FRANKLIN EDUARDO MELO SANTIAGO

SULFUR-SELENIUM INTERACTION ON BIOFORTIFICATION OF ROCKET AND LETTUCE PLANTS

LAVRAS – MG

2019

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para a obtenção do título de Doutor.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Santiago, Franklin Eduardo Melo.
Sulfur-selenium interaction on biofortification of rocket and lettuce plants / Franklin Eduardo Melo Santiago. - 2019.
81 p. : il.

Orientador(a): Maria Ligia de Souza Silva.
Coorientador(a): Luiz Roberto Guimarães Guilherme.
Tese (doutorado) - Universidade Federal de Lavras, 2019.
Bibliografia.

1. Agronomic biofortification. 2. Eruca sativa. 3. Lactuca sativa. I. Silva, Maria Ligia de Souza. II. Guilherme, Luiz Roberto Guimarães. III. Título.

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APROVADA em 15 de Agosto de 2019.

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À Deus por sua presença em todos os momentos.

Aos meus pais Antônia Betânia Santiago Melo e Edvaldo Oliveira Ximenes pelo amor imenso e incondicional.

À minha vó Maria do Socorro Santiago Melo e ao saudoso Tio Ovídio (in memorian) pelo incentivo e inspiração.

Á minha amada esposa, Flávia Santiago, por todo amor e companheirismo.

DEDICO

AGRADECIMENTOS

À Deus, pelo discernimento, carinho e amor, que mesmo imerecido me sustentou até aqui.

À Universidade Federal de Lavras (UFLA), Departamento de Ciência do Solo (DCS) e ao Programa de Pós-Graduação em Ciência do Solo (PPGCS) por proporcionarem toda estrutura e suporte para realização das pesquisas e pela oportunidade desta formação.

À todos os docentes e servidores do PPGCS por criarem um ambiente amigável e estimulante para busca do conhecimento.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado. Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo financiamento das pesquisas.

Ao Instituto Federal de Educação, Ciência e Tecnologia do Piauí (IFPI), pela liberação das atividades docentes para dedicação ao curso de doutorado.

À professora Dra. Maria Ligia de Souza Silva pela confiança e orientação em todas as etapas do doutorado.

Ao professor Ph.D. Luiz Roberto Guimarães Guilherme pela coorientação e colaboração imprescindíveis para conclusão deste trabalho.

À Dra. Li Li pela oportunidade e todos os ensinamentos durante o estágio doutoral na Cornell University-USDA.

Ao amigo Fabrício Ribeiro de Andrade pela ajuda na condução dos experimentos e por toda parceria na pesquisa.

Aos membros da banca examinadora, composta por Dr. Guilherme Lopes, Dra. Cynthia de Oliveira, Dr. Fabricio Willian Ávila, Dr. Milton Ferreira de Moraes pela disponibilidade e contribuições dadas na avaliação deste trabalho.

À toda minha família que através do amor, carinho e incentivo sempre foram a minha maior fonte de inspiração para superar todos os obstáculos da vida.

À Flavia Santiago, minha amada esposa, companheira de todas as horas, por seu amor, carinho e dedicação, que me encoraja a continuar nesta jornada.

À todos os colegas do DCS pelos inúmeros momentos de descontração compartilhados que tornaram inesquecível toda essa temporada em Lavras-MG: Fábio, Soraya, Cristiane, Sara, Bárbara, Fabrício, Ferreira, Jodean, Leandro, Rodrigo, Cristiano, Guilherme, Samara, Aline, Anita e Sérgio.

Enfim, a todos que, direta e indiretamente, contribuíram para a conclusão desta importante caminhada.

Muito obrigado!

RESUMO

O selênio (Se) é essencial à saúde de humanos e animais. Entretanto, uma grande parcela da população mundial sofre com a deficiência de Se. Nesse sentido, a biofortificação agronômica com Se têm-se mostrado uma eficiente abordagem para o aumento da sua ingestão. Apesar de não ser essencial ao metabolismo das plantas, o Se pode conferir benefícios ao desenvolvimento e produção das culturas. Porém, as plantas possuem diferentes capacidades acumulativas e respostas aos efeitos benéficos do Se. Além disso, como análogo do enxofre (S), o Se compartilha das mesmas vias de absorção, translocação e assimilação do S em plantas, que podem afetar a concentração de Se nos tecidos vegetais. Assim, foram realizados dois experimentos em condições de casa de vegetação com os objetivos de: (i) avaliar o efeito da interação Se x S sobre o crescimento, fotossíntese, sistema antioxidante e qualidade nutricional de plantas de rúcula cultivadas sob diferentes tratamentos de Se e S aplicados via solo e foliar; e (ii) comparar o efeito dos tratamentos de Se e S nas respostas de crescimento e bioquímicas entre plantas acumuladoras de Se (rúcula) versus plantas não acumuladoras (alface). Os resultados do primeiro experimento demonstraram que a aplicação foliar de Se foi o método mais eficiente para aumentar a concentração de Se em plantas de rúcula. Apesar do efeito regulador que o S impõe sobre a absorção de Se tenha reduzido a concentração de Se na parte aérea, a adubação sulfatada mostrou-se essencial para manutenção produtiva e qualidade nutricional, além de melhorar as defesas antioxidantes e a atividade fotossintética de plantas de rúcula enriquecidas com Se. Isso indica a importância da manutenção de níveis adequados de S e Se em plantas biofortificadas. Os resultados do segundo experimento indicam que a maior tolerância que a rúcula apresentou ao tratamento com Se em relação à alface pode ser devido à maior capacidade redox no sistema antioxidante, demonstrada pela maior atividade da ascorbato peroxidase (APX) e maior conteúdo de glutationa (GSH) e não-proteico tiols (NPT). Além disso, níveis superiores de Se em proteínas foram observados em plantas de rúcula do que em alface, indicando que as plantas acumuladores de Se podem ter maior tolerância a selenoaminoácidos em proteínas, o que presumivelmente afeta a função normal de enzimas e proteínas.

