, 2001). Desde sua ingestão, o alimento passa por diversos processos para que os nutrientes possam ser utilizados (KONG; SINGH, 2008). No trato digestivo, o alimento é processado e uma fração do conteúdo nutricional é extraída e solubilizada, constituindo a fração acessível. Desta, somente uma parcela é efetivamente absorvida e atinge a circulação sanguínea, sendo considerada fração biodisponível.

Os polifenóis, fitatos e taninos são alguns dos exemplos de substâncias que podem inibir a absorção de cátions, diminuindo a biodisponibilidade destes

(CAMPION et al., 2013; ENGLE-STONE et al., 2005; GLAHN et al., 2002a; JIN et al., 2009; TAKO et al., 2014).

Técnicas *in vivo* e *in vitro* tem sido utilizadas para estimar a biodisponibilidade de Fe (DEWIVEDI, 2012). O modelo *in vitro* mais conhecido utiliza células humanas cultivadas, Caco-2, que simulam a digestão gastrointestinal em humanos (GLAHN et al., 1998). Esse modelo é rápido, eficaz e bem comparável com os estudos humanos (GLAHN et al., 1998). As células Caco-2 têm sido utilizadas para a determinação da biodisponibilidade de Fe em várias culturas, como por exemplo, o milho, arroz, trigo e feijão (ARIZA-NIETO et al., 2007; EAGLING et al., 2014; GLAHN et al., 2002b; TAKO et al., 2013), sendo assim uma importante ferramenta complementar à biofortificação e à avaliação da nutrição humana.

3 CONSIDERAÇÕES GERAIS

A importância do feijão na dieta da população de países em desenvolvimento e a necessidade de acesso de grupos de baixo poder aquisitivo a alimentos com elevados teores de elementos essenciais à saúde, principalmente Fe, Se e Zn, torna o consumo dessa leguminosa um importante aliado no combate às deficiências nutricionais. A biofortificação é, portanto, um processo altamente viável para aumentar a qualidade nutricional dos produtos agrícolas.

Ressalta-se que, além do enriquecimento dos alimentos com esses elementos, é necessário avaliar a biodisponibilidade de minerais para o organismo humano, bem como os fatores que afetam essa biodisponibilidade, como, por exemplo, os fatores antinutricionais.

Dessa forma, com o intuito de contribuir com informações relevantes sobre biofortificação, biodisponibilidade de Fe e interação entre os três elementos estudados, foram conduzidos vários trabalhos que são apresentados a seguir.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Iron, zinc, and selenium content and iron bioavailability in common bean varieties

(Artigo elaborado segundo normas da revista Annals of Botany - Versão preliminar)

ORIGINAL ARTICLE

Iron, zinc and selenium nutrition and iron bioavailability in common bean varieties

MARISLAINE A. DE FIGUEIREDO[1,2,3], PAULO F. BOLDRIN[1,2,4], JON J. HART[1], MESSIAS J. B. DE ANDRADE[3], LUIZ R. G. GUILHERME[4], RAYMOND P. GLAHN[1], LI LI[1,2,*]

[1] Robert W. Holley Center for Agriculture and Health, USDA-ARS, Cornell University, Ithaca, NY 14853, USA, [2] Plant Breeding and Genetics section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA, [3] Department of Agriculture, Federal University of Lavras, Zip Code 3037, Lavras, MG 37200-000, Brazil, [4] Department of Soil Science, Federal University of Lavras, Zip Code 3037, Lavras, MG 37200-000, Brazil.

Running title: Biofortification of common bean varieties with Fe, Se and Zn

* **For correspondence.** Email: ll37@cornell.edu

ABSTRACT

Background and Aims The common bean (*Phaseolus vulgaris*) is the most important legume crop. It represents a major source of micronutrients and has been targeted for essential mineral enhancement (i.e. biofortification). To gain a better understand micronutrient nutrition and accumulation, we investigated the effects of iron (Fe), zinc (Zn), and selenium (Se) treatments on plant growth and grain mineral content in common bean cultivars. We also examined phytic acid and polyphenol levels and their association with Fe bioavailability in the Zn and Se fortified grains.

Methods We selected 10 cultivars of both carioca and black bean groups, and grew them hydroponically under control conditions. Following Fe, Zn, and Se treatments, the mineral contents in various organs were analyzed using an inductively coupled plasma trace analyzer emission spectrometer and the Fe bioavailability in grains was assessed using Caco-2 cell model.

Key results Common bean plants were found to have relative good ability to adapt Fe nutrient status as their biomasses in general were not affected by low Fe nutrition. However, they were less adaptable to Zn nutrient status changes because their growth was greatly suppressed by both low and high Zn supplementation. Grains of common bean varieties maintained relatively consistent Fe, Zn, and Se levels during various stages of pod development. Se and Zn treatments had minimal effects on phytic acid and polyphenol levels. The Fe bioavailability in general was not greatly affected in grains treated with Se and Zn. However, the inhibitory polyphenol compounds profoundly reduced Fe bioavailability.

Conclusions Our results provide guides for Fe, Se, and Zn enhancement in common bean and suggest it is possible to simultaneously biofortify with these

nutrients without greatly affecting mineral bioavailability for improving human health.

Key words: *Phaseolus vulgaris*, micronutrient, polyphenol, phytic acid, Fe bioavailability, genetic variation.

INTRODUCTION

Micronutrient deficiency is a widespread human health problem. It is estimated that approximately one third of the world population is at risk of nutritional iron (Fe) deficiency, which causes about half of all cases of anemia (Benoist *et al.*, 2008). Almost the same proportion of the population is deficient in zinc (Zn) (Hotz and Brown, 2004; Alloway, 2009), and between half and one billion people worldwide are affected by selenium (Se) deficiency (Combs Jr., 2001). While a number of strategies are employed globally to reduce these micronutrient deficiencies for human health, enhancement of their content in food crops via agronomic or genetic methods provides a complimentary and sustainable way to help combat micronutrient malnutrition.

The common bean (*Phaseolus vulgaris*) is the most important grain legume in human diets and represents about half of the grain legumes consumed worldwide. Common bean is a central part of the basic diet for a large number of the world's population, particularly in Africa and Latin America countries (Broughton *et al.*, 2003; Blair, 2013). It provides a rich source of proteins, carbohydrates, vitamins, and minerals (Tharanthan and Mahadevamma, 2003; Costa *et al.*, 2006). As the common bean accumulates Fe and Zn at much higher levels than cereal crops such as rice, wheat, and corn, there is great opportunity to biofortify the common bean with these nutrients and thus meet the target levels of Fe and Zn that can have nutritional impact (Petry *et al.*, 2015).

Both Fe and Zn are essential for plants and serve critical roles for the function of many plant enzymes and proteins (Broadley *et al.*, 2012). Fe and Zn are limiting factors for plant growth (Hänsch and Mendel, 2009; Briat *et al.*, 2015), and their content in plants can be directly associated with crop yield and the seed nutritional quality (Cakmak, 2008; Blair *et al.*, 2010; Khoshgoftarmanesh *et al.*, 2010). Se is not essential for plants, but is known to

be beneficial for some plant growth (Pilon-Smits *et al.*, 2009). Se at low dosages given as Na_2SeO_4 or Na_2SeO_3 has been demonstrated to promote plant growth in various crops grown in soil or hydroponic solution in controlled conditions (Hartikainen *et al.*, 2000; Rios *et al.*, 2010; Ramos *et al.*, 2011*a*; Souza *et al.*, 2013). While plants can effectively take up Zn and Se from soil and transport up into leaves and grains, they are less efficient at uptake and transport of Fe (White and Broadley, 2009; Kobayashi and Nishizawa, 2012). Genetic variation within a crop species also greatly affects a plant's ability to accumulate and utilize these minerals (Rosa *et al.*, 2010; Ramos *et al.*, 2011*b*; Yan *et al.*, 2011; Souza *et al.*, 2014).

Considering the importance of common bean and of these three minerals to human health, as well as the importance of better understanding of these micronutrients nutrition for crop biofortification, we investigated the effects of Fe, Zn, and Se treatments on plant growth and grain mineral content in common bean cultivars grown hydroponically under control conditions. The Fe, Zn, and Se levels were evaluated in grains and pod walls during seed development to uncover the mineral accumulation patterns during grain development. In addition, grain phytate content and polyphenolic profile, which are known to affect Fe bioavailability, were examined in response to increased Zn and Se supplementation. The Fe bioavailability in the Zn and Se biofortified grains was evaluated using *in vitro* Caco-2 cell model, a simulated gastrointestinal digestion of foods in humans (Glahn *et al.*, 1998). The result indicated that it is possible to simultaneously biofortified common beans with essential minerals without impact on nutrient bioavailability.

MATERIALS AND METHODS

Plant materials, growth conditions, and treatments

Seeds of common bean cultivars were obtained from EMBRAPA (Rice and Beans, Santo Antônio de Goiás, Brazil) and IAPAR (Londrina, Brazil). These varieties were selected based on their good agronomic and genetic characteristics, as determined by growth performance in various locations in Brazil. Two groups of common bean were used in this work: the carioca cultivars of BRS Notável (B), Pérola (C), IPR Tangará (D), BRS Requinte (E), BRS Estilo (F), BRS Pontal (G), BRSMG Madrepérola (H), and BRS Cometa (J), and the black cultivars of BRS Supremo (A) and BRS Grafite (I).

To grow common bean plants hydroponically, seeds were surface-sterilized in 10% NaOCl for ten min, rinsed in distilled milli-Q water, and germinated on moistened filter paper at 22 ºC for three days. The young seedlings were first transferred to a large tray containing modified Hoagland and Arnon`s solution (1950). $Fe(NO_3)_3$-HEDTA was used in place of $FeSO_4$-EDTA as a chelate buffer. $Fe(NO_3)_3$ was equilibrated with H_3HEDTA (N-2-hydroxyethylethhylenediamine-N,N',N'-triacetic acid) before adding to the nutrient solution. The young seedlings were grown for three days before treatments.

In the seedling experiments, the uniform six-day-old seedlings were transplanted into 2.2 L dark pots containing the nutrient solution. The treatments applied consisted of control (modified Hoagland and Arnon`s solution), minus iron, minus zinc, plus 5 µM Na_2SeO_4, and plus 50 µM $ZnCl_2$. The nutrient solutions were changed twice a week. After 15 days of treatments, plants were harvested separately in shoots and roots, weighed for fresh weight, and dried at 65 ºC for three days for mineral analysis.

In the grain experiments, seeds of BRS Supremo (black beans), BRSMG Madrepérola and BRS Cometa (light brown beans) were used. Young seedlings were transferred and grown hydroponically in 10 L dark pots in the greenhouse for three months until seed mature. The treatments, applied during flowering, consisted of control (modified Hoagland and Arnon`s solution), plus 5 μM Na_2SeO_4 and plus 5 and 10 μM $ZnCl_2$. The nutrient solutions were changed once a week during vegetative growth and twice a week during treatments. The seed pods were harvested at different developmental stages (17, 20, 23, 26, 30 days after pollination [DAP] and mature) and separated into grains and pod walls. Both were dried at 65 ºC for three days, ground, and analyzed for their mineral contents.

For both seedling and grain experiments, plants were grown with constant aeration in a greenhouse at 24 ^{o}C (± 2 ^{o}C) under 14-h day length. The design was completely randomized and treatments had three replicates.

Mineral analysis

The total mineral contents of plant materials were determined using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model Thermo Scientific ICAP 6000 trace element analyzer, San Jose, CA). Each sample was analyzed in triplicate.

Individual dried samples were weighed (between 0.2 and 0.5g) into borosilicate glass tubes and predigested in 4 mL HNO_3/$HClO_4$ (60/40% v/v) and 0.25 mL of 40 ppm of Yttrium solution (internal standard to correct drift in ICP) overnight at room temperature. Contents were carefully swirled to submerge plant material. Samples were then heated to 120 ºC for two hours before 0.5 mL of HNO_3 was added. The temperature of the block was increased to 190 ºC for 30 min. Digested samples were cooled to room temperature and mineral ash digests were solubilized with 20 mL of 18 MΩ water. Blanks and internal

yttrium standards were used to confirm the accuracy of the analytical results. All samples were prepared and analyzed following a rigid quality assurance/ quality control (QA/QC) protocol to ensure accurate and reliable analytical results.

Phytate analysis

Phytate concentration was determined in seeds using a commercially available kit (standard assay procedure of K-PHYT 08/14 Megazyme, Ireland) with colorimetric determination of phosphorus (P) following the manufacturer's instructions. Briefly, 1.0 g of ground bean grain was extracted with 20 mL of HCl (0.66 M), followed by an enzymatic dephosphorylation reaction with phytase and alkaline phosphatase enzymes. The color reagent prepared from ascorbic acid (10% w/v) in sulphuric acid (1 M) and ammonium molybdate (5% w/v) was used to precipitate the solution. Trichloroacetic acid (50% w/v) was added to stop the reaction. Samples were then centrifuged at 10,000 g for ten min and the supernatant was pipetted for colorimetric determination of P. A calibration curve was constructed to calculate P concentration and, subsequently, phytic acid concentration. Absorbance (at 655 nm) was determined to calculate P concentration, given as grams of P per 100 g of sample material. Phytate concentration (g $100g^{-1}$) was calculated based on P concentration divided by 0.282. Oat flour was used as control.

Analysis of polyphenols by LC-MS

Samples (1.0 g) of bean seed coats from three cultivars (BRS Supremo, BRSMG Madrepérola and BRS Cometa) were extracted with 5 mL of MeOH:H_2O (50:50 v/v). They were vortexed to submerge all dried materials, shaken for 30 min, sonicated at 30 ^oC for 15 min, vortexed again, and centrifuged at 3,700 g for 12 min. Supernatants (2 to 3 mL) were filtered through 0.2 µm PTFE syringe filters and used for analysis.

Polyphenol analysis was carried out based on method as described (Hart *et al.*, 2015) by LC-MS using an Agilent 1220 Infinity LC (Agilent Technologies, Santa Clara, CA) coupled to an Advion expressIon^{L} CMS (Advion, Inc., Ithaca, NY). For LC analysis, 5 µL samples of extract were injected and passed through a BEH Shield RP18 1.7 µm 2.1 x 100 mm column (Waters) at 0.5 mL min^{-1}. Mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 86.7 to 84.4% A in 90 sec, 84.4 to 81.5% A in 12 sec, 81.5 to 77% A in 168 sec, 77 to 55% A in 30 sec, 55 to 46% A in 60 sec, 46 to 86.7% A in 12 sec and a 48 sec hold at 86.7% A for a total seven min run time. ESI mass spectrometry was performed in negative ionization mode with a scan speed of five/s in the mass range from 50 to 1200 Da.

LC-MS data were analyzed by Advion Data Express software (Version 2.1.24.3, Advion, Ithaca, NY). Individual polyphenols in seed coat samples were tentatively determined by mass and their identities were confirmed by comparison of LC retention times with authentic standards. A standard curve to determine concentrations was constructed for each identified polyphenol.

Analysis of Fe bioavailability using Caco-2 cells

Iron bioavailability was assessed using an established *in vitro* digestion/Caco-2 cell culture model (Glahn *et al.*, 1998). Briefly, bean samples were autoclaved in 18 MΩ water (1:3 w/v) at 120 °C for 30 min. The cooked samples were lyophilized and ground. They were applied to the upper chamber of two-chambered inserts in contact with Caco-2 cell cultures growing on the bottoms of wells of culture plate and incubated for two hours. Inserts were then removed and replaced with 1 mL of minimum essential medium (MEM) and incubated for 22 hours at 37 °C. Following incubation, cells were washed twice with buffer and lysed by addition of 18 MΩ water. Ferritin concentrations

(expressed as ng ferritin/mg protein) were measured with an Enzyme Immunoassay Procedure for the Quantitative Analysis of Serum Ferritin (Spectro Ferritin MT Assay, Ramco Laboratories, Stafford, TX). The samples were analyzed along with various quality and reference controls, i.e. blank digests (baseline), plus $FeCl_3$, and plus $FeCl_3$/Ascorbic acid (AA). Each bean sample was analyzed in triplicate with the presence or absence of AA.

Statistical analysis

The data from each experiment were subjected to individual analysis of variance using the Statistical Analysis Software Sisvar® (Ferreira, 2011). Significant treatment difference was obtained through comparing the means by Scott-Knott test at 5% probability.

RESULTS

Common bean plant growth in responses to Fe, Zn, and Se treatments

To examine common bean plant growth in responses to Fe, Zn, and Se exposures, the biomass production of ten cultivars under controlled growth conditions was measured after 15 days of cultivation in the nutrient solution with different treatments. Plant biomass production varies depending on a number of factors such as variety, age and nutrient status. The ten selected Brazil cultivars showed variable dry weight with up to 2-fold difference in their shoots and 3-fold difference in their roots when grew in the control solution (Fig. 1A and B).

Iron is essential for plant growth. Withdrawal of Fe for 15 days following three days of seedling growth in the nutrition solution appeared to exert minimal effect on shoot growth in all common bean cultivars except cultivar D (Fig. 1A). Such treatment also did not reduce root growth except four cultivars (i.e. D, H, I, J) (Fig. 1B). The results indicated a general adaption of common bean plants to reduced Fe nutrient status.

When these cultivars were treated with 5 μM Na_2SeO_4, their shoot biomasses were significantly increased in five cultivars (i.e. B, C, E, H, and I), decreased in two cultivars (i.e. G and J), and remained unchanged in the other three cultivars (Fig. 1A). Similar variation with positive and negative responses to Se treatment was also observed for root biomasses. These results show genetic variation among these common bean varieties in response to selenate treatment.

Interestingly, a general suppression of plant growth was observed in nearly all cultivars when Zn was withdrawn from or supplied in high concentration to the nutrition solution, especially the shoot growth (Fig. 1A). The shoot biomasses were dramatically reduced in all cultivars except cultivar F

with minus Zn treatment. In contrast, the root biomasses in half of cultivars were not affected following the withdrawal of Zn in the nutrient solutions (Fig. 1B). The growth in all cultivars was negatively influenced by high concentration of Zn supply. The data indicate that common bean plant growth was sensitive to Zn nutrient status changes.

Fe, Se, and Zn accumulation in common bean cultivars

To examine the capacity of various common bean cultivars to accumulate minerals in response to Fe, Se, and Zn treatments, the mineral content in shoots and roots of these ten cultivars were examined by ICP. The total Fe content in shoots and roots of these cultivars exhibited over 3-fold difference (Fig. 2A and D), showing genetic variation in their capacity to uptake and accumulate Fe. While withdrawal of Fe for 15 days generally exerted minimal effect on shoot growth (Fig. 1A), the total Fe contents were dramatically reduced in all these cultivars (Fig. 2A). The reduction ratio varied from 2.8- to 6.6-fold. The total Fe contents were also dramatically decreased in roots of all cultivars (Fig. 2D). Se treatment exerted various effects on total Fe levels in both shoots and roots of these common bean varieties. The total Fe content was enhanced in shoots in three of these cultivars (i.e. A, C, F) and reduced in two (i.e. E, J) (Fig. 2A), but remained unchanged in roots in over half of these varieties following 5 μM Na_2SeO_4 treatment (Fig. 2D). While withdrawal of Zn for 15 days following three days of seedling growth in full nutrition solution appeared not to affect Fe levels in both shoots and roots in over half of these cultivars, the Fe accumulation was favored by the high concentration of zinc in the roots with 3- to 9-fold difference in these cultivars (Fig. 2D). In the shoot, an enhanced Fe level was observed in four cultivars in the presence of high dose of Zn (i.e. A, B, C, F) (Fig. 2A).