Palavras-chave: *Eruca sativa*; *Lactuca sativa*; biofortificação agronômica; sulfato; interações iônicas; selenato de sódio; qualidade nutricional; defesas antioxidantes.

ABSTRACT

Selenium (Se) is essential to the humans and animals health. However, yet a great fraction of the world's population suffers from Se deficiency. In this sense, agronomic biofortification with Se has been shown to be an efficient approach to increase Se intake. Although not essential to plant metabolism, Se can confer benefits on crop development and yield. However, plants have different accumulative capacities and responses to Se. In addition, as a sulfur (S) analogue, Se shares uptake, translocation and assimilation pathways with S, which can affects Se concentration in plants. Thus, two experiment were performed under greenhouse conditions aimed to: (i) evaluate the effect of S x Se interactions on growth, photosynthesis, antioxidant system and nutritional quality of rocket plants grown under different S and Se treatments applied via soil and foliar; and (ii) compare the effect of Se and S treatments on growth and biochemical responses between Se accumulative (rocket) versus non-accumulative (lettuce) plants. The results of the first experiment showed that Se foliar application was the most efficient method to increase Se concentration in rocket plants. Although the regulatory effect that S imposes on the Se uptake has reduced shoot Se concentration, S fertilization showed to be essential for maintaining yield and nutritional quality, as well as to improve antioxidant defenses and photosynthetic activity of rocket plants enriched with Se. This fact evidence the importance of maintaining adequate S and Se levels in biofortified plants. The results of the second experiment indicate that the greater tolerance that rocket presented to Se treatment when compared with lettuce, might be due to higher redox capacity, demonstrated by the greater ascorbate peroxidase (APX) activity and higher glutathione (GSH) and non-protein thiols (NPT) contents. In addition, much higher levels of Se in proteins were observed in rocket than lettuce, indicating that Se accumulators might have higher tolerance to selenoamino acids in proteins, which presumably affect the normal function of enzymes and proteins.

Keywords: *Eruca sativa* Mill; *Lactuca sativa*; agronomic biofortification; sulfate; sodium selenate; nutritional quality; antixodant defenses.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Projeções apontam que até o ano de 2050 será necessário dobrar a produção de alimentos para suprir a demanda da crescente população mundial, estimada em 9,5 bilhões de pessoas (ONU, 2018). Para cumprir o desafio de fornecer alimentos em quantidades suficientes, há tempos a agricultura vem lançando mão de tecnologias para aumentar a produtividade das culturas, como o uso de corretivos, fertilizantes, defensivos e cultivares mais produtivas. Entretanto, o ganho em produtividade têm apresentado relação inversa ao conteúdo de minerais e vitaminas nas porções comestíveis das plantas (WHITE; BROADLEY, 2009; LYONS; CAKMAK, 2012).

Mudanças no paradigma da produção de alimentos são necessárias, em que a agricultura não se concentre em apenas produzir mais alimentos, mas também forneça alimentos de melhor qualidade nutricional. Isso contribuirá ainda mais na redução dos altos índices de mortalidade e doenças relacionadas a má-nutrição, que atingem mais de 2 bilhões de pessoas pela ingestão insuficiente de minerais e vitaminas, como ferro, zinco, iodo, selênio e vitamina A (WELCH; GRAHAM, 2004; GRAHAM et al., 2007).

O selênio (Se) é um micronutriente para animais e humanos. A sua atuação no metabolismo dos mamíferos se dá pela síntese de mais de 25 selenoproteínas que desempenham funções importantes nos sistemas antioxidante, imunológico, reprodutivo e hormonal (JONES et al., 2017). Para humanos, a ingestão diária recomendada é de 55-70 μg Se, com tolerância máxima de 400 μg (INSTITUTE OF MEDICINE OF USA, 2000). Níveis de ingestão abaixo do recomendado estão associados a diversas doenças e disfunções fisiológicas, como cardiopatias, doença de Keshan-Beck (osteocondropatia crônica e degenerativa), hipertireoidismo, aumento de infecções virais e desenvolvimento de câncer (RAYMAN, 2012). Estima-se que mais de 1 bilhão de pessoas no mundo sofram com algum grau de deficiência de Se devido ao consumo de alimentos com baixa concentração de Se (WHITE; BROADLEY, 2009; WINKEL et al., 2011).

A concentração de Se nos alimentos está relacionado diretamente à disponibilidade e dinâmica do elemento no solo (COMBS, 2001; KABATA-PENDIAS, 2011). Em geral, o teor disponível de Se na maioria dos solos do mundo são baixos e desuniformes, com média de 0,4 mg kg^{-1}. Por outro lado, áreas seleníferas

podem apresentar concentrações acima de 1.200 mg kg^{-1}. A desuniformidade na geodisponibilidade de Se está condicionada especialmente a composição química do material de origem. Rochas ígneas e metamórficas, em geral, apresentam baixas concentrações de Se, enquanto em rochas sedimentares, tais como arenitos, argilitos, siltitos e folhelhos, apresentam concentrações mais expressivas (KABATA-PENDIAS; MUKHERJEE, 2007). Alguns estudos em solos brasileiros evidenciam desuniformes e baixos teores de Se. Gabos et al. (2014) e Matos et al. (2017) verificaram que solos dos estados de São Paulo e Minas Gerais apresentam, respectivamente, faixas que variam de 0,08 a 1,61 mg dm^{-3} e 0,3 a 5,97 mg dm^{-3} de Se.

O selenato (Se^{6+}) e o selenito (Se^{4+}) são as principais formas inôrganicas de Se encontrados no solo e predominantemente absorvidas pelas plantas (FISHBEIN, 1991; KABATA-PENDIAS; MUKHERJEE, 2007). A dinâmica das espécies químicas de Se no solo está em função de fatores como o potencial redox, pH, teores de óxidos de Fe e Al e pelo conteúdo de argila e matéria orgânica, as quais influenciam diretamente sua disponibilidade para as plantas (WANG; CHEN, 2003). Assim, o selenato é a forma mais solúvel, móvel e biodisponível, uma vez que é fracamente adsorvido pelas superfícies das partículas do solo, especialmente em condições de solos oxidantes e alcalinos. Por outro lado, o selenito é adsorvido mais fortemente via complexo de esfera interna em solos argilosos e com elevadas concentrações de óxidos de Fe e Al, reduzindo sua disponibilidade para as plantas (GOH; LIM, 2004; MONTEIL; LESSA et al., 2016; ARAUJO et al., 2018).

Embora não seja essencial ao metabolismo das plantas, a aplicação de baixas doses de Se têm demonstrado efeitos benéficos sobre o crescimento, produtividade e qualidade das culturas (DJANAGUIRAMAN et al., 2005; RAMOS et al., 2010, SCHIAVON et al., 2017). Acredita-se que grande parte dos benéficios do Se no desenvolvimento vegetal decorra da sua atuação no sistema de defesa antioxidante. Estudos têm observado que a aplicação de Se aumenta a atividade de enzimas antioxidantes, como dismutase do superóxido (SOD), catalase (CAT), ascorbato peroxidase (APX) e peroxidase (POD), que atuam no combate aos danos às membranas celulares ocasionado pelas espécies reativas de oxigênio (EROs) sob condições de estresse (PENNANEN; XUE; HARTIKAINEN, 2002; NAWAZ et al., 2015).

As plantas são classificadas por suas diferenciadas capacidades em acumular

Se nos tecidos em: hiperacumuladoras, acumuladoras secundárias e não-acumuladoras. As hiperacumuladoras acumulam mais que 1000 mg kg^{-1} Se na máteria seca e crescem bem em solos ricos com Se, a exemplo das espécies do gêneros *Astragalus*, *Stanleya*, *Morinda*, *Neptunia*, *Oonopsis* e *Xylorhiza* (KOPSELL; RANDLE; MILL, 2000). As acumuladoras secundárias podem acumular até 1000 mg kg^{-1} Se na máteria seca e apresentam sinais de toxidez em níveis acima disso, a exemplo da *Brassica juncea*, *Medicago sativa*, brócolis e rúcula. Entretanto, a maioria das culturas agrícolas são reconhecidas como não-acumuladoras e não toleram concentrações de Se acima de 100 mg kg^{-1} Se na máteria seca, a exemplo do arroz, trigo, feijão e alface (TERRY et al., 2000; DHILLON; DHILLON, 2003; WHITE et al., 2004).

Considerando a baixa disponibilidade de Se nos solos e sua essencialidade no metabolismo humano, a biofortificação das culturas alimentares é uma eficiente estratégia para mitigar a deficiência de Se na saúde humana (CAKMAK, 2008). A biofortificação consiste no aumento dos teores de Se nas partes comestíveis das plantas, podendo ser realizada através de abordagens genéticas, com seleção de cultivares com maior potencial de absorção e acúmulo de Se, bem como por práticas agronômicas pela utilização de fertilizantes enriquecidos com o Se (WHITE; BROADLEY, 2009).

Países como a Finlândia, Nova Zelândia e Reino Unido foram pioneiros em programas de fertilização com Se no mundo, adicionando-o aos fertilizantes NPK ou por aplicação foliar (ARTHUR, 2003). Com essa iniciativa, esses países conseguiram aumentar o teor de Se no plasma sanguíneo da população e reduzir expressivamente a incidência de doenças relacionadas a sua deficiência (EUROLA et al., 2003). No Brasil, as pesquisas com a biofortificação com Se têm avançado e recentemente o Ministério da Agricultura, Pecuária e Abastecimento publicou a Instrução Normativa - nº 46, a qual definiu que os fertilizantes que contenham Se devem asssegurar teor total mínimo de 30 mg kg^{-1} de Se (BRASIL, 2016).

Nesse sentido, a biofortificação com Se tem sido eficiente para aumentar o teor do elemento em diferentes culturas como arroz (BOLDRIN et al., 2012; ZHANG et al., 2014), milho (CHILIMBA et al., 2012; LONGCHAMP et al., 2015), trigo BROADLEY et al., 2010; GALINHA et al., 2014), alface (RIOS et al., 2008; RAMOS et al., 2011), tomate (PEZZAROSSA et al., 2014; BUSINELLI et al., 2015), brócolis (ÁVILA et al., 2014; BAÑUELOS et al., 2015; MANH, 2017) e rúcula

(DALL'ACQUA et al., 2019).

Para além do aumento do teor de Se, a biofortificação das plantas tem aumentado a qualidade dos alimentos pela maior produção de fitoquímicos e de metabólitos benéficos à saúde (MALAGOLI et al., 2015). Resultados de estudos anteriores indicam que a aplicação de Se contribuiu para o aumento do conteúdo de vitamina C, sólidos solúveis, carotenoides, antocianinas e flavonoides (PEZZAROSSA et al., 2014; LI et al., 2018). Além disso, nas plantas as formas monometiladas de Se, tais como Se-metilselenocisteína e γ-glutamil-Se-metilselenocisteína, atuam como agentes anticarcinogênicos (DONG et al., 2001; ÁVILA et al., 2014; HATFIELD et al., 2014).