As expected, Se accumulation was observed in shoots and roots of these cultivars treated with 5 μM Na_2SeO_4 (Fig. 2B and E). Over 2-folds of variation between these cultivars were observed in both shoots and roots. Noticeably, the roots accumulated much more Se than shoots.

Zinc content in both shoots and roots of these cultivars was not affected by minus Fe or 5 μM Na_2SeO_4 treatment (Fig. 2C and 2F). Withdrawal of Zn in the nutrient solution resulted in low but not significant reduction in Zn content in both shoots and roots of all cultivars, which might be due to the small difference in the Zn concentration present in control (0.365 μM) and -Zn (0) solution. Noticeably, following treatment with 50 μM $ZnCl_2$, the Zn levels were dramatically increased with at least 30- and 100-fold changes in shoots and roots, respectively, of these cultivars (Fig. 2C and 2F), indicating their high capacity in accumulating Zn. Moreover, much higher Zn levels were observed in roots than shoots in these cultivars exposed to high level of Zn treatment.

In all cases with these treatments, it appeared that roots contained significant more Fe, Se and Zn content than shoots (Fig. 2). For the Fe values, the biggest difference between shoots and roots was when the cultivars were treated with Zn, exhibiting 18- to 47-fold difference. The cultivars treated with selenate exhibited 2- to 5-fold difference in the Se content. Zn values varied from 3- to 10-fold difference when more Zn was added in the nutrient solution.

Mineral content in pod walls and grains

To examine the mineral level changes during pod development in the common beans, three cultivars including a black bean variety BRS Supremo (A) were grown hydroponically until mature. The pods were harvested at different development stages (17, 20, 23, 26, 30 DAP and mature) and separated into pod walls and grains before ICP analysis.

As shown in Figure 3 and Supplementary Data Table S1, the total Fe content in pod walls of the three cultivars showed a general decrease during pod development either treated with or without Se and Zn. Supplementation of Se and Zn exhibited various effects on total Fe content in pod walls. Se treatment enhanced Fe levels in pod walls of two cultivars during most stages of development.

All cultivars responded positively to the addition of Se and Zn (Fig. 3 and Supplementary Data Table S1). A general decrease in Se content during pod development was observed (Fig. 3). However, when the cultivars were subjected to additional 5 or 10 μM $ZnCl_2$, Zn content in pod walls was relatively consistent during pod development, indicating that the addition of this micronutrient in the nutrient solution may favor its contineous accumulation during pod development.

The grains of these common bean cultivars in general contained relatively consistent level of Fe, Se, and Zn during grain development (Fig. 4 and Supplementary Data Table S2), indicating the capacity of accumulation of these minerals in grains. Fe content was not dramatically affected by the addition of Se and Zn in the nutrition solution. However, Zn levels were enhanced during grain development with additional 5 μM $ZnCl_2$ supply although further addition of $ZnCl_2$ did not cause more accumulation (Fig. 4).

In comparison, grains contained more Fe, Se and Zn content than pod walls (Figs. 3 and 4). These cultivars showed approximately 2- to 12-fold more Fe in grains at 17 DAP and mature stages, respectively. Grains following selenate treatment exhibited 2.1- to 5.1-fold difference in the Se content compared with pod walls. The Zn values varied from 1.1- to 2.9-fold difference with additional Zn in the nutrient solution. It was noted that the grains of these common bean cultivars in general exhibited less genetic variation in their

capacity to accumulate Fe, Se, and Zn in comparison with other tissues, as shown in other crops (Souza *et al.*, 2014).

Grain phytic acid and polyphenol levels

Both phytic acid and polyphenols are known to affect the minerals availability (Glahn *et al.*, 2002; Jin *et al.*, 2009; Tako *et al.*, 2014; Hart *et al.*, 2015). Their levels in mature seeds of BRS Supremo, BRSMG Madrepérola and BRS Cometa following Se and Zn treatments were examined.

Seeds of these three cultivars contained slight variable levels of phytic acid (Fig. 5). The addition of Se or Zn to the nutrient solution exhibited no significant effect on the phytic acid concentration in the black bean cultivar, BRS Supremo (A), but the additional supplementation of Zn significantly reduced phytic acid level in the light brown cultivars (Fig. 5).

The polyphenol content and composition were analyzed in these three common beans. Eight polyphenols were detected in the different samples: 3,4-dihydroxybenzoic acid, catechin, myricetin 3-glucoside, quercetin 3-glucoside, kaempferol 3-glucoside; myricetin, quercetin and kaempferol. While the light brown beans contained mainly three polyphenols, i.e. catechin, kaempferol 3-glucoside, and kampferol, the black beans (BRS Supremo) contain five additional major polyphenols (Fig. 6).

The levels of these major polyphenolic compounds in general did not change dramatically in responses to Se and Zn treatments in the three cultivars although variations existed (Fig. 6). Catechin and kaempferol 3-glucoside contents remained consistent in BRS Cometa (Fig. 6A) and decreased in BRSMG Madrepérola cultivar (Fig. 6B) following Se treatment. Their levels remained unaffected in response to Zn treatment. Kampferol concentrations varied in response to Se and Zn treatment (Fig. 6A and B). The major polyphenol contents in the black bean variety BRS Supremo varied with no clear

pattern in response to Se and Zn treatments (Fig. 6C). However, the total polyphenol contents were not greatly altered in these three cultivars exposed to Se or Zn.

Grain iron bioavailability in response to Se and Zn treatment

The Fe bioavailability in the grains of these three cultivars was assessed using an *in vitro* digestion Caco-2 cell culture model that simulates gastric and intestinal digestion (Glahn *et al.*, 1998). The grains were first cooked prior *in vitro* digestion. Ascorbic acid (AA) as Fe bioavailability enhancer (Glahn *et al.*, 1999; Jin *et al.*, 2009) was added in the replicate samples. As shown in Fig. 7, the black bean cultivar BRS Supremo had very low Fe bioavailability in comparison with the light brown bean varieties. In the absence of AA, the black bean had approximately 5-folds less Fe bioavailability than the light brown beans. In the presence of AA, up to 20-folds Fe bioavailability difference existed between the black and light brown beans (Fig. 7).

In general, Fe bioavailability was not affected by Se and Zn treatments in the absence of AA in both black and light brown beans (Fig. 7). In the presence of AA, the ferritin formation was not significantly changed in black bean BRS Supremo in response to Se and Zn treatments. While Se treatment slightly increased Fe uptake in one light brown cultivar and showed no effect on the other cultivar, more Zn supplementation slightly decreased Fe uptake in these two light brown bean varieties (Fig. 7).

DISCUSSION

Common bean is a widely consumed grain for a large number of the world's population and represents an important source of essential minerals in human diets. There is much advantage to biofortify common bean with nutrients to achieve overall Fe, Zn, and Se level increases in diets due to its good capacity to accumulate them. In addition, significant genetic variability for grain Fe and Zn content exists among common bean varieties (Talukder *et al.*, 2010), which offers the opportunity for further enhancing these nutrient content. A good understanding of the effects of mineral supplement on plant growth, nutrient content, and bioavailability among various genotypes is critical for such endeavor.

Fe, Zn, and Se treatments exhibit different effects on common bean plant growth

Plant mineral nutrient status greatly affects crop growth and production. Common bean plants were found to respond differently to Fe, Zn, and Se supplementations. We observed no reduced biomass in almost all cultivars when the plants were exposed to the modified Hoagland and Arnon`s solution without Fe supplement for 15 days, despite that the Fe content in leaves and roots was only on average 22% or 11%, respectively, of that in control (Fig. 1 and Fig. 2A and 2D). This indicates that common bean plants retained relative good ability to adjust wide range of Fe nutrition to support their growth. Consistently, no difference in root and shoot dry weight was observed in cucumber plants growing under –Fe and +Fe conditions (Dell'Orto *et al.*, 2000). However, a decreased biomass was reported in soybean plants exposed to –Fe solution for 14 days (Zocchi *et al.*, 2007), showing different sensitive to Fe nutrient status among plants.

In contrast to response to Fe nutrition status, the growth of common bean plants appeared to be less adaptable to Zn nutrient status changes. Both Zn omission and high level supplementation influenced negatively the plant growth. Plant growth in nearly all the cultivars was significantly reduced when Zn was withdrawn from the nutrition solution despite that the plants still contain an average of over 50% of Zn in comparison with controls (Fig. 1 and Fig. 2C and 2F). Similarly, plant growth in all cultivars was greatly suppressed with the addition of 50 μM of Zn. In other studies, although genotypic variation is observed, enhanced plant growth is reported with increased Zn supplementation even at the same concentration of Zn supplement (Souza *et al.*, 2013). It is likely that common beans are less tolerant to high concentration of Zn fertilization.

The beneficial effect of Se supply at low concentrations on promoting biomass production was observed in seven common bean cultivars. The growth stimulated effect of Se is consistent with previous studies in plant species, such as broccoli, lettuce and wheat (Ramos *et al.*, 2011*a*, *b*; Souza *et al.*, 2013).

Common bean plants show genetic variation with accumulation of different minerals

As expected, all cultivars responded coordinately to accumulate nutrients in shoot and roots following the Fe, Se and Zn treatments. Elimination of Fe from the nutrient solution dramatically reduced total Fe content in shoots and roots of all cultivars (Fig. 2A and D). Interestingly, Fe accumulation in roots was observed to be greatly increased by high Zn concentration (Fig. 2D). A previous study reports a strong positive correlation between Zn and Fe contents in common bean seeds (Hacisalihoglu *et al.*, 2005), but such high fold of increases was not reported previously. In contrast, a general antagonistic effect between Fe and Zn is observed in other crop species (Ghasemi-Fasaei and Ronaghi, 2008; Souza *et al.*, 2013).

While Se supplement led to Se accumulation in common bean, the common bean cultivars had a very low genetic variation with less than 2-fold changes in both shoots and roots (Fig. 2B and E). In other studies, over 3-fold genetic variations are reported in shoot tissues (Ramos *et al.*, 2011*a*; Souza *et al.*, 2013). The low fold change of Se content in common bean cultivars could be due to their limited genetic variation for Se accumulation.

Noticeably, the common bean plants accumulated high levels of Zn when exposed to 50 μM of Zn, especially in roots (Fig. 2C and 2F). The Zn contents were much higher than other plants with similar treatments (Sagardoy *et al.*, 2009; Souza *et al.*, 2013). Such high level of Zn accumulation might be toxic to common beans, which explained the reduced biomass production in the 50 μM Zn treated plants.

Fe, Zn and Se concentrations are relative consistent in grains during pod development

The Fe, Zn, and Se accumulation in grains and pod walls, the tissue that receiving limited attention for its role in re-distribution of nutrients to the developing grains, exhibited different patterns during seed development (Fig. 3 and Fig. 4). Both Se and Zn enhancements were achieved when three cultivars were subjected to Se or Zn treatments. During pod development from 17 DAP to mature, the Fe, Zn, and Se concentration in grains remained relatively constant in the tested cultivars except Se content in BRS Cometa line, showing a genetic variation of accumulation. In contrast, a general trend of decrease in these mineral levels was observed in pod walls during pod development, particularly at mature stage, except the Zn level in pod walls with additional supplementation of Zn. Moreover, Fe, Zn, and Se accumulated much more in grains than pod walls with an average of 72%, 57%, and 50%, respectively, during all stages of pod development.

Pod walls have been indicated to be significant source of grain minerals in a number of plant species (Hocking and Pate, 1977; Waters and Grusak, 2008; Sankaran and Grusak, 2014). Mineral loss from pod walls is reported to account for the minerals being remobilized to grains. In peas at mature stage, a range from 75 to 95% of total minerals including Fe and Zn in pods are partitioned to the seeds, and the pod walls contribute 9-40% of the total seed mineral content (Sankaran and Grusak, 2014). Similarly in some legume seeds, pods are estimated to provide 4-39% of the total accumulation of some seed essential minerals including Fe and Zn (Hocking and Pate, 1977). Both Fe and Zn contents in pod walls of these three common bean cultivars were dramatically reduced during grain mature in control (Fig. 3), suggesting a remobilization of these minerals to the grains. While additional Zn supplementation led to increased Zn level in the pod walls, it appeared not to translate into much enhanced Zn level in the grains. The results suggest that other factors limit the remobilization of additional Zn into the grains.

The Fe and Zn levels to be achieved in the biofortified bean crop, established by the Harvest Plus program, are 107 μg g^{-1} DW and 56 μg g^{-1} DW, respectively (Bouis and Welch, 2010). All these common bean cultivars examined had high capacities to take up and accumulate Fe and Zn. They can provide adequate amounts of these nutrients in a small portion of consumption.

Fe bioavailability is affected by specific polyphenol compounds and not greatly changed by Se and Zn biofortification

Black beans are known with low Fe bioavailability in comparison with the white bean varieties (Tako *et al.*, 2014; Hart *et al.*, 2015). In consistent with this, we showed here that the black grain cultivar BRS Supremo exhibited much lower Fe bioavailability than the light brown BRSMG Madrepérola and BRS Cometa cultivars in the Caco-2 cell model in the presence or absence of ascorbic

acid, an enhancer to increase the Fe bioavailability (Glahn *et al.*, 1999; Jin *et al.*, 2009).

Both phytic acid and polyphenols are known to affect mineral bioavailability in food crops (Glahn *et al.*, 2002; Jin *et al.*, 2009; Tako *et al.*, 2014). The phytic acid and polyphenol contents in grains of bean cultivars were analyzed to relate to their Fe bioavailability. Although the black bean variety had much low Fe bioavailability, it contained similar level of total phytic acid as the light brown cultivars (Fig. 5), indicating that phytic acid was not the main cause for the low Fe bioavailability in the black bean variety. The black and light brown common bean cultivars exhibited different polyphenol profiles (Fig. 6). While the light brown beans contained mainly catechin, kaempferol 3-glucoside, and kampferol, the black beans (BRS Supremo) had five additional major polyphenols of 3,4-dihydroxybenzoic acid, myricetin 3-glucoside, quercetin 3-glucoside, myricetin and quercetin. A recent detail analysis of individual polyphenols on Fe uptake reveals different abilities of various polyphenols in inhibiting or promoting Fe uptake in the cell model (Hart *et al.*, 2015). The three major polyphenols found in the light brown bean varieties are reported to clearly promote Fe uptake and the five additional polyphenols found in the black bean variety inhibit Fe uptake (Hart *et al.*, 2015). Thus, the presence of these polyphenol inhibitors detected only in the black bean cultivar BRS Supremo was likely responsible for its low Fe bioavailability.

Biofortifying these common bean cultivars with Se in general did not affect Fe bioavailability and promoted it slightly in one variety in the presence of ascorbic acid (Fig. 7). Enriching of these cultivars with Zn also did not affect Fe bioavailability except slight decrease in two light brown varieties in the presence of ascorbic acid (Fig. 7). Both Se and Zn additions showed no dramatic effects on the total levels of phytic acid and polyphenols in grains of these common beans. The slight decrease in Fe bioavailability in the presence of

ascorbic acid following Zn treatment in the two light brown cultivars could be resulted from other antinutritional compounds that interact with ascorbic acid, like tannins (Engle-Stone *et al.*, 2005; Jin *et al.*, 2009). However, the Fe bioavailability in these common bean cultivars was not greatly affected by both Se and Zn treatments in the absence of ascorbic acid. The results suggest the potential to simultaneously biofortify common beans with Se and Zn with minimal effect on Fe bioavailability.

In conclusion, the common bean cultivars exhibited genotypic variation in plant growth and mineral accumulation in both seedlings and grains in responses to Fe, Se, and Zn treatments. Common bean plants appeared to retain relative good ability to adjust Fe nutrition, but are less adaptable to Zn nutrient status changes. The plant biomass in general was not affected by low Fe status, but greatly suppressed by low or high Zn concentration. Se supply at low concentrations had beneficial effect on biomass production. All cultivars responded positively to accumulate Fe, Se and Zn following the mineral treatments.

Low dosages of Se and Zn supplements were sufficient to increase the Se and Zn contents in grains of common beans. During the pod development, the grains maintained relative consistent Fe, Se, and Zn levels and the pod walls gradually reduced these mineral contents, probably due to remobilization of them from pod walls to grains.

While both phytic acid and polyphenols affect Fe bioavailability, the presence of inhibitory polyphenol compounds appeared to exert profound effect on reducing Fe uptake as shown in the black bean variety. The Fe uptake was not dramatically affected by both Se and Zn treatments, indicating that it is possible to enhance these minerals without greatly affecting Fe bioavailability. Thus, common bean cultivars have the potential to be simultaneously biofortified with these essential nutrients for improving human health.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1, total Fe, Se and Zn content in pod walls of three cultivars of common beans subjected to Se and Zn treatments during pod development. Table S2, total Fe, Se and Zn content in grains of three cultivars of common beans subjected to Se and Zn treatments during pod development.

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FIGURE LEGENDS

Fig. 1. Biomass production of common beans subjected to different Fe, Se and Zn treatments for 15 days. (A) Shoots. (B) Roots. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls. Cultivars A to J represents varieties as shown in Materials and methods.

Fig. 2. Total Fe, Se, and Zn content in shoots (A, B, C) and roots (D, E, F) of ten cultivars of common beans subjected to different nutrient treatments. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

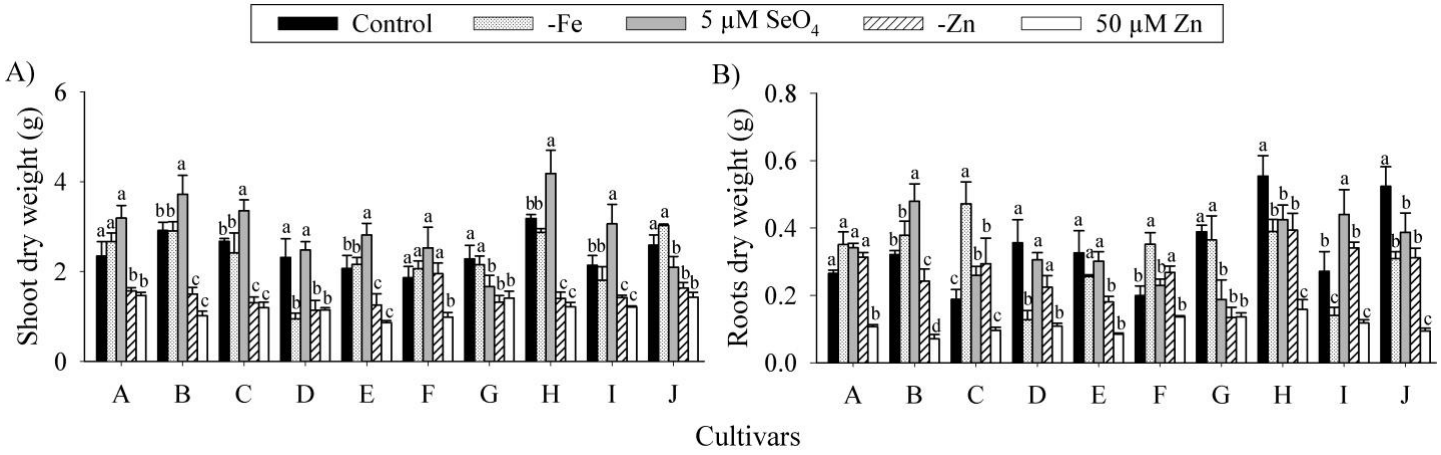
Fig. 3. Total Fe, Se and Zn content in pod walls of three cultivars of common beans subjected to Se and Zn treatments during various stages of pod development. Error bars indicate standard error of the mean (SEM) (n = 3). DAP: days after pollination

Fig. 4. Total Fe, Se and Zn content in grains of three cultivars of common beans subjected to Se and Zn treatments during grain development. Error bars indicate standard error of the mean (SEM) (n = 3). DAP: days after pollination

Fig. 5. Phytic acid content in mature seeds of three cultivars of common beans subjected to Se and Zn treatments. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

Fig. 6. Polyphenol concentration in mature seeds of common beans subjected to Se and Zn treatments. A. BRS Cometa; B. BRS-MG Madrepérola; C. BRS Supremo (black bean variety). Error bars indicate standard error of the mean (SEM) (n = 5). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

Fig. 7. Caco-2 cell ferritin formation in response to *in vitro* digests of three cultivars of cooked beans with or without ascorbic acid (AA) added immediately prior to experiment. Digests contained a constant Fe:ascorbic acid molar ratio of 1:20. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters represent significant difference at $p \leq 0.05$ for treatments.