A seleção da cultura a ser enriquecida com Se também é um fator-chave para o sucesso dos programas de biofortificação, optando-se por culturas com alto consumo e que componham a alimentação básica da população em geral, a exemplo do arroz, milho e trigo (LYONS; CAKMAK, 2012). Nesse sentido, hortaliças, como alface e rúcula, representam uma valiosa opção para biofortificação, pois além de serem ricas em minerais, flavonoides e vitaminas A, C e E, são também alimentos funcionais com importantes propriedades nutracêuticas que podem ser potencializadas com a aplicação de Se (PUCCINELLI; MALORGIO; PEZZAROSSA, 2017).

O enxofre (S) desempenha papel fundamental na nutrição e produção das culturas agrícolas, constituindo aminoácidos, proteínas e coenzimas, que juntamente com o nitrogênio (N), desempenha importante função na composição do RNA e DNA, controle hormonal e diferenciação celular (DROUX, 2004). Além disso, o S está associado ao sistema de defesa das plantas na resistência a estresses abióticos e bióticos, especialmente na síntese de compostos de defesa que incluem o enxofre elementar, o sulfeto de hidrogênio, a glutationa, as fitoquelatinas, glucosinolatos e vários outros metabólitos secundários (CAPALDI et al., 2015).

Nas plantas, devido à grande similaridade química entre Se e S, o ânion selenato (SeO_4^{2-}) é absorvido, translocado e assimilado pelas vias metabólicas do ânion S (SO_4^{2-}). Assim, o Se pode substituir o S na síntese de aminoácidos como cisteína e metionina, formando selenocisteína e selenometionina. Dessa forma, o sulfato presente na rizosfera pode inibir a absorção de Se pelas raízes por competição direta por transportadores, podendo reduzir significativamente a concentração de Se na parte aérea das plantas (WHITE et al., 2004; SCHIAVON et al., 2015; LIU et al., 2017, GUPTA; GUPTA, 2017). Por outro lado, estudos mostram que baixas

concentrações de Se podem aumentar a absorção de S nas plantas, sugerindo que o aumento da acumulação de S por tratamentos com selenato é devido sua ação em mimetizar a privação de S para estimular a expressão de transportadores de sulfato que são regulados sob deficiência de S (BOLDRIN et al. 2016; CABANNES et al. 2011; HARRIS et al. 2014).

Inúmeros estudos são conduzidos em todo o mundo buscando compreender formas mais eficientes, seguras e viáveis para biofortificação das plantas com Se, com foco na avaliação de fontes, doses e formas de aplicação de Se em diferentes culturas agrícolas. Porém, o conhecimento de alguns fatores que podem influenciar diretamente o processo de enriquecimento das plantas com Se, a exemplo da interação metabólica entre Se e S e os mecanismo de tolerância das plantas ao Se, ainda são escassos e pouco compreendidos. Nesse contexto, os objetivos deste trabalho foram: (i) avaliar o efeito da interação Se x S sobre o crescimento, fotossíntese, sistema antioxidante e qualidade nutricional de plantas de rúcula cultivadas sob diferentes tratamentos de Se e S aplicados via solo e foliar; e (ii) comparar o efeito dos tratamentos de Se e S nas respostas de crescimento e bioquímicas entre plantas acumuladoras de Se (rúcula) versus plantas não acumuladoras (alface).

A tese está dividida em dois capítulos apresentados na forma de artigos para publicação em revistas científicas. No primeiro capítulo, intitulado *Sulfur nutrition enhances photosynthesis, antioxidant activity and nutritional quality of selenium enriched rocket plants,* foram avaliados as respostas de crescimento e bioquímicas de plantas rúcula sob diferentes formas de aplicação de Se na presença ou ausência da aplicação do S ao solo. No segundo capítulo, com o título *Selenium biofortitication differentially affects sulfur metabolism in rocket (Eruca sativa Mill) and lettuce (Lactuca sativa),* foram avaliados os mecanismos básicos de tolerância ao Se entre plantas de rúcula e alface sob tratamentos de Se e S.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Sulfur nutrition enhances photosynthesis, antioxidant activity and nutritional quality of selenium enriched rocket plants

(Formatado de acordo com as normas do periódico *Journal of Plant Nutrition and Soil Science*)

Sulfur nutrition enhances photosynthesis, antioxidant activity and nutritional quality of selenium enriched rocket plants

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Abstract

Selenium (Se) is an essential element for human and animal health, and considered beneficial to higher plants. Selenium uptake in selenate form by plants occurs through sulfur (S) transporters. Therefore, Se accumulation in plants can be significantly affected by the S nutrition. However, there is a lack of information about the growth and biochemical responses of rocket plants to S x Se interaction. This study aimed to evaluate the effect of S x Se interaction on growth, photosynthesis, antioxidant system, and nutritional quality of rocket plants grown under different S and Se treatments applied via soil and foliar. The results showed that Se foliar application was the most efficient method to increase Se concentration in rocket plants. There was a significant regulatory effect of S on the shoot Se concentration. The

combination of S and Se application increased the concentration of vitamin C, soluble solids and reduced titratable acidity. The S x Se interaction improved the antioxidant and photosynthetic system, by increasing the antioxidant enzymes activities that provided protective effect against oxidative damages. Although the regulatory effect that S imposes on the Se uptake has reduced shoot Se concentration, S fertilization showed to be essential for maintaining yield and nutritional quality, as well as to improve antioxidant defenses and photosynthetic activity in rocket plants enriched with Se.