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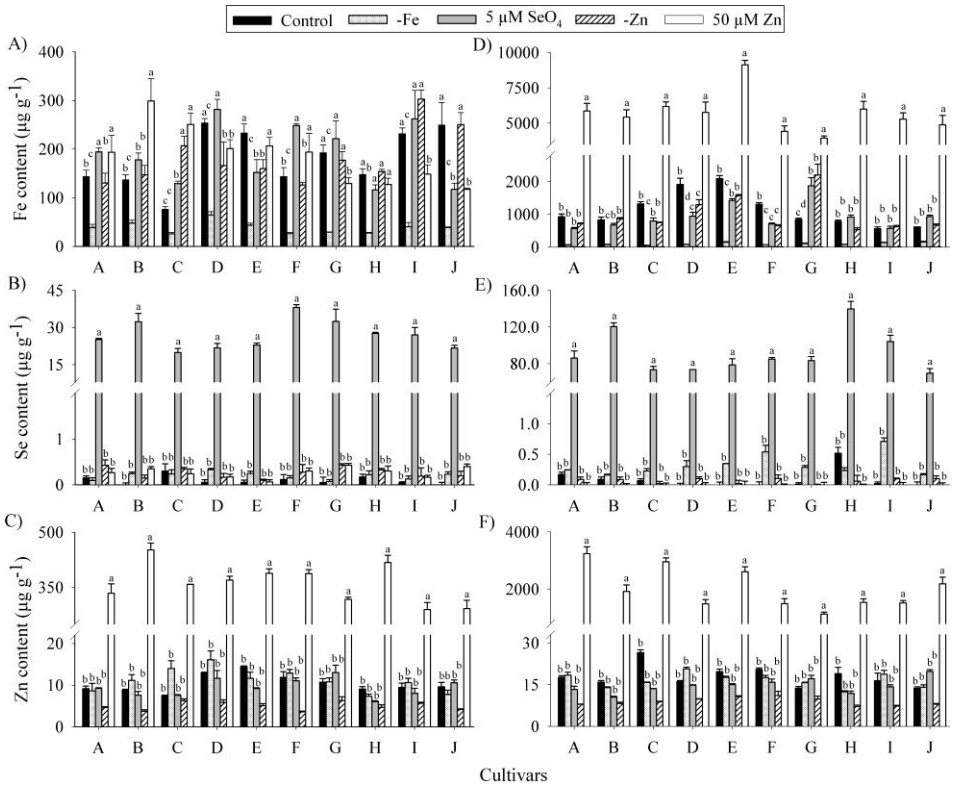

Fig. 2. Total Fe, Se, and Zn content in shoots (A, B, C) and roots (D, E, F) of ten cultivars of common beans subjected to different nutrient treatments. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

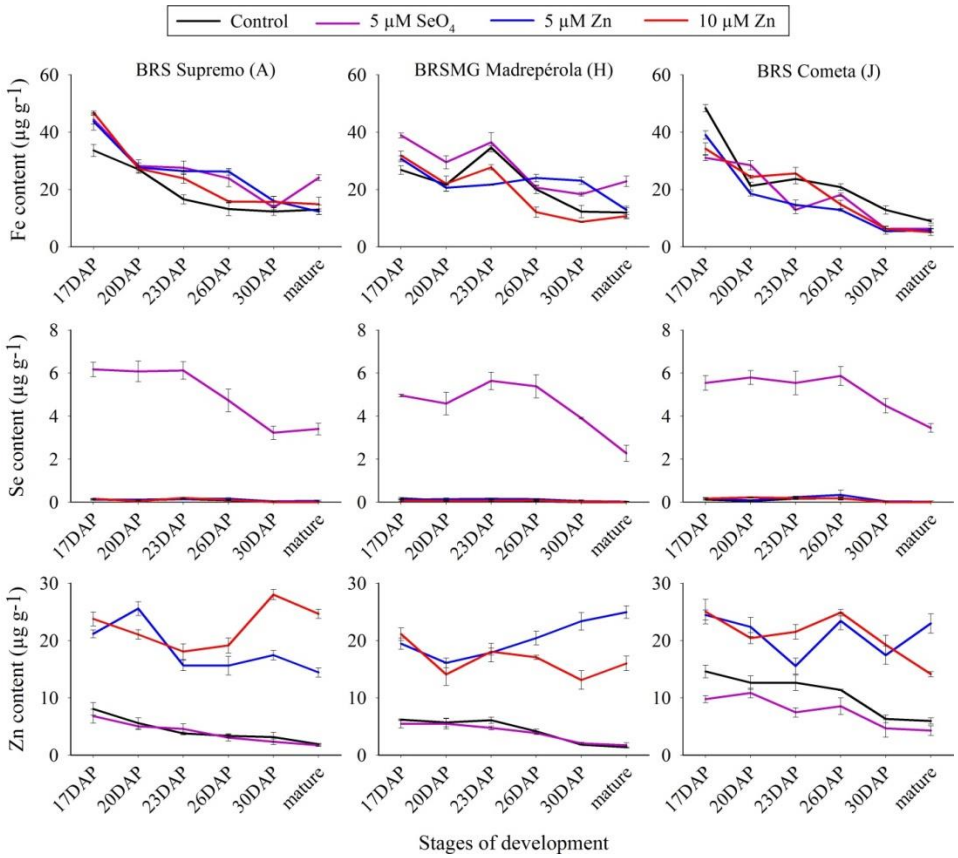

Fig. 3. Total Fe, Se and Zn content in pod walls of three cultivars of common beans subjected to Se and Zn treatments during various stages of pod development. Error bars indicate standard error of the mean (SEM) (n = 3). DAP: days after pollination

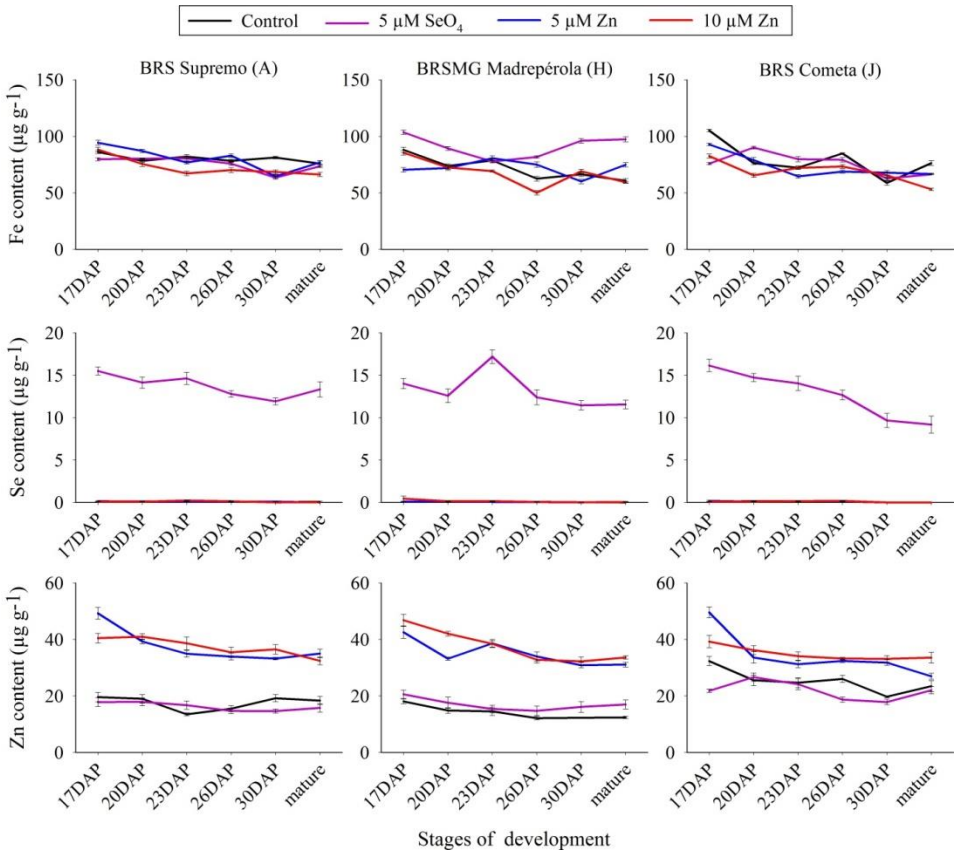

Fig. 4. Total Fe, Se and Zn content in grains of three cultivars of common beans subjected to Se and Zn treatments during grain development. Error bars indicate standard error of the mean (SEM) (n = 3). DAP: days after pollination

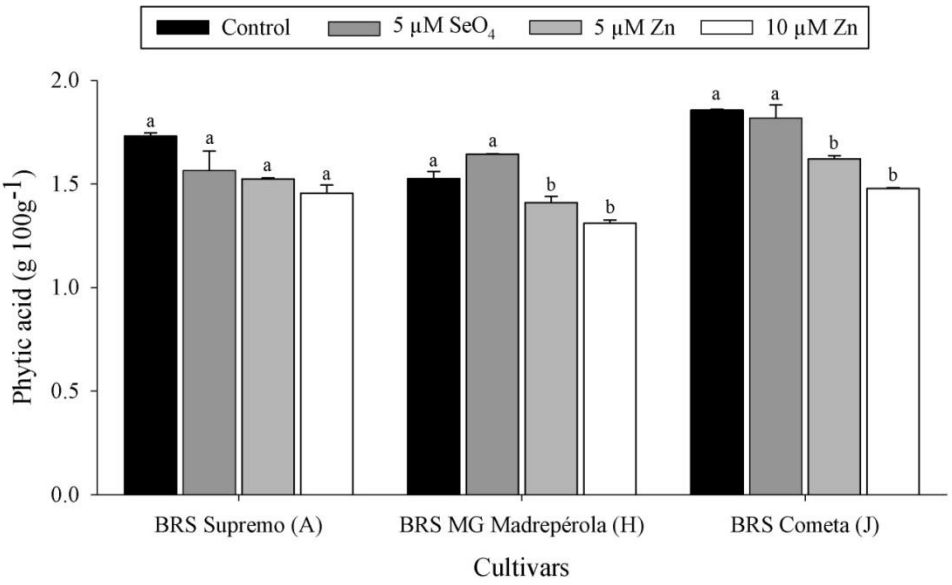

Fig. 5. Phytic acid content in mature seeds of three cultivars of common beans subjected to Se and Zn treatments. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

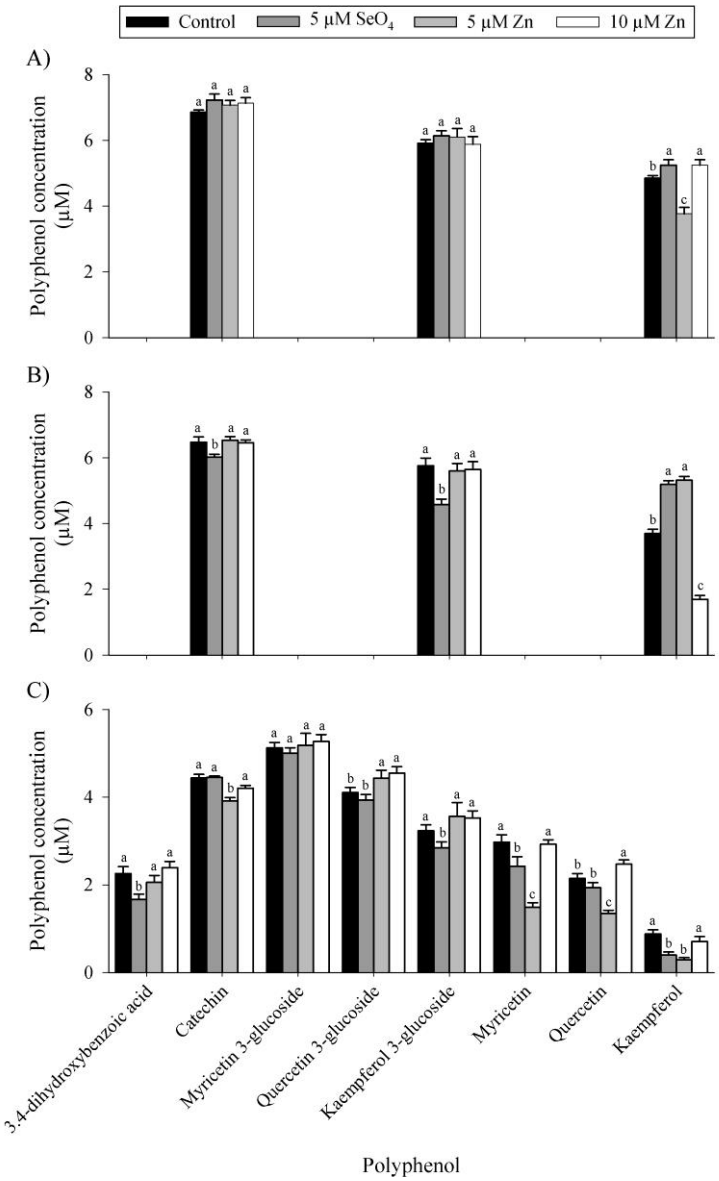

Fig. 6. Polyphenol concentration in mature seeds of common beans subjected to Se and Zn treatments. A. BRS Cometa; B. BRSMG Madrepérola; C. BRS Supremo (black bean variety). Error bars indicate standard error of the mean (SEM) (n = 5). Different letters above the column indicate significant difference at $p \leq 0.05$ in comparison with controls.

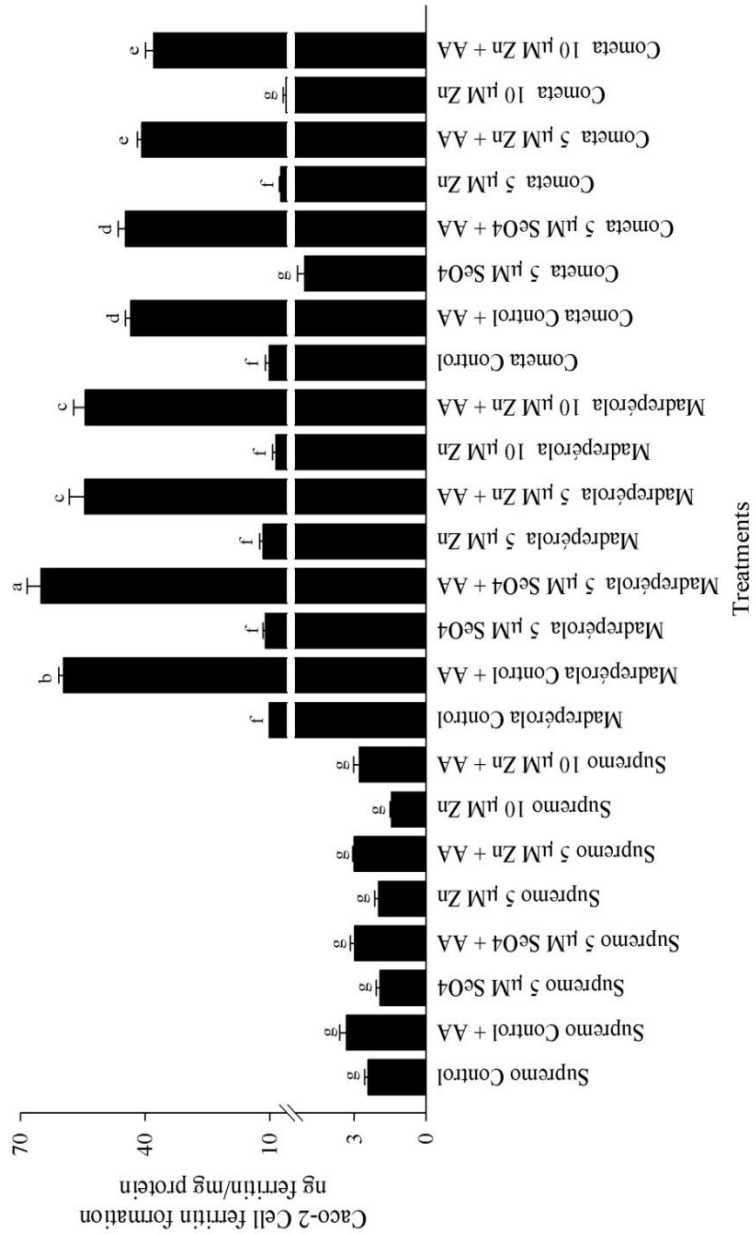

Fig. 7. Caco-2 cell ferritin formation in response to *in vitro* digests of three cultivars of cooked beans with or without ascorbic acid (AA) added immediately prior to experiment. Digests contained a constant Fe:ascorbic acid molar ratio of 1:20. Error bars indicate standard error of the mean (SEM) ($n = 3$). Different letters represent significant difference at $p \leq 0.05$ for treatments.

Table S1 Total Fe, Se and Zn content in pod walls of three cultivars of common beans subjected to Se and Zn treatments during pod development.