Keywords: *Eruca sativa* Mill, fuctional foods, selenate, sulfur fertilization, ionic interaction

1 Introduction

Selenium (Se) is an essential micronutrient for human and animal health. Selenium is recognized for its specific roles in acting on antioxidant system and in reducing the risk of some types of cancer, such as gastrointestinal and prostate (*Gandin* et al., 2018; *Short* et al., 2018). A large part of the world population suffers from Se nutritional deficiency, which leads to numerous physiological dysfunctions and health problems (*Lyons* and *Cakmak*, 2012).

The low Se intake by the population is related to the consumption of foods containing low Se levels. Selenium concentration in soils is non-uniform and strongly related to organic matter, pH, redox potential, and soil texture (*Reis* et al., 2017). Brazilian tropical soils show a great variation on Se concentration ranging from 0.08 to 1.61 mg Se kg^{-1} (*Gabos* et al., 2014). Agronomic biofortification of crops by the addition of Se fertilizes has been considered a good strategy to mitigate the Se malnutrition in the human diet (*Lyons*, 2018). This technique has been shown to increase Se levels in several foods, such as rice, corn, wheat, lettuce, carrot, tomato, and kale (*Ramos* et al., 2010; *Ávila* et al., 2014; *Pezzarossa* et al., 2014; *Zhang* et al., 2014; *Longchamp* et al., 2015; *Boldrin* et al., 2016).

Agronomic biofortification of crops with Se has gained interest due to the increase of food quality (*Malagoli* et al., 2015; *Puccinelli* et al., 2017). Studies indicate that the nutritive and organoleptic characteristics of fruits and vegetables can be positively influenced by increases in vitamin C, soluble solids, carotenoids, anthocyanins, and flavonoids (*Schiavon* et al., 2013; *Ávila* et al., 2014; *Pezzarossa* et al., 2014; *Li* et al., 2018).

The Se uptake by plants can be influenced by the presence of others elements in the soil, such as sulfur (S). Due to the chemical similarity between S and Se, selenate is uptake and metabolized via sulfate transporters and assimilation pathways, replacing it in the synthesis of amino acids such as cysteine and methionine (*Chang* et al., 2008; *Gupta* and *Gupta*, 2017). The selectivity to selenate or sulfate transports depends on the species and the concentration of these elements in soil and plant (*White,* 2016; *White,* 2018). Thus, the sulfate presents in the rhizosphere may inhibit selenate uptake by roots (*White* et al., 2004; *Schiavon* et al., 2015; *Liu* et al., 2017). In contrast, some studies indicate that Se may increase the sulfate uptake at low concentrations (*Cabannes* et al., 2011; *Harris* et al., 2014; *Boldrin* et al., 2016). The metabolic interaction between S and Se in plants still needs to be better understood, and physiological responses are needs to elucidate how S can affect the nutritional quality of the Se-enriched crops.

Vegetables represent a valuable option to Se-enriched foods, since they comprise a group of functional foods with good health benefits. Rocket (*Eruca sativa* Mill.) is an leaf vegetable belonging to the Brassicaceae family, which has a peculiar spicy taste, rich source of iron, potassium, flavonoids and vitamin A and C (*Kim* et al., 2007; *Martínez-Sánchez* et al., 2008). In addition, rocket plants have large S and Se cumulative capacity and synthesize important phytochemicals, such as methylselenocysteine and selenoglucosinolates (*Chun* et al., 2017; *Wiesner-Reinhold* et al., 2017).

However, the information that guides Se biofortification and the interaction between S and Se in rocket is still scarce. Thus, this study aimed to evaluate how S nutrition affects the growth and biochemical responses of rocket plants biofortified with Se via soil or foliar application.

2 Materials and Methods

2.1 Plant materials and experimental designs

The study was performed under greenhouse conditions at the Department of Soil Science of the Federal University of Lavras, Brazil. Each experimental plot consisted of a pot with 4 kg with Distroferric Red Latossol (Oxisol) collected in the 0-0.2 m depth.

The soil was air dried and sieved in a 4 mm mesh, being chemically and physically characterized according to the methodology proposed by *Embrapa* (2009): pH (H_2O) = 5.0; O.M. = 28.7 g kg^{-1}; phosphorus (Mehlich^{-1}) = 1.13 mg dm^{-3}; potassium = 54 mg dm^{-3}; calcium = 1.5 cmolc dm^{-3}; magnesium = 0.2 cmolc dm^{-3}; sulfur = 9.1 mg dm^{-3}, aluminum = 0.4 cmolc dm^{-3}; H+Al = 6.3 cmolc dm^{-3}; P-rem = 12,93 mg L^{-1}; clay = 710 g kg^{-1}; silt = 140 g kg^{-1} and sand = 150 g kg^{-1}. The Se concentration was 0.2 mg kg^{-1}, determined from the digestion by method USEPA 3051A (*USEPA*, 1998). Liming was applied to raise the base saturation to 70%, calcine lime applied to 24.9% Ca, 8.4% Mg and PRNT of 94.5% and then incubated for 30 days with moisture at 60% the total pore volume.

The experimental design was completely randomized, with six treatments: control (absence of S and Se); S via soil (60 mg kg^{-1}); Se via soil (0.25 mg kg^{-1}); Se via foliar (106 µmol L^{-1}); S via soil (60 mg kg^{-1}) + Se via soil (0.25 mg kg^{-1}) and S via soil (60 mg kg^{-1}) + Se via foliar (106 µmol L^{-1}), with four replications. The sources used were sodium selenate (Sigma-Aldrich, Saint Louis, USA) and calcium sulfate. The rate of Se via soil was based on

the studies of *Ramos* et al. (2012) and *Santiago* et al. (2018) in tropical soils, while for via foliar 12.5% the Se rate applied to the soil was considered.