Content	Stages of development	BRS Supremo				BRSMG Madrepérola				BRS Cometa			
		Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn
Fe ($\mu g\ g^{-1}$)	17DAP	33,60 Ab	44,37 Aa	43,73 Aa	46,80 Aa	26,81 Bc	38,87 Aa	30,68 Ab	31,81 Ab	48,38 Aa	31,01 Ac	39,01 Ab	34,21 Ac
	20DAP	26,99 Ba	28,17 Ba	27,63 Ba	27,26 Ba	21,70 Cb	29,48 Ba	20,59 Bb	21,98 Bb	21,23 Bc	28,46 Aa	18,53 Bc	24,38 Bb
	23DAP	16,56 Cb	27,55 Ba	26,41 Ba	23,88 Ba	34,55 Aa	36,43 Aa	21,67 Bc	27,70 Ab	23,66 Ba	12,90 Cb	14,61 Cb	25,58 Ba
	26DAP	13,15 Cb	23,87 Ba	26,22 Ba	15,77 Cb	20,05 Ca	20,64 Ca	23,99 Ba	12,08 Cb	20,81 Ba	18,14 Ba	12,84 Cb	14,75 Cb
	30DAP	12,29 Ca	13,68 Ca	15,98 Ca	15,65 Ca	12,25 Dc	18,33 Cb	23,06 Ba	8,69 Cc	12,84 Ca	6,25 Db	5,42 Db	6,25 Db
	MATURE	12,93 Cb	24,12 Ba	12,26 Cb	14,84 Cb	11,94 Db	22,83 Ca	12,87 Cb	10,68 Cb	8,95 Da	6,30 Da	5,71 Da	5,08 Da
CV (%)		12,46				11,81				11,84			
Se ($\mu g\ g^{-1}$)	17DAP	0,12 Ab	6,17 Aa	0,12 Ab	0,15 Ab	0,17 Ab	4,96 Aa	0,12 Ab	0,06 Ab	0,12 Ab	5,54 Aa	0,17 Ab	0,16 Ab
	20DAP	0,08 Ab	6,08 Aa	0,11 Ab	0,04 Ab	0,07 Ab	4,58 Ba	0,13 Ab	0,06 Ab	0,05 Ab	5,79 Aa	0,08 Ab	0,22 Ab
	23DAP	0,14 Ab	6,12 Aa	0,15 Ab	0,19 Ab	0,06 Ab	5,64 Aa	0,15 Ab	0,06 Ab	0,16 Ab	5,54 Aa	0,23 Ab	0,19 Ab
	26DAP	0,08 Ab	4,73 Ba	0,15 Ab	0,10 Ab	0,04 Ab	5,38 Aa	0,13 Ab	0,08 Ab	0,18 Ab	5,87 Aa	0,33 Ab	0,17 Ab
	30DAP	0,03 Ab	3,23 Ca	0,03 Ab	0,00 Ab	0,00 Ab	3,90 Ca	0,04 Ab	0,04 Ab	0,02 Ab	4,48 Ba	0,03 Ab	0,01 Ab
	MATURE	0,01 Ab	3,40 Ca	0,06 Ab	0,00 Ab	0,00 Ab	2,26 Da	0,02 Ab	0,02 Ab	0,01 Ab	3,45 Ca	0,03 Ab	0,01 Ab

Content	Stages of development	BRS Supremo				BRSMG Madrepérola				BRS Cometa			
		Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn
CV (%)			26,72				28,78				24,93		
Zn ($\mu g\ g^{-1}$)	17DAP	8,06 Ab	6,81 Ab	21,16 Ba	23,76 Ba	6,18 Ab	5,45 Ab	19,45 Ba	21,12 Aa	14,60 Ab	9,76 Ac	24,45 Aa	25,02 Aa
	20DAP	5,59 Ac	5,03 Ac	25,56 Aa	21,04 Cb	5,71 Ab	5,47 Ab	16,09 Ca	14,08 Ca	12,64 Ab	10,85 Ab	22,36 Aa	20,41 Ba
	23DAP	3,79 Bc	4,58 Ac	15,65 Cb	18,07 Ca	6,09 Ab	4,75 Ab	17,87 Ca	18,05 Ba	12,63 Ac	7,47 Ad	15,54 Bb	21,51 Ba
	26DAP	3,36 Bc	3,05 Bc	15,61 Cb	19,13 Ca	4,16 Ac	3,85 Ac	20,42 Ba	17,07 Bb	11,37 Ab	8,53 Ab	23,41 Aa	24,87 Aa
	30DAP	3,15 Bc	2,33 Bc	17,44 Cb	28,00 Aa	1,83 Bc	2,05 Ac	23,36 Aa	13,10 Cb	6,32 Bb	4,68 Bb	17,43 Ba	19,24 Ba
	MATURE	1,90 Bc	1,72 Bc	14,43 Cb	24,65 Ba	1,39 Bc	1,72 Ac	22,94 Aa	15,00 Cb	5,95 Bc	4,29 Bc	22,99 Aa	14,14 Cb
CV (%)			13,00				14,68				14,08		

Different upper case letters in the column and lower case in the line indicate significant difference between treatments at $p<0.05$.

DAP: days after pollination

CV: coefficient of variation

Table S2 Total Fe, Se and Zn content in grains of three cultivars of common beans subjected to Se and Zn treatments during pod development.

Content	Stages of development	BRS Supremo				BRSMG Madrepérola				BRS Cometa			
		Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn
Fe (µg g^{-1})	17DAP	86,14 Ab	79,76 Ac	94,32 Aa	88,29 Ab	88,07 Ab	103,58 Aa	70,36 Bc	85,53 Ab	105,1 Aa	75,83 Bd	92,81 Ab	82,51 Ac
	20DAP	78,32 Bb	80,25 Ab	87,08 Ba	75,49 Bb	73,80 Bb	89,31 Ca	71,93 Bb	72,42 Bb	76,31 Cb	90,10 Aa	79,05 Bb	65,45 Cc
	23DAP	82,06 Aa	80,39 Aa	76,82 Ca	67,20 Cb	78,67 Ba	77,75 Da	80,60 Aa	69,20 Bb	72,23 Cb	79,80 Ba	64,54 Cc	72,04 Bb
	26DAP	78,33 Bb	75,80 Bb	82,90 Ba	70,15 Cc	62,55 Cc	81,68 Da	74,96 Bb	50,04 Dd	84,88 Ba	79,24 Bb	68,76 Cd	73,33 Bc
	30DAP	81,23 Aa	63,42 Cb	64,57 Db	68,50 Cb	66,34 Cb	96,08 Ba	60,07 Cc	69,08 Bb	58,79 Db	62,69 Cb	67,84 Ca	65,51 Ca
	MATURE	75,87 Ba	73,50 Ba	76,95 Ca	66,21 Cb	60,71 Cc	97,35 Ba	74,83 Bb	59,34 Bc	76,27 Ca	66,31 Cb	66,71 Cb	53,14 Dc
CV (%)		3,88				4,30				3,69			
Se (µg g^{-1})	17DAP	0,07 Ab	15,50 Aa	0,14 Ab	0,08 Ab	0,09 Ab	14,01 Ba	0,09 Ab	0,44 Ab	0,09 Ab	16,14 Aa	0,18 Ab	0,08 Ab
	20DAP	0,10 Ab	14,13 Ba	0,11 Ab	0,09 Ab	0,08 Ab	12,58 Ca	0,12 Ab	0,13 Ab	0,15 Ab	14,73 Ba	0,09 Ab	0,10 Ab
	23DAP	0,10 Ab	14,62 Ba	0,07 Ab	0,22 Ab	0,09 Ab	17,19 Aa	0,05 Ab	0,14 Ab	0,14 Ab	14,04 Ca	0,14 Ab	0,14 Ab
	26DAP	0,09 Ab	12,79 Ca	0,08 Ab	0,13 Ab	0,07 Ab	12,40 Ca	0,06 Ab	0,06 Ab	0,10 Ab	12,66 Da	0,16 Ab	0,18 Ab
	30DAP	0,03 Ab	11,92 Da	0,09 Ab	0,01 Ab	0,03 Ab	11,45 Da	0,03 Ab	0,02 Ab	0,01 Ab	9,66 Ea	0,00 Ab	0,00 Ab
	MATURE	0,08 Ab	13,34 Ca	0,01 Ab	0,02 Ab	0,00 Ab	11,55 Da	0,06 Ab	0,04 Ab	0,00 Ab	9,18 Ea	0,00 Ab	0,00 Ab

Content	Stages of development	BRS Supremo				BRSMG Madrepérola				BRS Cometa			
		Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn	Control	5µM SeO_4	5µM Zn	10µM Zn
CV (%)		15,48				18,72				20,41			
Zn (µg g^{-1})	17DAP	19,60 Ac	17,88 Ac	49,27 Aa	40,49 Ab	18,08 Ac	20,62 Ac	42,55 Ab	46,83 Aa	32,36 Ac	21,82 Ad	49,58 Aa	39,26 Ab
	20DAP	19,03 Ab	17,92 Ab	39,29 Ba	40,99 Aa	14,87 Bc	17,62 Ac	33,21 Cb	42,06 Ba	25,58 Bb	26,68 Ab	33,61 Ba	36,23 Aa
	23DAP	13,50 Bb	16,75 Ab	34,94 Ca	38,66 Aa	14,56 Bb	15,42 Ab	38,62 Ba	38,46 Ca	24,69 Bb	24,18 Ab	31,25 Ba	34,11 Ba
	26DAP	15,52 Bb	14,73 Ab	33,97 Ca	35,47 Ba	12,15 Bb	14,75 Ab	34,00 Ca	32,82 Da	26,02 Bb	18,72 Bc	32,39 Ba	33,25 Ba
	30DAP	19,21 Ab	14,65 Ac	33,25 Ca	36,51 Ba	12,29 Bc	16,16 Ab	30,88 Ca	32,25 Da	19,74 Cb	17,86 Bb	31,82 Ba	33,16 Ba
	MATURE	18,38 Ab	15,79 Ab	35,01 Ca	32,43 Ba	12,42 Bc	17,00 Ab	31,14 Ca	33,64 Da	23,50 Bc	22,02 Ac	26,97 Cb	33,57 Ba
CV (%)		9,05				8,86				8,68			

Different upper case letters in the column and lower case in the line indicate significant difference between treatments at $p<0.05$.

DAP: days after pollination

CV: coefficient of variation

ARTIGO 2

Biofortification with iron, selenium and zinc, and their interactions in common bean seedlings

(Artigo elaborado segundo normas da revista Plant Science - Versão preliminar)

Biofortification with iron, selenium and zinc, and their interactions in common bean seedlings

Marislaine Alves de Figueiredo[a,b,c], Messias José Bastos de Andrade[c], Luiz Roberto Guimarães Guilherme[d], Li Li[a,b,*]

[a] Robert W. Holley Center for Agriculture and Health, USDA-ARS, Cornell University, Ithaca, NY 14853, USA.

[b] Plant Breeding and Genetics section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA

[c] Department of Agriculture, Federal University of Lavras, Zip Code 3037, 37200-000 Lavras, MG, Brazil

[d] Department of Soil Science, Federal University of Lavras, Zip Code 3037, 37200-000 Lavras, MG, Brazil

* Corresponding authors: Email: ll37@cornell.edu; Tel: +1-607-255-5708; Fax: +1-607-255-1132

E-mails:

marislaine_alves@yahoo.com.br (M.A. de Figueiredo); mandrade@dag.ufla.br (M.J.B. de Andrade); guilherm@dcs.ufla.br (L.R.G. Guilherme); ll37@cornell.edu (L. Li)

Abstract

Iron (Fe), selenium (Se) and zinc (Zn) deficiencies are a big challenge affecting about one third of the population worldwide. Biofortification of common beans, a legume crop nutritionally rich and mainly consumed in developing countries, can improve the content of these minerals and reduce micronutrient malnutrition. We investigated if some common bean cultivars consumed in USA or in Brazil can be biofortified with Fe, Se and Zn without compromising the accumulation of each other. As Zn supply improved Fe accumulation in roots but did not promote its translocation to shoots, hydroponic experiments were carried out and gene expression and microscopy analyses were done to understand this effect. There were interactions between Fe, Se and Zn. Fe supplementation at higher concentration decreased Zn content, but did not affect Se nutrition. Zn omission reduced Se content in some cultivars. The common bean cultivars had high ability to accumulate Se and Zn when these minerals were supplemented. When Zn was added at 20 µM, Fe was prone to be precipitated prior to crossing root membranes, accumulating in roots epidermis. Zn fertilization may decrease Fe nutrition, although no Fe deficiency symptom was observed. Then, common beans have potential to be biofortified simultaneously with Fe, Se and Zn without causing significant negative effects on plant accumulation of these elements.

Keywords

Microscopy analyses, gene expression, *Phaseolus vulgaris*, Fe localization, micronutrients

1 Introduction

Biofortification, through agronomic or genetic approaches, is a strategy to combat mineral deficiencies of elements such as iron (Fe), selenium (Se) and zinc (Zn) [1]. Micronutrients deficiencies are a major cause of malnutrition that affects more than 2 billion people worldwide [2,3].

Zn and Fe are essential elements for plant growth and human health [4] and their interaction has been addressed in some studies concerning plant nutrition of different crops [5-8]. Selenium is also essential to human health but is considered a beneficial element for plants [9]. These micronutrients have important functions in many metabolic pathways and in cell protection (redox-active), acting also as cofactors in enzymes, gene regulation, reproduction and others [4,9,10].

Common bean (*Phaseolus vulgaris* L.) is a staple food crop [11,12] feeding over half a billion people of Latin America and Africa [13]. In Brazil, this crop is consumed in great quantities, reaching an average of 18 kg/capita/year [11,14,15]. Its consumption in North America and Europe is less than 5 kg/capita/year, yet in some African countries it reaches more than 40 kg/capita/year [11,16]. In fact, common beans represents about 65% of protein consumed and 32% of energy supply [17].

Despite the recent gains in hunger reduction observed in Africa over the years by FAO, approximately 795 million people are undernourished, especially in developing countries, where smallholder agriculture plays an important role in food availability [18]. As common bean is nutritionally rich and cultivated mainly by family farmers [11] it represents an essential crop to provide food and, consequently, nutrients to people, thus improving human health in such regions.

In order to contribute to an improvement of the nutritional quality of beans, the goal of this research was to verify the interaction between Fe, Se and Zn in enriched seedlings of common bean cultivars consumed in USA or in Brazil. Since Zn supplementation stimulated Fe accumulation in roots but did not enhance its translocation to shoots, several hydroponic experiments and analyses were carried out to understand Zn influence on Fe nutrition in this legume crop.

2 Materials and Methods

2.1 Plant growth and mineral treatments

General

Seeds of common bean genotypes were obtained from IAPAR (Londrina, Brazil), EMBRAPA Rice and Beans (Santo Antônio de Goiás, Brazil) or commercially in Ithaca - USA (Navy and Pinto Beans). The seeds were surface-sterilized in 10% NaOCl for few minutes, rinsed in distilled MΩ water and germinated on moistened filter paper for three or four days. Germinated seeds were transferred to a box containing modified Hoagland and Arnon`s solution [19] with 50% ionic strength. $Fe(NO_3)_3$ $9H_2O$, H_3HEDTA and KOH were used instead of $FeSO_4$ $7H_2O$, EDTA and NaOH. $Fe(NO_3)_3$ was equilibrated with H_3HEDTA (N-2-hydroxyethylethhylenediamine-N,N',N'-triacetic acid). The young seedlings were transplanted to 2.2 L dark pots containing the same modified Hoagland and Arnon`s solution. Nutrient solutions were changed twice a week. Plants were grown hydroponically with constant aeration in a greenhouse at 24 ^oC (± 2 ^oC) under 14-h day length. Shoots and roots were harvested separately, dried at 65 ^oC for three days, and ground. Fe, Se and Zn contents were analyzed by an inductively coupled plasma (ICP) trace

analyzer emission spectrometer (model Thermo Scientific ICAP 6000 trace element analyzer, San Jose, CA). Se content was analyzed just in experiments with Se treatments. The design was completely randomized and treatments had three replicates.

Experiment 1

Two common bean cultivars consumed in USA (Navy and Pinto Beans) and seven treatments (1 - Control: modified Hoagland and Arnon`s solution (0.365 µM Zn; 30 µM Fe); 2 - 50 µM $ZnCl_2$; 3 - 50 µM $ZnCl_2$ + 10 µM Na_2SeO_4; 4 - 50 µM $ZnCl_2$ + 10 µM Na_2SeO_3; 5 - 100 µM $ZnCl_2$; 6 - 100 µM $ZnCl_2$ + 10 µM Na_2SeO_4; 7 - 100 µM $ZnCl_2$ + 10 µM Na_2SeO_3) were evaluated in the first experiment. After 20 days, the plants were harvested separately in shoots and roots, and the Fe, Se and Zn contents were analyzed by ICP.

Experiment 2

Ten Brazilian common bean cultivars, BRS Supremo (A), BRS Notável (B), Pérola (C), IPR Tangará (D), BRS Requinte (E), BRS Estilo (F), BRS Pontal (G), BRSMG Madrepérola (H), BRS Grafite (I) and BRS Cometa (J) were grown hydroponically. The A and I cultivars belong to the black group and the other cultivars have light brown seeds. Six treatments were evaluated (1 - Control: modified Hoagland and Arnon`s solution (0.365 µM Zn; 30 µM Fe); 2 - Fe absence; 3 - Zn absence; 4 - 5 µM Na_2SeO_4; 5 - 5 µM Na_2SeO_4 – Zn; 6 - 10 µM $ZnCl_2$). After 22 days, the plants were harvested separately in shoots and roots, and the Fe, Se and Zn contents were analyzed by ICP.

Experiment 3

Following experiment 2, another experiment was carried out with three cultivars – BRS Supremo (black beans), BRSMG Madrepérola and BRS Cometa

(light brown beans). They were selected based on results from the second experiment. The plants were kept in the modified Hoagland and Arnon`s solution as control treatment (0.365 µM Zn) or exposed to the nutrient solution containing $ZnCl_2$ at three different concentrations (5, 10 or 20 µM). After 20 days, the plants were harvested separately in shoots and roots. The roots were first washed with distilled water, followed by washing with 0.5 mM $CaCl_2$ and finally with distilled water again. The Fe and Zn contents in both shoots and roots were analyzed by ICP.

Experiment 4

The last experiment was carried out with the same cultivars used in the third experiment. Six treatments were evaluated (1 – Control: modified Hoagland and Arnon`s solution (0.365 µM Zn; 30 µM Fe); 2 - 20 µM $ZnCl_2$; 3 - 60 µM $Fe(NO_3)_3$ $9H_2O$; 4 - 20 µM $ZnCl_2$ + 60 µM $Fe(NO_3)_3$ $9H_2O$; 5 - Fe absence; 6 - Zn absence). After 22 days, the young roots were collected, immediately frozen in liquid nitrogen and stored at -80 ºC for qRT-PCR. The roots were first washed with distilled water, followed by 0.5 mM $CaCl_2$ solution and, finally, distilled water again. Some pieces of the roots also were collected for Fe staining and microscope analysis. The plants were harvested separately in shoots and roots and Fe and Zn were analyzed by ICP.

2.2 Analysis of Fe, Se and Zn contents by ICP

In all experiments, the total Fe, Se and Zn contents in dry tissues were determined using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model Thermo Scientific ICAP 6000 trace element analyzer, San Jose, CA). Briefly, dried tissue samples were weighed (between 0.2 and 0.5g) and pre-digested with 4 mL of mixture $HNO_3/HClO_4$ (60/40% v/v) and 0.25 mL of 40 ppm of Yttrium solution (internal standard to correct drift in ICP)

overnight at room temperature. The mixture into the tubes was slowly swirled and then, the samples were heated to 120 ºC for two hours and 0.5 mL of HNO_3 was added. The temperature of the block was increased to 190 ºC for 30 min. The samples were cooled to room temperature and diluted with 20 mL of MΩ water. Blank and an internal standard Yttrium were used to substantiate the accuracy of the analytical results obtained. The experimental samples were prepared and analyzed following a rigid quality assurance/quality control (QA/QC) to ensure accurate and reliable analytical data. Each bean sample was analyzed in triplicate.