Treatments with S and Se to the soil application occurred before planting, while the Se foliar application was carried out 35 days after emergence. The foliar application volume was 15 mL per pot. The Se application via foliar did not reach the soil immediately after spraying. Untreated plants with Se solution via foliar application were sprayed with distilled water. Base fertilization consisted of the application of 100 mg of N, 400 mg of P_2O_5, 150 mg of K_2O, 0.5 mg of B, 1.5 mg of Cu, 0.1 mg of Mo e 5.0 mg of Zn per kg^{-1} of soil.

Rocket (*Eruca sativa* Mill.) seeds of commercial variety (Giant Lead Broad) were obtained from Isla Seeds (Poá, Brazil, 100% purity) and used in this study. Seeds were germinated in each pot, at 15 days after emergence (DAE), 12 plants with uniform size were kept. Cover fertilizer was performed with 200 mg of N and 200 mg of K_2O kg^{-1} of soil, divided into three applications. Irrigations of the pots were carried out to keep soil moisture close to field capacity.

Fifty days after the emergency, growth parameters and gas exchange were recorded. The leaves for the chemical, physiological and nutritional analysis were collected.

2.2 Shoot and root biomass analysis

The plants were separated into shoots and roots, and nine plants were individually weighed for their fresh weights. The leaf area were measured by using a scanner (modelo LI-3100C, LiCOR, Nebraska, USA). The roots were washed until completely detached from the soil. Thereafter, shoots and roots of the plants were dried in an oven at 55ºC to a constant mass, and weighed to determine dry mass.

2.3 Photosynthetic pigments and SPAD index

Photosynthetic pigments (chlorophylls and carotenoids) from plant tissues were extracted in 80% acetone solution and analyzed according to the method described by *Arnon* (1949) and *Duke* and *Kenyon* (1986). SPAD index was determined on three leaves per pot using a portable chlorophyll meter (SPAD-502, MINOLTA).

2.4 Gas exchange

Gas exchange was evaluated analyses using a portable infra-red gas exchange analyzer (model IRGA - LICOR 6400, LiCOR, Nebraska, USA). The following parameters were determined: the CO_2 assimilation rate as expressed by area (*A* - µmol CO_2 m^{-2} s^{-1}), transpiration (*E* - mmol H_2O m^{-2} s^{-1}), stomatal conductance (*gs* - mol H_2O m^{-2} s^{-1}), and the internal CO_2 concentration in the substomatal chamber (*Ci*- µmol CO_2 mol air^{-1}). The conditions imposed for the measurements were 1.000 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), which was provided by LED lamps, 380 ppm of CO_2. The mean relative humidity was 65% and room temperature was 24ºC at the time of the evaluations. The readings were performed on cloudy day, between 9 and 11 o'clock in the morning, taking the last fully expanded leaf by default.

2.5 Determination of minerals elements

The shoot dry mass was ground in a Wiley mill, and then 11 elements (nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, boron, iron, zinc, cooper and manganese) were determined according to *Embrapa* (2009). The Se concentration was determined by the USEPA method 3051A (*USEPA*, 1998). For quality analytical control, the analyses were certified using BCR 402 standard (White Clover) (Sigma-Aldrich, Saint Louis, USA), which

presented a minimum of 90% recovery, quantified by atomic absorption spectroscopy with electrothermal atomization in graphite furnace.

2.6 Hydrogen peroxide and lipid peroxidation concentration

Five leaves per pot were collected, immediately frozen in liquid nitrogen and stored in a freezer at -80ºC until use.

Hydrogen peroxide (H_2O_2) concentration was determined by measuring the absorbance at 390 nm, where aliquots of the supernatant were added to reaction medium containing 2.5 mM potassium phosphate buffer (pH 7.0) and 500 mM potassium iodide (*Velikova* et al., 2000). Leaf H_2O_2 concentrations were calculated based on a standard curve, and the results expressed in nmol g^{-1} FW (fresh weight). Lipid peroxidation was determined by the production of thiobarbituric acid (TBA), especially malondialdehyde (MDA), according to *Buege* and *Aust* (1978), and the results were expressed in nmol g^{-1} FW.

2.7 Antioxidant enzymes activity

To quantify the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in the leaves, the extract was obtained from liquid N_2 maceration of 0.2 g of leaves and addition of the buffer (pH 7.8), 0.1 mM EDTA (pH 7.0), 0.01 M ascorbic acid and 10% polyvinylpyrrolidone –PVPP (*Biemelt* et al., 1998).

SOD (EC.1.15.1.1) activity was determined by the ability of the enzyme to inhibit the photoreduction of nitrotetrazolium blue (NBT) (*Giannopolitis* and *Ries*, 1977). Thus, an aliquot of the supernatant was added to the incubation medium composed of 50 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin. The tubes with the reaction medium and samples were illuminated for 7 minutes with a 20 W fluorescent lamp. For the control, the same reaction medium without illumination was

performed. The analysis were carried out at 560 nm, and a unit of SOD corresponded to the ability to inhibit 50% the NBT photoreduction.

CAT (EC 1.11.1.6) was evaluated according to *Havir* and *McHale* (1987). An aliquot of the enzyme extract was added to the incubation medium containing 100 mM potassium phosphate (pH 7.0) previously incubated at 30 °C. Immediately prior to readings, 12.5 mM hydrogen peroxide was added to start the reaction. The activity of this enzyme was determined by the decrease in absorbance at 240 nm for 3 minutes, monitored by the consumption of hydrogen peroxide, and the molar extinction coefficient used was 36 mM^{-1} cm^{-1}.