2.3 RNA extraction, cDNA isolation and qRT-PCR analysis

In experiment 4, total RNA from roots of plants grown in control solution or in supplied solution with 20 µM $ZnCl_2$ was extracted using Trizol reagent following the manufacturer's instruction (Invitrogen). Reverse transcription into cDNA was done on 5 µg of RNA using Superscript III reverse transcriptase (Invitrogen). The synthesized cDNA quality was checked based on the amplification of common bean Actin gene using semi-quantitative PCR. Quantitative real-time PCR was run in triplicate with two biological repeats. The relative gene expression was calculated following the methodology described previously [20].

2.4 Staining for Fe and microscopy images

Fe localization in roots of common bean plants of the fourth experiment subjected to control and plus Zn treatments was detected using Perls blue stain [21]. This staining was done according to the protocol's instructions based on a procedure described in the literature [22]. Briefly, pieces of roots were collected and fixed for 1-2 h under vacuum (500 mbar) in the methanol:chloroform:glacial acetic acid (6:3:1) solution. The fixing solution was removed and roots were

washed three times with distilled water. The pre-warmed staining solution [4% $K_4Fe(Cn)_6$ and 4% HCl solutions mixed in a 1:1 (v:v) proportion] was added and incubated for 30 min under vacuum. After removing the staining solution, the roots were washed three times with distilled water. Thin transverse and longitudinal sections were obtained by hand. Images were collected on a Leica DM5500 Epifluorescence microscope using three different objectives (10X dry, 20X immersion and 40X dry). The color CCD camera Retiga-2000R connected to the Qcapture Pro 6.0 acquisition software was used to process the images.

2.5 Ion speciation by Visual MINTEQ program

The ionic species in control and Zn supplied solutions from experiment 4 were evaluated in Visual MINTEQ program (Version 3.1, Jon Petter Gustafsson, KHT, Div. of Land and Water Resources Engineering, Stockholm, Sweden).

2.6 Statistical analysis

All data were subjected to statistical analysis of variance using the Statistical Analysis Software Sisvar® [23]. The means were compared by the Scott-Knott test at a level of 5% of probability.

3 Results

3.1 Seedlings treated with Fe, Se and Zn

3.1.1 Effect of different Zn doses and Se forms on Fe, Se and Zn accumulation in common bean seedlings

In experiment 1, seedlings of Navy and Pinto Beans cultivars were evaluated after growing in hydroponic conditions for two weeks in solutions

supplemented with two high doses of $ZnCl_2$ (50 and 100 μM), without or with 10 μM of Se in two forms (selenate and selenite). Fe content in shoots of common bean plants exposed to control solution was higher than in plants exposed to Zn-enriched solution (Fig. 1A). On the other hand, Fe content in roots and Zn content in whole bean plants had the lowest values when submitted to the control solution (Fig. 1B, C and F).

The lower Zn dose combined with selenate increased Se content in shoots for both cultivars (Fig. 1B). Generally, higher Fe, Se and Zn contents were observed in roots grown in solutions with higher Zn doses, regardless of Se form used (Fig. 1D, E and F).

The second experiment was conducted to analyze the effect of different Fe, Se and Zn treatments in ten Brazilian common bean cultivars. All of them responded positively to Se and Zn addition (Fig. 2). In more than half of cultivars, Zn omission adversely affected Se nutrition (Fig. 2B and E). In shoots, 10 μM of $ZnCl_2$ affected negatively Fe content in seven cultivars, which did not happen in roots (Fig. 2A and D). As expected, Fe elimination reduced Fe content. Positive effect of Fe suppression in Zn shoots content was observed in all cultivars. In roots, this effect was observed in only 3 cultivars – A, B and I (Fig. 2 C and F).

3.1.2 High Zn dose influenced Fe and Zn content

In experiment 3, as show in Fig. 3A, the cultivars exhibited similar Fe content in shoots when exposed to increasing doses of Zn. The highest Zn dose applied in the nutrient solution provided the highest Fe content in roots of all cultivars (Fig. 3C). Greater Zn content was obtained at higher Zn doses (Fig. 3B and D). BRSMG Madrepérola and BRS Cometa cultivars had not difference in Zn content in shoots supplied either at 10 or 20 μM $ZnCl_2$.

Seedlings of the fourth experiment were evaluated in relation to Fe and Zn content (Fig. 4). Fe elimination drastically reduced this element's content in shoots but not in roots, which were not statistically different from the treatments without additional Zn (Fig. 4A and C). As showed in all experiments, Zn supply promoted Fe accumulation in the roots for all cultivars analyzed (Fig. 4C). The range of Fe accumulation in roots treated with 20 µM $ZnCl_2$ supplemented or not with 60 µM $Fe(NO_3)_3$ varied from 3060 to 4147 µg g^{-1}dry weight (Fig. 4C). In the light brown bean cultivars, Fe supplied with Zn reduced the Fe content when compared with the solution treated just with Zn. Interestingly, withdrawal of Fe on nutrition solution had a positive effect on Zn content in shoots.

3.2 Genes related to Zn and Fe uptake and transport in plants

In experiment 4, to examine why Zn supply stimulated the Fe content in roots, the relative expression of some genes involved in Zn and Fe uptake and transport in plants were measured by qRT-PCR (Fig. 5). Most genes were upregulated in roots subjected to Zn supply at 20 µM $ZnCl_2$ in nutrition solution. The expression of some Zn transporter genes (*ZIP1, ZIP3* and *Zrt2*) were significantly downregulated in roots of both light brown bean cultivars treated with Zn (Fig. 5B and C). *FRO2*, *YS1* and *Zrt1* were highly upregulated under Zn supply. The Fe regulated transporter (*IRT1*) was significantly increased in roots of the three cultivars under Zn-enriched solution. The black beans cultivar had approximately 2- and 3-fold more relative *IRT1* expression compared with BRS Cometa and BRSMG Madrepérola, respectively. BRSMG Madrepérola had the lowest relative gene expression.

3.3 Perls stain detect Fe localization in roots of common bean seedlings

As show in Fig. 6, transverse and longitudinal roots sections were made after being fixed in Perls staining solution. Strong blue colors, which indicate Fe

localization, appeared in whole longitudinal roots subjected to Zn supplemented solution (Fig. 6B). On the other hand, roots under control solution had just blue dots uniformly distributed (Fig. 6A). Transverse roots sections showed that a Prussian blue precipitate accumulated in the epidermal tissues of roots exposed do Zn supply. In contrast, Fe localization in roots subjected to control treatment was observed in the vascular system (Fig. 6A).

3.4 Ion speciation program Visual MINTEQ

The control and Zn-enriched solutions were analyzed by the chemical equilibrium program, Visual MINTEQ. As showed in Table 1, EDTA was more complexed with Zn when this micronutrient was added in solution. In contrast, less EDTA was complexed with Fe. The percentage of dissolved Fe (not precipitated) in control solution was higher than in the Zn-supplied solution, while the fraction of Zn in "dissolved" form was almost the same in both solutions (Table 2).

4 Discussion

Agronomic biofortification was a strategy used to reach a nutritional enhancement of common beans in this study. We observed the interactions among three important minerals to human health, Fe, Se and Zn.

In addition to genetic variation found among bean cultivars, in all experiments the plants were positively affected by Se and Zn supplementation. The Se content varied from 13 to 385 μg g^{-1} dry weight of shoot and from 41 to 302 μg g^{-1} dry weight of roots depending on Se and Zn dosages applied in the nutrient solution, chemical species of Se and cultivar. The same was observed for Zn content. A variation of 52 to 714 μg g^{-1} dry weight of shoots and of 241 to 2511 μg g^{-1} dry weight of roots was observed depending of Zn and Fe dosages

applied in the nutrient solution, chemical species of Se and cultivar. Positive effect of Se and Zn supply in seedlings and genetic variation were also observed in other food crops aiming biofortification like in broccoli, wheat and lettuce [8,24-26].

Selenium accumulation in shoots from selenate treatment was higher than from selenite in both Navy Beans and Pinto Beans cultivars. Other studies reported consistent results showing better effect of selenate in accumulating Se than selenite [24,26-28]. Inversely, in roots, selenite supply had more positive effect on Se accumulation. Selenite has a limitation to be taken up by shoots because its tendency to accumulate in roots [29-31]. Pinto Beans cultivar had over 3-fold Se content than Navy Beans when submitted to selenate supply, showing to be more efficient in accumulating Se.

In more than half of the cultivars, Zn omission in the nutrient solution with Se supply decreased Se accumulation in common bean plants, i.e., bean plants show a higher capacity to accumulate this mineral when in the presence of Zn. Other researchers reported synergistic interaction between Zn and Se [8].

Withdrawal of Fe dramatically reduced total Fe content in plants of all common bean cultivars. Schmidt et al. [32] also found negative effect in Fe content in plants following Fe omission. However, Zn elimination of nutrient solution affected negatively Zn accumulation just in shoots. So, under the absence of Zn, this micronutrient was not translocated to shoots and remained at the same content as in roots exposed to control.

The Fe nutrition in roots was greatly increased by Zn supply with different doses. In contrast, Fe content in shoots of plants exposed to control solution was greater than or equal to Fe content obtained in plants exposed to Zn-enriched solution. These results indicate that Fe was not translocated to shoots in the same proportion that was accumulated in roots. Other studies have reported Fe suppression by Zn treatment, i.e., an antagonistic effect between

these micronutrients [7,8,33-35]. In contrast, positive correlation between Fe and Zn contents was found in common bean seeds [5].

To investigate the cause of Zn supply stimulating Fe accumulation but not its translocation to shoots, we evaluated, in three cultivars, the gene expression of some transporters and uptake genes besides microscopy analysis of roots to verify the Fe localization.

Common bean, as a legume crop, is included in strategy I group of plants in relation to Fe acquisition [36,37]. So, the reduction of ferric to ferrous Fe on the root surface is necessary. This reduction is performed by *FRO2* gene [38,39]. In all cultivars evaluated in this work Zn supplied in the solution increased relative *FRO2* expression in common bean roots, showing a possible cause of the synergistic effect of Zn in Fe accumulation in root surface.

Other genes also are involved in Fe uptake, like *YS1* [40], *FIT1*, *bHLH038* and *bHLH101* [41]. In BRS Supremo cultivar all these genes were upregulated in roots exposed to Zn supply at 20 µM $ZnCl_2$. *YS1* also was upregulated in the other two cultivars, but the others genes had different behavior in each cultivar. Despite the fact the black bean roots exhibit the highest relative gene expression related to Fe uptake, the Fe content was similar to the light brown bean cultivars. Fukao et al. [34] reported an increase of *FRO2* levels under excess Zn, but a decrease of Fe content in roots and shoots.

IRT1 is responsible for Fe uptake in addition to Fe and Zn transport [42,43]. Our results showed an increase of *IRT* expression in roots grown in Zn supplementation solution. The expression of this gene has been previously reported to enhance under Fe deficiency, but it can also be regulated by Zn [44].

FPN1, *NAP14* and *NRAMP3* are important to Fe transport [45]. Their expressions were significantly upregulated in roots exposed to 20 µM $ZnCl_2$, except for *FPN1* and *NRAMP3* in the BRS Cometa cultivar.

There are some genes responsible for Zn uptake, like *ZIP1*, *ZIP3*, *ZIP4* [46,47] and its transport, as *Zrt1* and *Zrt2* [48,49]. *ZIP1* and *ZIP3* are expressed in roots in response to Zn deficiency [47]. Both genes were downregulated in roots of the light brown bean cultivars (BRSMG Madrepérola and BRS Cometa) submitted to Zn-enriched solution. In contrast, they were upregulated in black bean cultivar (BRS Supremo).

No difference was observed between BRS Supremo and BRS Cometa in relation to Zn content but BRSMG Madrepérola had the highest Zn content in roots in the presence of 20 µM $ZnCl_2$. In relation to Zn transport, the Zn supplementation increased the *Zrt1* expression in all cultivars but decreased *Zrt2* in the light brown bean cultivars. This can explain the higher Zn content in shoots of BRS Supremo cultivar exposed to Zn supply.

Gene expression did not clearly explain the fact that Zn stimulates Fe accumulation in roots, but not its translocation to shoots. Then, transverse and longitudinal sections of roots exposed to control and Zn-enriched solutions were evaluated. A strong blue color, which indicates Fe localization, was observed in longitudinal roots subjected to Zn-supplemented solution. However, transverse sections showed Fe localization concentrated in the roots epidermis and not in the vascular system, which did not occur in roots grown in control solution.

Based on these results, control and Zn-enriched solution were analyzed with the aid of a speciation program, Visual MINTEQ, to verify whether there was some mineral precipitation.

When Zn concentration increases from 0.365 µM to 20 µM, less EDTA is complexed with Fe and more is complexed with Zn, i.e., more Fe is prone to be precipitated as shown by the equilibrated mass distribution of Fe. Under such circumstances, Fe uptake is reduced if Zn concentration increases because Fe is precipitated prior to crossing root membranes.

These results indicate that Zn fertilization at high concentrations increased Zn content and may negatively affected Fe nutrition in common bean plants, although no Fe deficiency symptom was observed. Maybe in poor Fe soils this negative effect could be more evident.

5 Conclusions

Se and Zn supplementation increase their contents in seedlings of common bean cultivars consumed by Americans and Brazilians.

The omission of Zn in the nutrient solution affects negatively Se accumulation when both minerals are combined. Although Zn stimulated the increase of Fe content in roots, its localization is only in the epidermis, not being transported to shoots in the same accumulated amount. In solution supplied with Zn, HEDTA is more complexed with Zn, and Fe is prone to be precipitate even before crossing root membranes. On the other hand, Zn supplied at lower concentration in control solution (0.365 μM) does not affect negatively Fe translocation, because its localization is in the vascular system.

Fe and Zn nutrition are not affected by Se supply. Withdraw of Fe increased Zn content mainly in shoots, did not affect Se accumulation, and decreased Fe content.

Zn fertilization may decrease Fe nutrition, although no Fe deficiency symptom was observed. Fe supply at lower concentration is recommended in order not to compromise Zn nutrition.

Then, common bean plants have potential to be biofortified simultaneously with Fe, Se and Zn without causing significant negative effects on plant accumulation of these elements.

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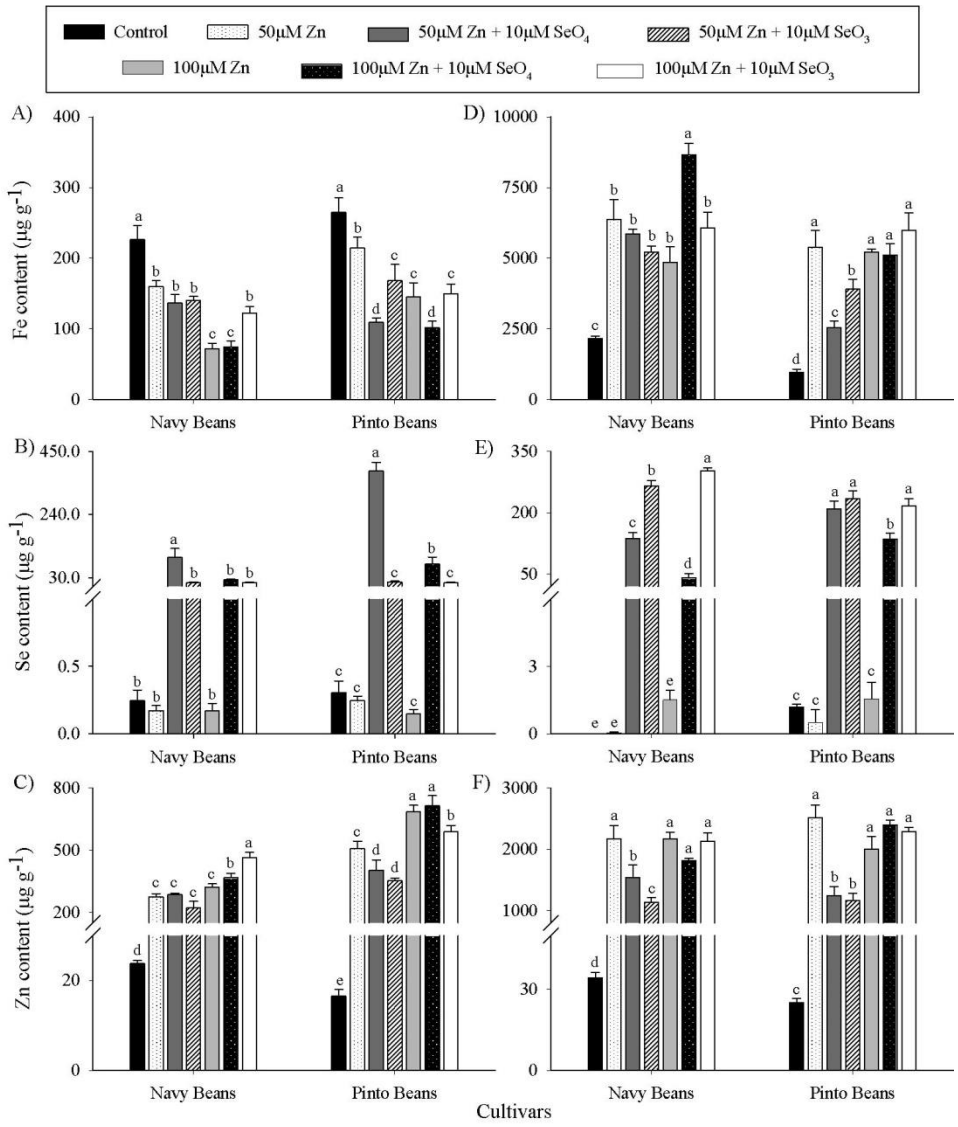

Fig. 1. Total Fe, Se and Zn content in shoot (A, B, C) and roots (D, E, F) of two common bean cultivars subjected to different Se forms and Zn doses. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column, for each cultivar, indicate significant difference at $p \leq 0.05$ by Scott-Knott test.

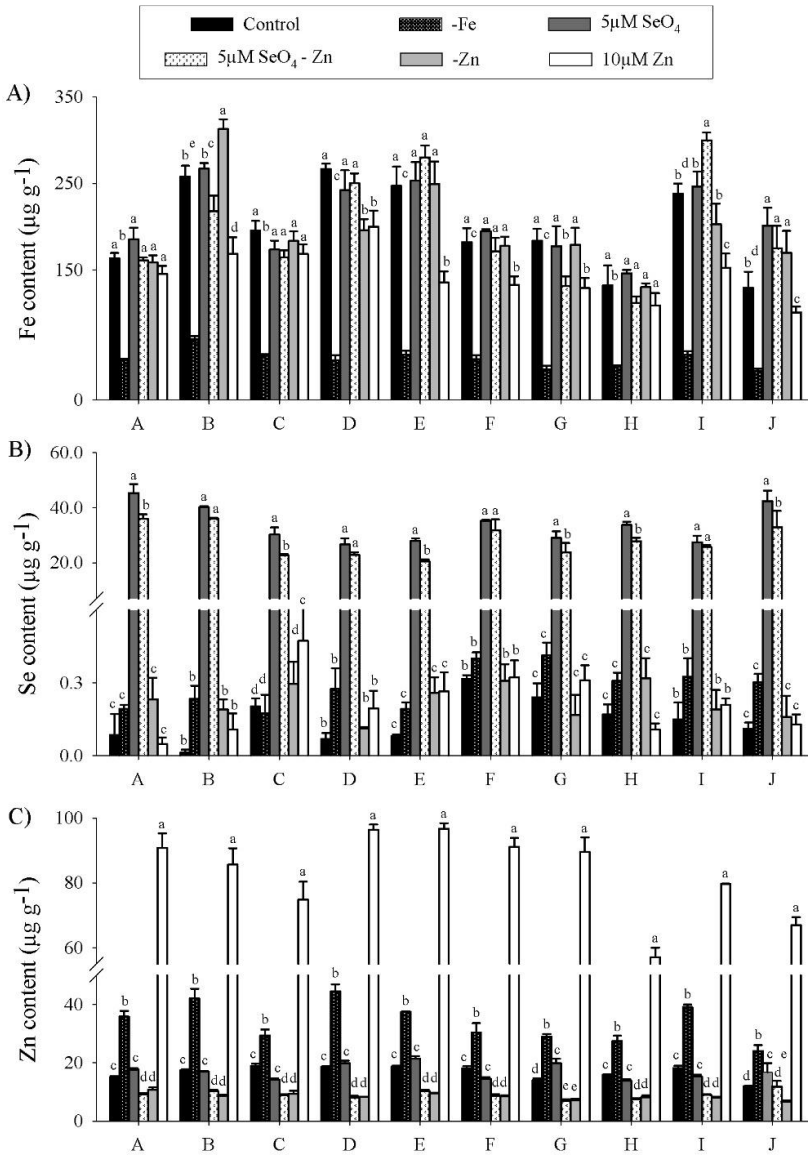

Fig. 2. Total Fe, Se and Zn content in shoot (A, B, C) and roots (D, E, F) of ten common bean cultivars subjected to different treatments of these minerals. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column, for each cultivar, indicate significant difference at $p \leq 0.05$ by Scott-Knott test.

A= cv. BRS Supremo; B= cv. BRS Notável; C= cv. Pérola; D= cv. IPR Tangará; E= cv. BRS Requinte; F= cv. BRS Estilo; G= cv. BRS Pontal; H= cv. BRSMG Madrepérola; I= cv. BRS Grafite; J= cv. BRS Cometa.

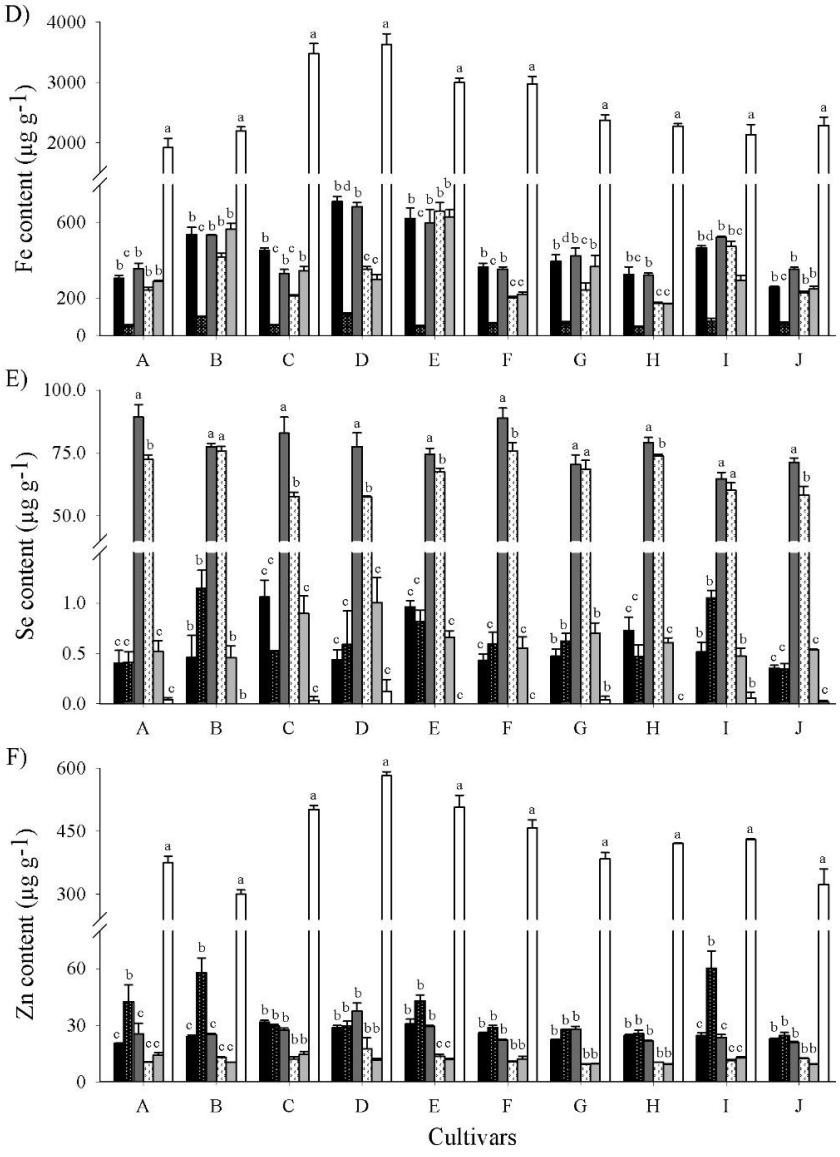

Fig. 2. (*Continued*).

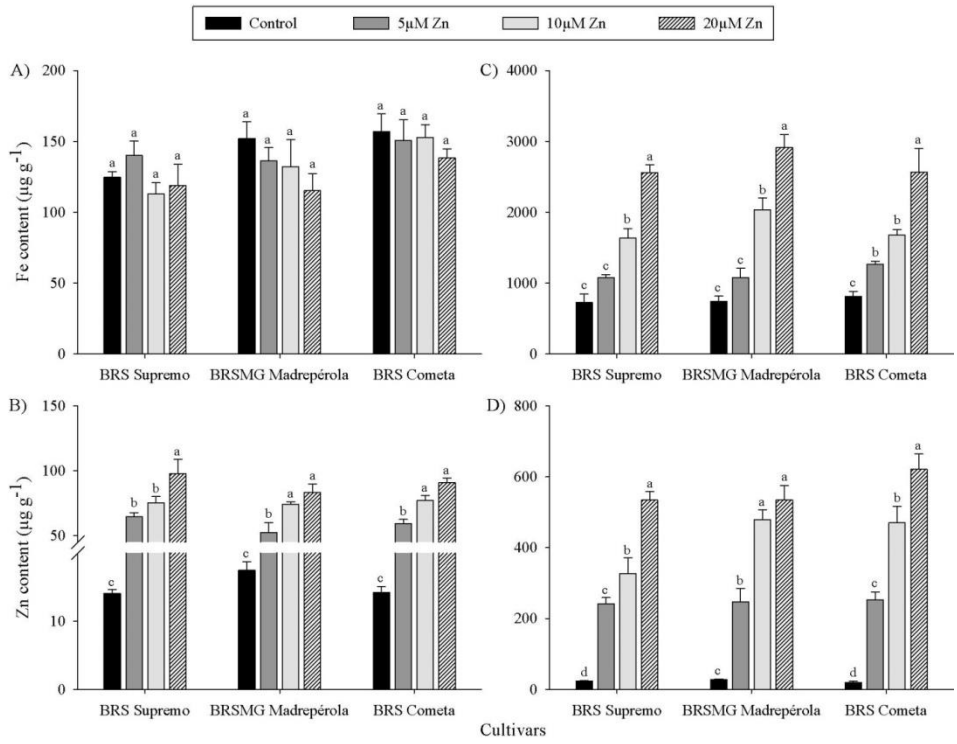

Fig. 3. Total Fe and Zn content in shoot (A, B) and roots (C, D) of three common bean cultivars subjected to different Zn dosages. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column, for each cultivar, indicate significant difference at $p \leq 0.05$ by Scott-Knott test.

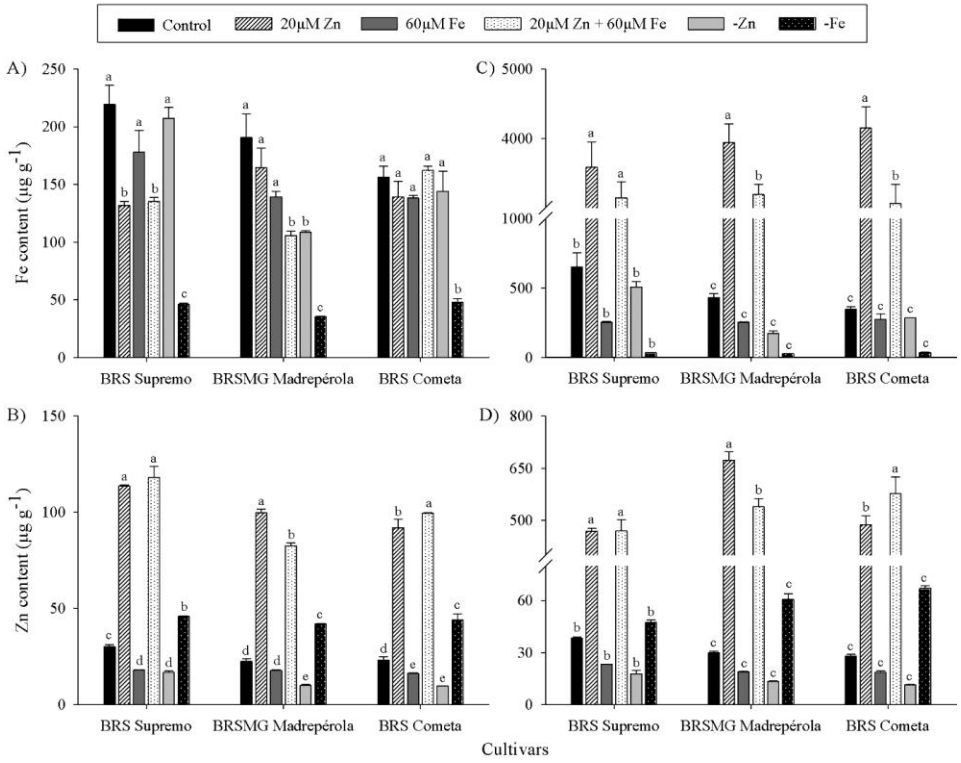

Fig. 4. Total Fe and Zn content in shoot (A, B) and roots (C, D) of three common bean cultivars subjected to different treatments of these minerals. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column, for each cultivar, indicate significant difference at $p \leq 0.05$ by Scott-Knott test.

Solutions: Control (0.365 µM Zn; 30 µM Fe); 20 µM Zn (30 µM Fe); 60 µM Fe (0.365 µM Zn); -Zn (30 µM Fe); -Fe (0.365 µM Zn).

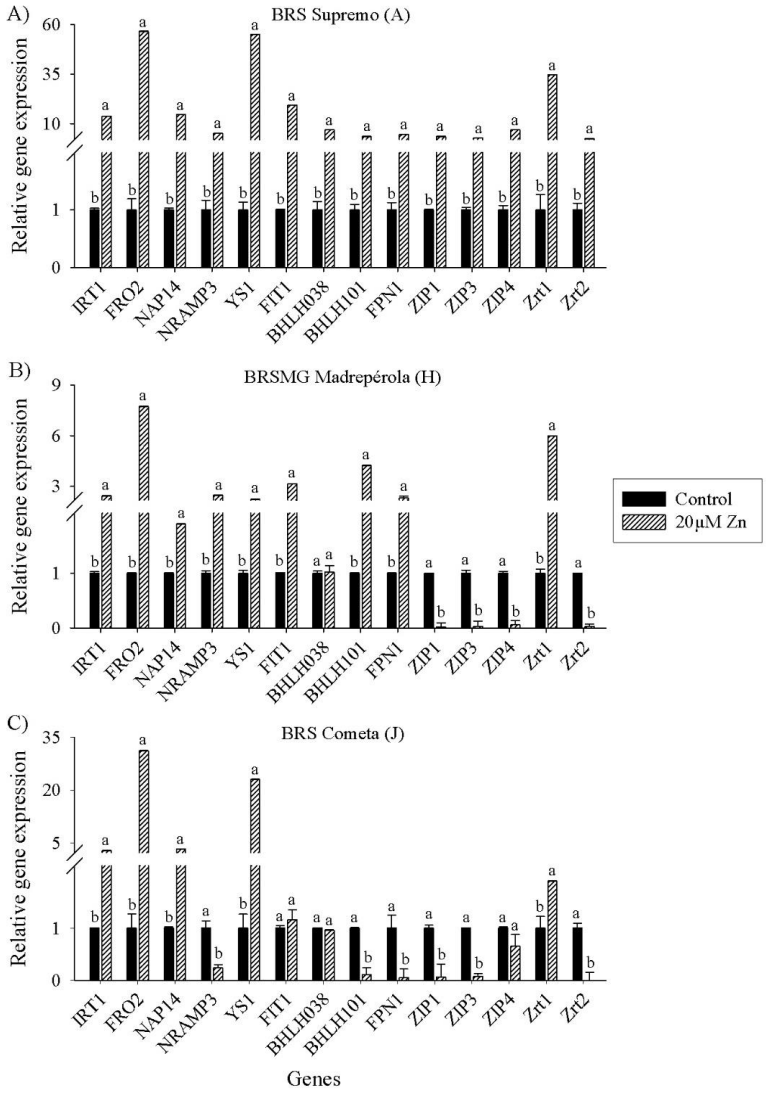

Fig. 5. Relative expression of genes in roots involved in Fe and Zn uptake and transport in plants in three common bean cultivars (BRS Supremo – A; BRSMG Madrepérola – B; BRS Cometa – C). Transcript levels of genes in roots of common bean cultivars treated with or without 20 µM $ZnCl_2$ were measured by qRT-PCR. Data are means of three technical trials with two biological repeats. Error bars indicate standard error of the mean (SEM) (n = 3). Different letters above the column, for each gene, indicate significant difference at $p \leq 0.05$ between plants treated with and without 20 µM $ZnCl_2$ by F test.

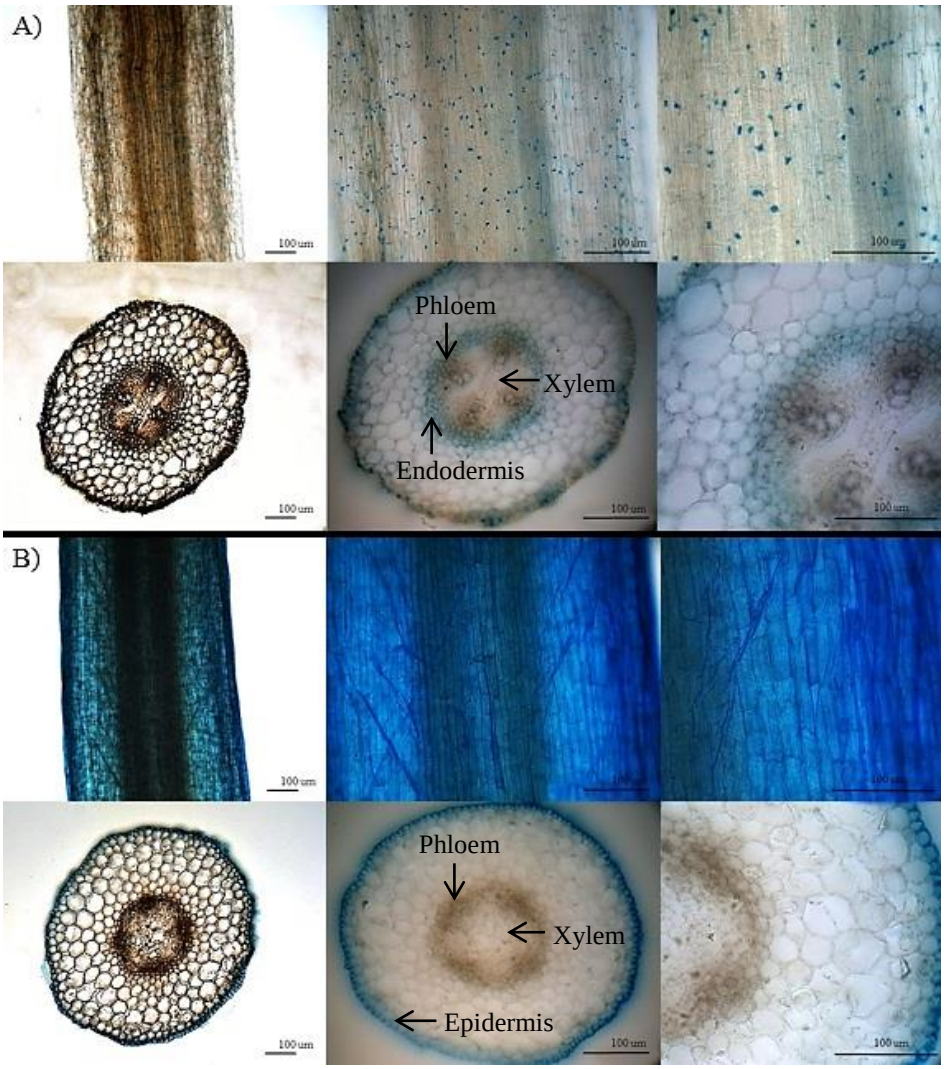

Fig. 6. Iron staining and localization in common bean (cv. BRS Supremo) roots at different objectives (10X – first column; 20X – second column; 40X – third column) and cuts (longitudinal – first and third lines; and transverse – second and fourth lines). The roots were treated without (Control - A) or with 20µM $ZnCl_2$ (B). The blue staining (Perl's) indicates the Fe presence. The Leica DM5500 Epifluorescence microscope was used to get the images.

Table 1
Percentage distribution of $HEDTA^{-3}$ among dissolved cations present in the nutrient solution as estimated by Visual MINTEQ.

	Control solution (0.365 µM Zn)	Zn-enriched solution (20 µM Zn)
$H2\text{-}HEDTA^{-}$	0.587	0.195
$H\text{-}HEDTA^{-2}$	1.274	0.422
$Zn\text{-}HEDTA^{-}$	1.217	66.662
$FeOH\text{-}HEDTA^{-}$	2.342	0.776
Fe-HEDTA (aq)	0.026	0.000
$Cu\text{-}HEDTA^{-}$	0.500	0.500
$Mn\text{-}HEDTA^{-}$	2.146	0.722
$Ca\text{-}HEDTA^{-}$	87.863	29.373
$Mg\text{-}HEDTA^{-}$	4.043	1.340

Table 2
Equilibrated mass distribution of Fe^{3+} and Zn^{2+} (%) in the nutrient solution as estimated by Visual MINTEQ.