APX (EC 1.11.1.11) activity was determined by monitoring the oxidation rate of ascorbate at 290 nm for 3 minutes. First, an aliquot of the enzyme extract was added to the incubation buffer, composed of 100 mM potassium phosphate (pH 7.0) and 0.5 mM ascorbic acid, previously incubated at 30 °C. Prior to the analysis, 0.1 mM hydrogen peroxide (H_2O_2) was added (*Nakano* and *Asada*, 1981). The molar extinction coefficient used was of 2.8 mM^{-1} cm^{-1}.

2.8 Vitamin C, brix and titratable acidity measurements

The vitamin C concentration was obtained by colorimetric method using 2,4-dinitrophenylhydrazine according to the method reported (*Strohecher* and *Henning*, 1967). The results was expressed as mg of ascorbic acid 100 g^{-1}. Soluble solids were obtained by directing analysis in the homogenized pulp juice and with digital bench refractometer (Optech model RMT), at room temperature, obtaining ºBrix values. The titratable acidity was determined by the titration method, as described by the Adolfo Lutz Institute (*IAL*, 2008). For the ratio between soluble solids (SS) and titratable acidity (TA) content, the SS/AT ratio was calculated.

2.9 Statistical analysis

Data were submitted to variance analysis ($P<0.05$) and the mean values were compared by the Scott-Knott test, using the statistical software R 3.2.3 (*R Core Team*, 2018). Pearson correlation analysis ($p < 0.05$) was also performed to identify any dependent variables that are correlated directly under the proposed treatments. The "corrplot" package was used to create the heat map, with the "cor" and "cor.mtest" functions to create coefficient and *p*-value matrices, respectively. To facilitate the visualization of significant correlations, asterisks were inserted into the heat map cells.

3 Results

3.1 Shoot and root biomass production

The both shoot and root biomass were increased with the S and Se application, regardless of the Se application form (Fig. 1). It was also observed that the plants submitted only to the Se soil or foliar application in the absence of S did not reduce their shoot and root biomass production in comparison with the control.

3.2 Photosynthetic pigments and SPAD index

The chlorophyll total concentration was increased with Se application in the presence of S. Both forms of Se application resulted in about 3.5 times higher than the control (Fig. 2A). However, the highest carotenoid concentrations were observed with individual application of S or Se. With the addition of S, the foliar Se application was greater than via soil (Fig. 2B).

In the presence of S, the foliar Se application showed higher SPAD index soil Se application (Fig. 2C). However, when Se was applied via foliar without S supplementation, it observed a reduction of 1.2 times in the SPAD index in relation to the same treatment in the presence of S.

3.3 Gas exchange

The Se application in the presence of S significantly increased the net photosynthetic rate (*A*), stomatal conductance (*gs*) and the transpiration rate (*E*) in the leaves. However, Se fertilization via soil and foliar in the absence of S did not improve these parameters, matching the control (Fig. 3A, B, C). Likewise, the S and Se application increased the intercellular concentration of CO_2 (*Ci*) in relation to only Se application (Fig. 3D).

3.4 Minerals elements

Se application via soil and foliar both in absence and presence of S resulted in higher Se concentration in shoot, which was 11 times higher in comparison to the untreated plants (Fig. 4A). Selenium concentration in plants cultivated with only foliar Se application was 2.2 times higher than those received only the Se via soil or combined with the S (Fig. 4A).

Shoot S concentration was higher in the treatments that received the element via soil, regardless of the Se application (Fig. 4B). Consequently, the highest Se/S ratio was obtained in the treatment with Se via foliar in the absence of S, being 1.9 times higher than the application via soil (Fig. 4C). In the treatments with S, the Se application via soil and foliar showed no statistical difference for Se/S ratio, and was on average 9 times lower in comparison to the treatment of Se via foliar (Fig. 4C).

The S and Se application also affected foliar concentration of N, P, K, B, Fe and Zn, but did not influence the uptake of Ca, Mg, Cu, and Mn (Table 1). The S and Se application via foliar increased N accumulation. The only Se application, independently of the form applied, reduced the P accumulate. The K and B accumulation were increased in treatments with only S. Se application, by itself or combined with S, reduced the uptake of Fe in relation to the treatments with S and control. The Zn accumulation was reduced when Se was applied via soil or combined with S.

3.5 Hydrogen peroxide and lipid peroxidation concentration

Hydrogen peroxide (H_2O_2) and lipid peroxidation (MDA) concentrations decreased with Se application (Fig. 5A, B). However, the lowest H_2O_2 and MDA concentrations were observed with the foliar Se application in the absence of S, representing a reduction of 2.7 and 2.0 times in comparison to treatments with only S and control, respectively. In the presence of S, both forms Se application produced H_2O_2 and MDA concentration similar to plants that received only Se via soil.

3.6 Antioxidant enzymes activity

The application of Se and S affected the activity of the antioxidant enzymes SOD and APX (Fig. 6), but did not influence the CAT activity. In the presence of S, the application of Se via soil and foliar increased the activity of SOD being similar to treatments with individual application of Se via foliar (Fig. 6A). On the other hand, when S was applied via soil, the SOD activity was similar to the control and the treatment with only S.

APX activity was 2.1 times higher when Se was applied via foliar in the absence of S in relation to the control and application of only S (Fig. 6B). While in the presence of S there was no difference between the forms of Se application for APX activity, matching the results found for the treatment of Se via soil.