	Control solution (0.365 µM Zn)		Zn-enriched solution (20 µM Zn)	
	Dissolved	Precipitated	Dissolved	Precipitated
Fe^{3+}	2.37	97.63	0.79	99.21
Zn^{2+}	100	0	100	0

ARTIGO 3

Adubação via solo e foliar na biofortificação agronômica de cultivares de feijoeiro-comum com zinco

(Artigo elaborado segundo norma NBR 6022 – ABNT 2003)

ADUBAÇÃO VIA SOLO E FOLIAR NA BIOFORTIFICAÇÃO AGRONÔMICA DE CULTIVARES DE FEIJOEIRO-COMUM COM ZINCO

Marislaine Alves de Figueiredo[a*], Dâmiany Pádua Oliveira[b], Luiz Roberto Guimarães Guilherme[b], Messias José Bastos de Andrade[a]

[a] Departamento de Agricultura, Universidade Federal de Lavras, Caixa Postal 3037, 37200-000 Lavras, MG, Brasil; [b] Departamento de Ciência do Solo, Universidade Federal de Lavras, Caixa Postal 3037, 37200-000 Lavras, MG, Brasil

* Autor correspondente: Email: marislaine_alves@yahoo.com.br

E-mails: marislaine_alves@yahoo.com.br (M.A. de Figueiredo); damiany.padua.oliveira@gmail.com (D.P. Oliveira); guilherm@dcs.ufla.br (L.R.G. Guilherme); mandrade@dag.ufla.br (M.J.B de Andrade).

RESUMO

A biofortificação agronômica é importante estratégia para aumentar o teor de nutrientes nos grãos, diminuindo as deficiências nutricionais em humanos, principalmente de ferro (Fe) e zinco (Zn), essenciais para as plantas e para a saúde humana. O feijoeiro-comum, consumido por milhões de pessoas no mundo, é uma cultura promissora para ser biofortificada, sendo uma boa fonte de Fe na dieta humana. Assim, objetivou-se avaliar o efeito da adubação de Zn, via solo e foliar, no teor e acúmulo de Fe e Zn nos grãos, e no rendimento de cultivares de feijoeiro-comum, bem como verificar a quantidade desses micronutrientes suprida pelo feijão biofortificado. Um experimento de campo foi conduzido em Patos de Minas - MG, em um solo com alto teor de fósforo. Foram avaliadas quatro cultivares (BRS Cometa, BRS Requinte, BRS Estilo e BRS Pontal), duas doses de Zn (0 e 4 kg ha^{-1} de Zn) aplicadas no sulco de semeadura e cinco doses de Zn via foliar (0, 100, 200, 400 e 800 g ha^{-1} de Zn) aplicadas no estádio de enchimento dos grãos. O rendimento de grãos e os teores e acúmulos de Fe e Zn nos grãos foram determinados. A biofortificação agronômica de cultivares de feijoeiro-comum com Zn, em solo com alta concentração de P e com nível de Zn fitodisponível considerado adequado, não influencia o rendimento de grãos, mas pode proporcionar maiores produtividades na próxima safra quando os grãos biofortificados forem utilizados como sementes. A aplicação conjunta de Zn via solo e foliar proporciona alto teor de Zn nos grãos das cultivares analisadas, representando 27% da ingestão diária recomendada de Zn. O maior teor de Fe nos grãos de feijão, obtido na cultivar BRS Cometa sem aplicação de Zn via foliar, supre 56% da necessidade diária de Fe. A cultivar BRS Pontal é mais responsiva ao Zn que as cultivares BRS Cometa, BRS Estilo e BRS Requinte. Relação antagônica entre Zn e Fe é verificada com a oferta crescente de Zn via foliar.

Palavras-chave: Fertilização. Micronutrientes. Biofortificação agronômica.

1 INTRODUÇÃO

O feijão comum (*Phaseolus vulgaris* L.), cultivado por pequenos e grandes agricultores, é uma das culturas de relevância social no Brasil, um dos maiores produtores e consumidores desta leguminosa. Juntamente com o arroz, o feijão é alimento básico na dieta da maioria dos brasileiros, formando uma combinação de alta qualidade nutricional. No mundo, aproximadamente 26 milhões de toneladas de feijão seco foram produzidas em 2014, sendo 3,3 milhões de toneladas produzidas no Brasil (FAOSTAT, 2016).

Os grãos de feijão, por serem de baixo custo e ricos em nutrientes, também estão presentes na dieta de grande parte da população de países em desenvolvimento (SIDDIQ; UEBERSAX, 2013). Na América Latina e na África estima-se que mais de 500 milhões de pessoas consomem feijão (CORTÉS et al., 2013). O consumo médio de feijão seco por pessoa no Brasil é de 50 g dia^{-1} (AGRIANUAL, 2015; BROUGHTON et al., 2003; IBGE, 2011). Os maiores valores de consumo diário são encontrados em países da África, como por exemplo, em Rwanda e em áreas rurais do Kenya (Kisii), com cerca de 132 e 181 g $pessoa^{-1}$, respectivamente (BROUGHTON et al., 2003).

A biofortificação agronômica refere-se ao aumento do teor dos nutrientes nas partes comestíveis das plantas por meio do uso de fertilizantes minerais (CAKMAK, 2008; WELCH, 2008). Essa prática é promissora na diminuição de deficiências nutricionais, principalmente de ferro (Fe) e zinco (Zn), que afetam bilhões de pessoas no mundo (KHUSH et al., 2012; STEIN, 2010; WHITE; BROADLEY, 2009). Dessa forma, o enriquecimento dos grãos de feijão com Fe e Zn é altamente viável, tendo em vista a introdução desses micronutrientes na cadeia alimentar e os benefícios proporcionados à saúde humana e às plantas (ABBASPOUR; HURREL; KELISHADI, 2014; BROADLEY et al., 2012; HOTZ; BROWN, 2004). Além disso, o consumo de

feijão tem sido relatado como benéfico no controle e prevenção de algumas doenças como, por exemplo, doenças cardíacas e câncer de cólon (CAMPOS-VEGA et al., 2013; HANGEN; BENNINK, 2002; JENKINS et al., 2012; MUDRYJ; YU; AUKEMA, 2014).

A ingestão diária recomendada (Estimated Average Requirement – EAR) de Fe e Zn varia de 3 a 8,1 e de 2,5 a 9,4 mg pessoa^{-1}, respectivamente, dependendo do gênero e da idade. Mulheres grávidas necessitam de maiores quantidades desses micronutrientes, sendo a ingestão diária recomendada de 23 mg dia^{-1}de Fe e de 10,5 mg dia^{-1} de Zn (INSTITUTE OF MEDICINE, 2006). Assim, levando-se em consideração as necessidades diárias desses nutrientes, estudos sobre biofortificação de grãos de feijão se fazem altamente necessários para suprimento dessa demanda.

Com esse estudo objetivou-se avaliar o efeito da adubação de Zn, via solo e foliar, no teor e acúmulo de Fe e Zn nos grãos, e no rendimento de cultivares de feijoeiro-comum, bem como verificar a quantidade desses micronutrientes suprida pelo consumo humano de feijão biofortificado.

2 MATERIAL E MÉTODOS

Um experimento de campo foi conduzido na safra primavera-verão 2013/2014, em um Latossolo Vermelho distrófico de textura argilosa e alto teor de fósforo (TABELA 1).

Tabela 1 - Resultado da análise química e granulométrica de amostras do solo utilizado, retiradas na camada de 0 a 20 cm de profundidade, antes da adubação, e altitude e coordenadas geográficas de Patos de Minas-MG.

Atributos*	Valores
pH (H_2O)	5,8 (Acidez média)
P-Mehlich-1 (mg dm^{-3})	23,93 (MB)
K (mg dm^{-3})	78,0 (B)
Ca ($cmol_c$ dm^{-3})	2,1 (M)
Mg ($cmol_c$ dm^{-3})	0,9 (M)
S (mg dm^{-3})	12,61 (MB)
Zn (mg dm^{-3})	1,98 (B)
Fe (mg dm^{-3})	41,2 (B)
Mn (mg dm^{-3})	57,88 (A)
Cu (mg dm^{-3})	10,08 (A)
B (mg dm^{-3})	0,10 (MBa)
Al ($cmol_c$ dm^{-3})	0,1 (MBa)
H + Al ($cmol_c$ dm^{-3})	4,52 (M)
SB ($cmol_c$ dm^{-3})	3,2 (M)
MO (dag kg^{-1})	3,99 (M)
V (%)	41,45 (M)
t ($cmol_c$ dm^{-3})	3,3 (M)
T ($cmol_c$ dm^{-3})	7,72 (M)
m (%)	3,03 (MBa)
Argila (dag kg^{-1})	53,0
Silte (dag kg^{-1})	21,0
Areia (dag kg^{-1})	26,0
Altitude (m)	833
Coordenadas geográficas (GPS)	-18,5881; -46,5141

*Análises realizadas no Laboratório de Fertilidade do Solo do Departamento de Ciência do Solo da Universidade Federal de Lavras de acordo com a metodologia da Embrapa (1997). Interpretação de acordo com Ribeiro; Guimarães; Alvarez V. (1999): MB=muito bom, A=alto, B=bom, M=médio, Ba=baixo e MBa=muito baixo.

O estudo foi realizado na estação experimental da Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) em Patos de Minas-MG, localidade que possui clima do tipo Cwa – tropical de altitude, com verão quente e úmido e inverno frio e seco (VIANELLO; ALVES, 1991). Na Figura 1 é apresentado um resumo dos dados de precipitação, temperatura e umidade durante o período de condução do experimento.

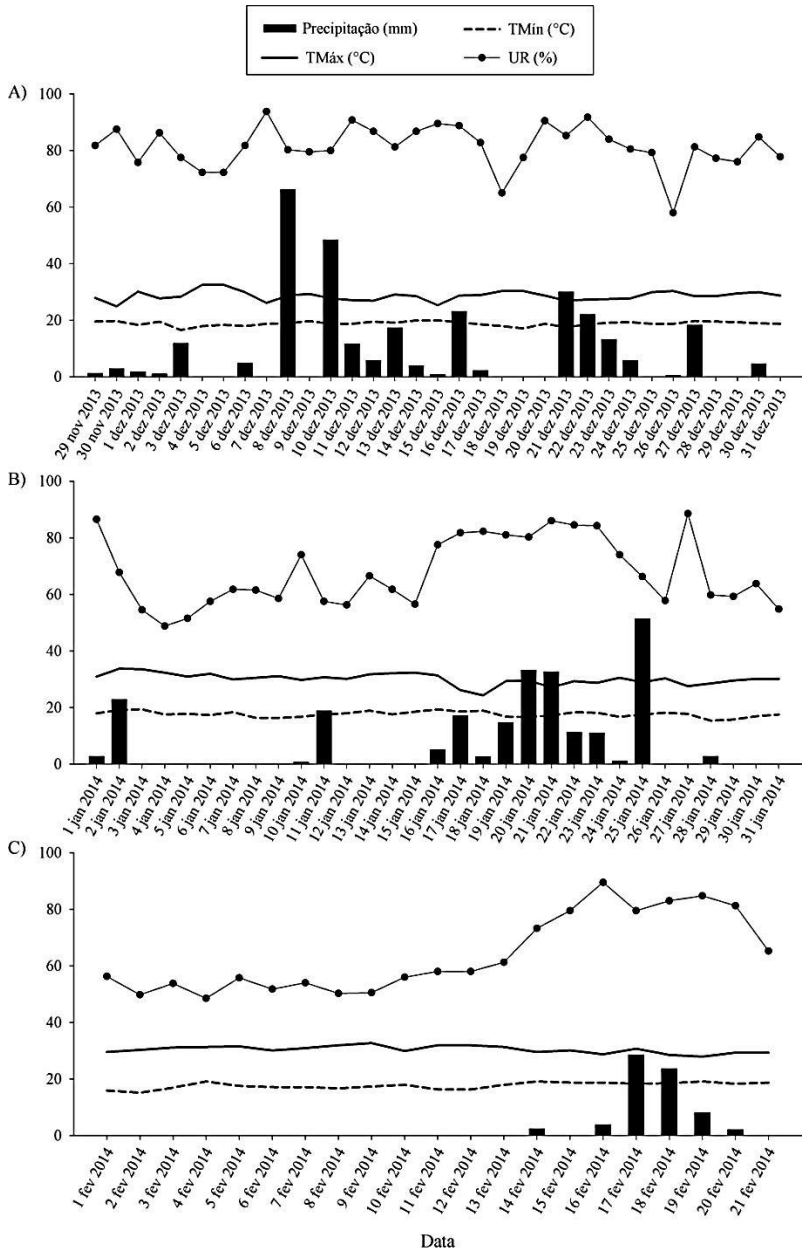

Figura 1 - Variação diária das médias de temperaturas (máxima e mínima), da precipitação pluvial e da umidade relativa entre os meses de novembro e dezembro de 2013 (A), janeiro (B) e fevereiro de 2014 (C), em Patos de Minas. Safra primavera-verão, 2013/14.

Fonte: Instituto Nacional de Meteorologia - INMET, Patos de Minas, 2016

A área de cultivo foi selecionada em função da alta concentração de P no solo, que de acordo com Ribeiro; Guimarães; Alvarez V. (1999), para solos com argila maior que 53 dag kg^{-1}, é considerada alta quando superior a 18 mg dm^{-3}. Adotou-se o sistema de plantio convencional, com a área preparada com uma aração e duas gradagens. O solo havia sido anteriormente cultivado com pastagem.

O delineamento experimental foi em blocos casualizados, com três repetições e os tratamentos dispostos em esquema fatorial 4x2x5, envolvendo quatro cultivares (BRS Cometa, BRS Requinte, BRS Estilo e BRS Pontal), duas doses de Zn aplicadas no sulco de semeadura (0 e 4 kg ha^{-1} de Zn) e cinco doses de Zn via foliar (0, 100, 200, 400 e 800 g ha^{-1} de Zn).

As cultivares de feijão comum utilizadas foram BRS Cometa, BRS Requinte, BRS Estilo e BRS Pontal, selecionadas dentre doze acessos multiplicados na safra outono de 2013, em Patos de Minas, e escolhidas em razão das concentrações de Zn nos grãos. Embora sejam todas do grupo carioca, as cultivares BRS Cometa e BRS Requinte apresentaram maiores concentrações de Zn que as cultivares BRS Estilo e BRS Pontal. Algumas características dessas cultivares são apresentadas na Tabela 2.

Tabela 2 - Principais características das cultivares de feijoeiro-comum em estudo.

Cultivar[1]	**Ciclo**[1] **(dias)**	**Massa de cem grãos**[1]	**Porte**[1]	**Tipo**[1]	**Destaque**[1]	**Teor de Zn no grão**[2] **(mg kg^{-1})**
BRS Cometa	85	25,0	Ereto	II	Precocidade e adaptação à colheita mecânica	38,32
BRS Requinte	90	24,0	Semi-ereto	II/III	Escurecimento tardio de grãos	38,25
BRS Estilo	90	26,0	Ereto	II	Adaptação à colheita mecânica direta e alta produtividade	31,57
BRS Pontal	90	26,1	Prostrado	III	Alta produtividade e resistência à antracnose e ao crestamento bacteriano	31,43

[1]Informações extraídas de Carneiro; Paula Jr.; Borém (2015); [2]Valores obtidos após multiplicação de grãos na safra outono-inverno 2013, em Patos de Minas-MG.

O zinco, fonte sulfato de zinco ($ZnSO_4.7H_2O$, com 20% de Zn), foi aplicado no sulco de semeadura juntamente com a adubação de base. A mesma fonte foi aplicada via foliar no estádio de enchimento dos grãos-R8, aos 60 dias após emergência (DAE). A distribuição da calda foi feita com pulverizador costal manual e o volume de calda foi equivalente a 400 L ha^{-1}.

Cada unidade experimental (12 m^2) foi constituída de 6 linhas de 4 m de comprimento, espaçadas de 0,5 m. As linhas 1 e 6 foram consideradas bordaduras e as linhas centrais, utilizadas para as avaliações. A semeadura foi manual, com densidade de 15 sementes por metro.

Todas as parcelas receberam, nos sulcos, adubação de base equivalente a 30 kg ha^{-1} de N (ureia), 50 kg ha^{-1} de P_2O_5 (superfosfato triplo) e 20 kg ha^{-1} de

K_2O (cloreto de potássio), em função do resultado da análise de solo e da recomendação de Chagas et al. (1999). Em cobertura foram aplicados 40 kg ha^{-1} de N (ureia), metade no estádio V3 e a outra metade em V4. Não foi realizada a calagem.

As plantas daninhas foram controladas por meio de capina manual. Não foi necessário controle de pragas ou doenças. O ensaio recebeu irrigação complementar, por aspersão convencional.

Na maturação-R9 foi determinado o rendimento de grãos (kg ha^{-1}), além do teor (mg kg^{-1}) e acúmulo (g ha^{-1}) de Fe e Zn nos grãos. O rendimento foi obtido a partir da massa total de grãos produzidos na parcela útil, corrigindo-se a umidade para 130 g kg^{-1}. A digestão das amostras foi feita em forno de microondas (Protocolo EPA 3051), baseando-se na metodologia descrita pela Agência de Proteção Ambiental dos Estados Unidos (UNITED STATES ENVIRONMENTAL PROTECTION AGENCY - USEPA, 1998). Os teores foram quantificados em espectrofotômetro de absorção atômica, seguindo metodologia de Malavolta; Vitti; Oliveira (1997). Os acúmulos de Fe e Zn nos grãos foram determinados multiplicando-se o rendimento de grãos pelos respectivos teores.

Os dados foram submetidos à análise de variância com o emprego do software Sisvar versão 4.0 (FERREIRA, 2011), após terem sido previamente submetidos aos testes de normalidade (Teste de Shapiro-Wilks) e de homocedasticidade de variâncias (Teste de Bartlett), utilizando o software R (R DEVELOPMENT CORE TEAM, 2008). Nos casos de efeito significativo de cultivares, realizou-se o agrupamento das médias pelo teste de Scott-Knott ($p \leq 0,05$). Quando houve efeito da aplicação de Zn via solo, a comparação se deu pelo teste F ($p \leq 0,05$), devido ao fator possuir apenas dois níveis. Nos casos de significância das doses de Zn via foliar, recorreu-se à análise de regressão, utilizando-se para a seleção das equações, os critérios de significância do

modelo (teste F), dos seus coeficientes (teste t) e o valor do coeficiente de determinação (R^2).

3 RESULTADOS

Na análise de variância dos dados verificou-se significância dos efeitos principais de cultivares (C) sobre todas as variáveis analisadas ($p \leq 0,05$), da aplicação de Zn via solo (S, $p \leq 0,01$) e de doses de Zn via foliar (F, $p \leq 0,05$) sobre o teor (TZn) e acúmulo de Zn (AZn). Ainda foram significativas as interações CxF sobre o teor (TFe, $p \leq 0,01$) e acúmulo de Fe (AFe, $p \leq 0,05$) e SxF sobre TZn ($p \leq 0,05$). Os coeficientes de variação indicaram boa precisão experimental para experimentos dessa natureza, inferiores a 20%.

As cultivares BRS Cometa e BRS Estilo apresentaram os maiores rendimentos de grãos e AZn, mas não sobressaíram quanto ao TZn, cujos valores médios foram inferiores aos das cultivares BRS Pontal e BRS Requinte (TABELA 3). A cultivar BRS Pontal também apresentou elevado AZn, mesmo com rendimento de grãos pouco menor, e seus valores se equivaleram aos das cultivares BRS Cometa e BRS Estilo.

Tabela 3 - Rendimento, teor e acúmulo de zinco nos grãos de cultivares de feijoeiro-comum.

Cultivares	Rendimento ($kg\ ha^{-1}$)	Teor de Zn ($mg\ kg^{-1}$)	Acúmulo de Zn ($g\ ha^{-1}$)
BRS Cometa	3569 A	36,15 B	128,2 A
BRS Requinte	3109 C	38,04 A	117,8 B
BRS Estilo	3749 A	35,03 B	131,4 A
BRS Pontal	3385 B	38,13 A	128,7 A
Média	3453	36,84	126,5

Médias seguidas pela mesma letra maiúscula na coluna pertencem ao mesmo grupo pelo teste Scott-Knott, ao nível de 5% de probabilidade.

A aplicação de zinco no solo proporcionou acréscimo no AZn. As doses foliares também interferiram nesse acúmulo, ocorrendo aumento nas médias com a elevação da dose de Zn foliar até 576,3 $g\ ha^{-1}$, quando se atingiu o AZn

máximo, de 135,5 g ha^{-1}. A partir dessa dose, entretanto, o acúmulo foi gradativamente reduzido (FIGURA 2).

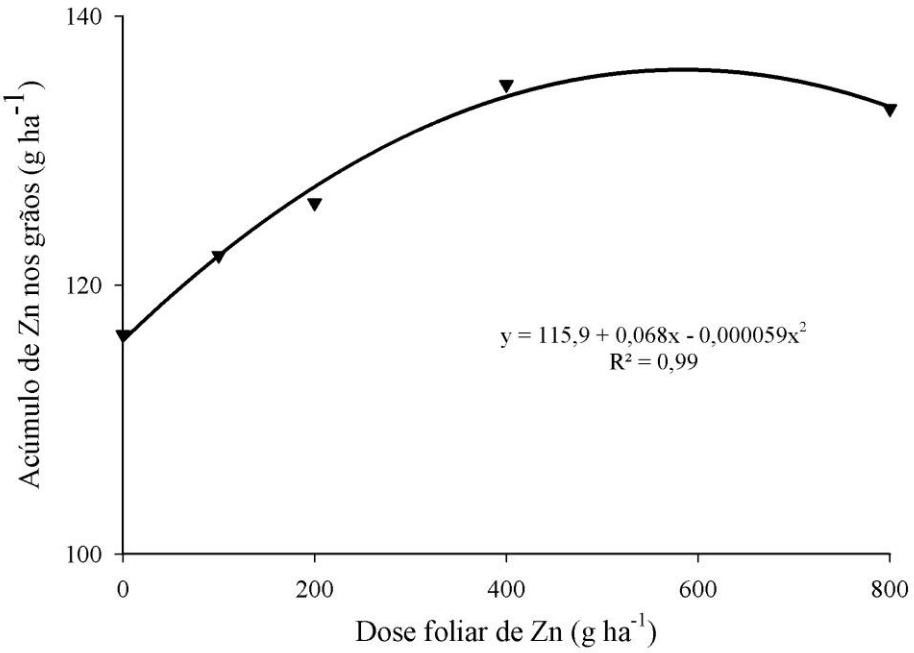

Figura 2 - Acúmulo de zinco nos grãos de feijão comum em função de doses de zinco aplicadas via foliar.

Em relação ao TZn, o efeito da aplicação de Zn no solo foi dependente da dose foliar e somente ocorreu incremento na presença da maior dose foliar de Zn, 800 g ha^{-1}. Do mesmo modo, as respostas às doses foliares estiveram fortemente condicionadas à complementação com o micronutriente no solo, de modo que o aumento da dose de Zn foliar elevou linearmente o TZn quando não se realizou aplicação via solo. Quando essa aplicação ocorreu, o TZn também se elevou, mas houve redução nos ganhos quando a dose foliar excedeu 731,6 g ha^{-1} (FIGURA 3).

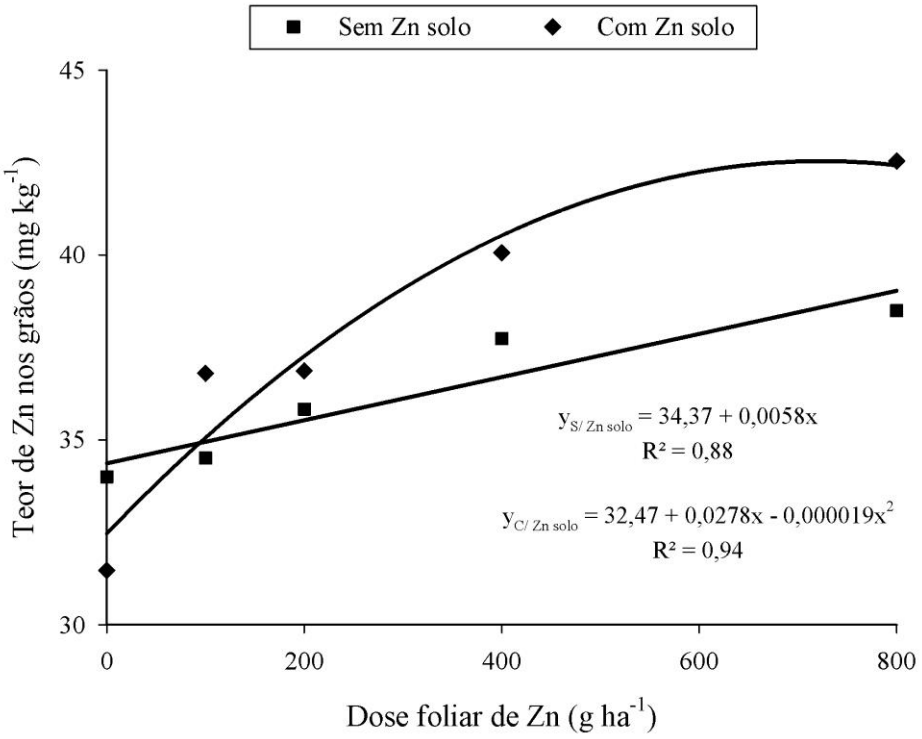

Figura 3 - Teor de zinco nos grãos de feijão comum em função da aplicação ou não de zinco via solo e doses de zinco via foliar.

Os TFe e AFe das cultivares variaram conforme a dose de Zn foliar e, em linhas gerais, dentro de cada dose, a concentração de Fe foi detectada em maior e menor valor nas cultivares BRS Cometa e BRS Estilo, respectivamente (TABELA 4). Apesar do bom comportamento da cultivar BRS Cometa, o incremento da dose de Zn foliar reduziu linearmente o TFe e AFe (FIGURAS 4 e 5). Já a cultivar BRS Pontal respondeu de forma quadrática ao fornecimento contínuo de Zn, atingindo, respectivamente nas doses 431,0 e 383,0 g ha^{-1}, os maiores TFe (74 mg kg^{-1}) e AFe (254,4 g ha^{-1}). O Fe presente nas demais cultivares não apresentou qualquer relação com a dose de Zn foliar.

Tabela 4 - Efeito de cultivares de feijoeiro-comum no teor e acúmulo de ferro nos grãos dentro de cada dose de zinco aplicada via foliar.

Doses de Zn foliar (g ha^{-1})	Teor de Fe (mg kg^{-1})			
	BRS Cometa	BRS Requinte	BRS Estilo	BRS Pontal
0	79,01 a	61,22 b	50,33 b	59,25 b
100	71,88 a	61,84 a	48,66 b	69,69 a
200	77,18 a	56,67 c	54,07 c	65,75 b
400	62,81 b	62,08 b	44,50 c	75,30 a
800	63,14 a	66,21 a	46,66 b	63,51 a
Média	70,80	61,60	48,84	66,70
	Acúmulo de Fe (g ha^{-1})			
	BRS Cometa	BRS Requinte	BRS Estilo	BRS Pontal
0	293,9 a	188,1 b	206,1 b	201,3 b
100	238,4 a	196,4 b	178,3 b	254,1 a
200	274,3 a	181,5 b	204,7 b	217,9 b
400	223,0 a	197,2 b	164,3 b	262,8 a
800	227,1 a	192,6 a	165,5 a	198,7 a
Média	251,3	191,2	183,8	227,0

Dentro de cada fator, médias seguidas pela mesma letra minúscula na linha pertencem ao mesmo grupo pelo teste Scott-Knott, ao nível de 5% de probabilidade.

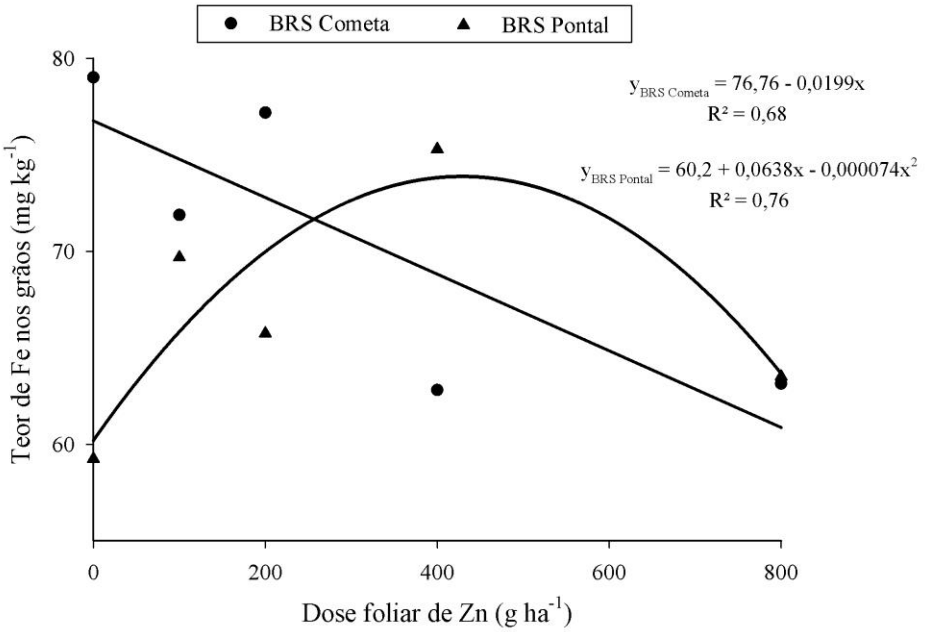

Figura 4 - Teor de ferro nos grãos de cultivares de feijoeiro-comum, BRS Cometa e BRS Pontal, em função de doses de zinco aplicadas via foliar.

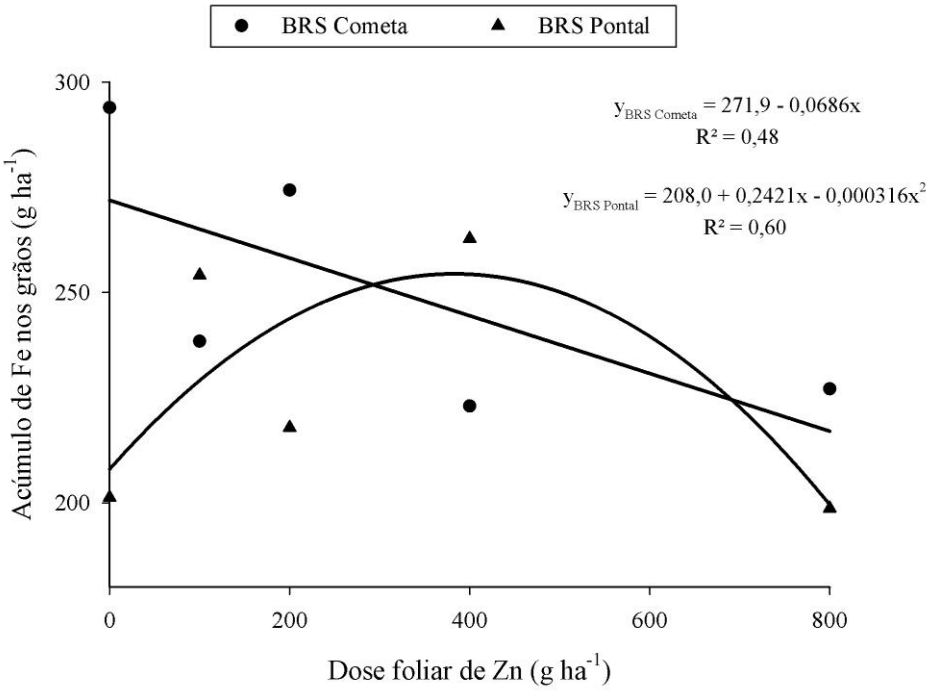

Figura 5 - Acúmulo de ferro nos grãos de cultivares de feijoeiro-comum, BRS Cometa e BRS Pontal, em função de doses de zinco aplicadas via foliar.

4 DISCUSSÃO

O alto rendimento médio obtido nesse estudo (3453 kg ha^{-1}) merece destaque por estar muito acima da média estimada pela Companhia Nacional de Abastecimento (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2014) para a mesma safra primavera-verão 2013/14 em Minas Gerais, da ordem de 1170 kg ha^{-1}. Os maiores valores dessa variável foram registrados nas cultivares de porte ereto tipo II (BRS Cometa e BRS Estilo), certamente compensando a maior produção de flores e/ou vagens esperadas em cultivares de hábito II/III (BRS Requinte) ou III (BRS Pontal), como descrito por Andrade (2010) e Santos et al. (2015). A diferença entre os rendimentos das cultivares foi considerável, alcançando até 11 sacos de 60 kg de feijão. Apesar do destaque dessas cultivares no rendimento e no AZn nos grãos, elas apresentaram os menores valores de TZn, que variaram de 5 a 9%.

O maior valor médio do TZn nos grãos secos (crus) de feijão das cultivares avaliadas nesse estudo foi de 42,64 mg kg^{-1}, obtido quando houve aplicação de Zn no solo juntamente com a dose foliar de Zn de 731,6 g ha^{-1}. Esse teor é 47% maior que o teor médio de Zn encontrado em grãos secos de feijão tipo carioca (29 mg kg^{-1}) (TACO, 2011) e 22,2% maior que o teor médio de Zn (34,9 mg kg^{-1}) encontrado nos grãos de feijão das cultivares analisadas antes da biofortificação agronômica.

O consumo de feijão representa uma grande contribuição para a nutrição humana (BROUGHTON et al., 2003; TARANATHAN; MAHADEVAMMA, 2003). Considerando o consumo médio de feijão seco no Brasil de 50 g $pessoa^{-1}$ dia^{-1} (AGRIANUAL, 2015; BROUGHTON et al., 2003; IBGE, 2011), os grãos de feijão biofortificados obtidos nesse estudo fornecem, em média, 2,13 mg de Zn dia^{-1}, ou seja, quase 27% do Zn médio requerido por um adulto diariamente (8 mg dia^{-1}) (INSTITUTE OF MEDICINE, 2006). Essa estimativa representa

um valor próximo do alvo estipulado por Bouis et al. (2011), que sugere um teor de Zn de 56 µg dia^{-1} de Zn em feijão seco, alcançando um suprimento de aproximadamente 40% da ingestão diária recomendada (EAR).

Aparentemente, a cultivar BRS Pontal foi a mais responsiva ao Zn, o que permitiu a obtenção de TZn mais expressivo que o da semente de origem e resultou em alto AZn. A ocorrência de efeito significativo da dose de Zn foliar sobre o TFe e AFe, confirma a responsividade dessa cultivar, que teve a assimilação de Fe no grão favorecida até à dose foliar de Zn aproximada de 400 g ha^{-1}. Isso mostra que o efeito da adubação de Zn sobre a absorção de Fe varia entre as cultivares e também entre as formas de aplicação de Zn, já que a aplicação no solo não causou o mesmo efeito. Por sua vez, o TZn da cultivar BRS Requinte, que já era elevado mesmo antes da condução do ensaio, não a favoreceu no AZn porque o rendimento de grãos da cultivar situou-se bem abaixo dos das demais.

De modo análogo, as doses foliares provavelmente só reduziram o Fe (TFe e AFe) da cultivar BRS Cometa porque as concentrações iniciais de Zn já eram elevadas, situação em que o incremento de Zn atuou negativamente sobre a absorção do Fe. É interessante ressaltar que, no caso específico da cultivar BRS Cometa e mesmo da cultivar BRS Pontal, prevaleceu a relação antagônica entre Zn e Fe, demonstrando a interferência do Zn na absorção de Fe, o que é frequentemente relatado na literatura (AREF, 2011; OLIVARES; PIZARRO; RUZ, 2007; SILVA; TREVIZAN, 2015; SOUZA et al., 2013).

De acordo com a tabela brasileira de composição de alimentos, o feijão cru tipo carioca possui teor médio de 80 mg de Fe por kg de feijão (TACO, 2011). Considerando a ingestão média diária de feijão seco descrita anteriormente e 79,01 mg kg^{-1} como o maior teor médio de Fe nos grãos de feijão, obtido na cultivar BRS Cometa sem aplicação de Zn via foliar, o consumo desse feijão fornece 3,95 mg de Fe por dia. Isto representa,

aproximadamente, 56% da IDR (EAR) de um adulto (7 mg dia^{-1}) (INSTITUTE OF MEDICINE, 2006), estimativa bem acima da sugerida por Bouis et al. 2011 (30% da EAR).

Apesar da adubação com Zn via solo ou foliar não aumentar a produtividade de grãos, houve um aumento significativo do teor de Zn nos mesmos. Grande parte dos agricultores familiares, principalmente no Brasil, utilizam os grãos colhidos para o próximo plantio (DIDONET, 2013; SILVA; WANDER, 2013), sendo a taxa de utilização de sementes de feijão comum no Brasil de apenas 19% na safra 2013/14 (ASSOCIAÇÃO BRASILEIRA DE SEMENTES E MUDAS, 2016). Assim, a utilização de grãos com maiores teores de nutrientes para semeadura na safra seguinte pode proporcionar plantas mais vigorosas e, consequentemente, mais produtivas (CARVALHO; NAKAGAWA, 2012; TRIGO et al., 1997).

5 CONCLUSÕES

A biofortificação agronômica de cultivares de feijoeiro-comum com Zn, em solo com alta concentração de P e com nível de Zn fitodisponível considerado adequado, não influencia o rendimento de grãos, mas pode proporcionar maiores produtividades na próxima safra quando os grãos biofortificados forem utilizados como sementes.

A aplicação de 4 kg ha^{-1} de Zn via solo e de 731,6 g ha^{-1} de Zn via foliar proporciona o maior teor de Zn nos grãos das cultivares analisadas, representando 27% da ingestão diária recomendada de Zn.

O maior teor de Fe nos grãos de feijão, obtido na cultivar BRS Cometa sem aplicação de Zn via foliar, supre 56% da necessidade diária de Fe.

A cultivar BRS Pontal é mais responsiva ao Zn que as cultivares BRS Cometa, BRS Estilo e BRS Requinte.

Nas cultivares BRS Cometa e BRS Pontal, de maneira geral, ocorre relação antagônica entre Zn e Fe com a aplicação de doses consideráveis de Zn via foliar.

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