3.7 Nutritionals parameters

Application of S and Se increased the nutritional characteristics of rocket. The highest concentrations of vitamin C and total soluble solids were found in the plants submitted to S supplementation only or when associated with the Se addition, independent of the Se application form (Fig. 7A, B).

In the presence of S and Se, in both forms of Se application, there was a 1.3-fold

reduction of the titratable acidity as compared to only the addition of Se and control (Fig. 7C). Likewise, the total soluble solids/titratable acidity ratio was increased in the treatments with S supplementation to the soil and Se via foliar, being higher in 1.2 times in relation to Se via soil, showing similar values to the treatment with S alone. Plants that received only Se soil and foliar obtained total soluble solids/titratable acidity ratio similar to the control (Fig. 7D).

In order to show the relationship among the growth and biochemistry parameters assayed in response to S and Se treatment, a heatmap was performed (Fig. 8). The application of S and Se clearly showed an augmented relationship with biomass production (SFW, SDW, LA and RDW), photosynthetic parameters (*A*, *E*, *gs*, *Ci*, Chl and SPAD), nutritional quality (Vit. C, SS and TA), and increased SOD and APX activity, and decrease H_2O_2 and MDA, as illustrated in Fig. 8.

4 Discussion

Selenium biofortification should be achieved without damage crop growth and yield, but ideally increasing its production parameters. In this sense, it was verified that the Se rates used in the present study was sufficiently adequate to promote nutritional enrichment with Se without causing toxicity and reducing the shoot and root biomass production (Fig. 1). On the other hand, it was observed that the S natural levels in the soil (9 mg dm^{-3}) were low and limiting to maintain the biomass production in such a way as to present great responsiveness to S fertilization, especially when there was the application of Se via soil or foliar.

The increase in photosynthetic parameters (*A*, *gs*, *E* and *Ci*), chlorophyll content and SPAD index in the plants that received S and Se correlated with the higher accumulation of N (Table 1), which, like S, is directly linked to synthesis of chlorophyll and other pigments (*Droux*, 2004), thus providing greater photosynthetic efficiency (Fig. 2). In addition, it is suggested that the action of S and Se in the antioxidant system provides protection against the

oxidative damage caused by reactive oxygen species (ROS) on chlorophyll biosynthesis (*Jiang* et al., 2017; *Yin* et al., 2019).

In presence of S, there was an effective action of Se on antioxidant system, reflected by the increase in the activity of SOD and APX enzymes and reduction in concentration of H_2O_2 and MDA (Fig. 5 and 6). The results showed the important roles of Se and S in plant resistance to oxidative stress by stimulating the activity of antioxidant enzymes that scavenge ROS and to mitigate oxidative damage to cell membranes (*Capaldi* et al., 2018). *Cheng* et al. (2016) found that S and Se application were important to increase the activity of glutathione peroxidase and catalase and to reduce lipid peroxidation in garlic plants. *Ríos* et al. (2008) and *Ramos* et al. (2010) verified that low levels of Se were effective in combating ROS by increasing SOD and CAT activity in lettuce.

The positive response of rocket plants to Se application (both soil and foliar) shows the benefit effect of Se. In addition, rocket plants showed good ability to accumulate Se in their tissues (Fig. 4A). However, the results showed an intense regulatory effect on the Se uptake and accumulation imposed by S, with a significant reduction of shoot Se concentration, independent of the Se application form. Sulfur and Se share the same pathways of uptake, translocation and metabolization in the plants. Selenate uptake through sulfate transporters can be inhibited by the sulfate present in the rhizosphere, and reduced Se concentration in the plant tissues (*White* et al., 2004; *Cabannes* et al., 2011; *Schiavon* et al., 2013; *Gupta* and *Gupta*, 2017). In this way, maintaining satisfactory levels of Se in biofortified plants is directly related to the need to ensure the proper balance of S:Se in plant nutrition.

Notably the foliar Se application was more efficient than via soil on shoot Se concentration (Fig. 4A). This result was expected, since the good mobility of selenate when applied to the leaves of the plants (*Ros* et al., 2016). Foliar fertilization has been shown to be more effective, safe and viable to promote Se enrichment in cereal crops and vegetables than

via soil (*Deng* et al., 2017; *Lyons*, 2018). In addition to the dynamics of Se with soil constituents, factors such as mineralogy, texture, pH, organic matter and oxy-reduction reactions can greatly affect the availability of Se to plants (*Stroud* et al., 2010; *Liu* et al., 2015; *Lessa* et al., 2016). The soil of the present study, which presents a very clayey texture (71% clay) and high Fe and Al oxides contents (237 and 262 g kg^{-1}, respectively), may have increased Se adsorption on the oxide surfaces, making it less available to plants in treatments with Se application via soil.

Application of S in the soil resulted in satisfactory levels of S in rocket shoot (Fig. 4B). However, the results did not indicate an increase in the concentration of S stimulated by Se, as verified in previous studies in broccoli (*White* et al., 2004; *Tian* et al., 2017), lettuce (*Ramos* et al., 2011) and strawberry (*Santiago* et al., 2018). Sulfur plays an important role in the metabolism and development of Brassicaceae, since important phytochemicals are generated from the S pathway, in particular glucosinolates, which are S rich secondary metabolites that act in the defense of plants and have a chemopreventive value for human health (*Aghajanzadeh* et al., 2014).

Fertilization with S and Se improved the nutritional quality of rocket plants (Fig. 7), suggesting that balanced nutrition with S and Se may have a positive influence on the nutrition and flavor, increasing their nutraceutical and taste properties. The beneficial effects of Se on plant quality is mainly due to its antioxidant action and regulation of carbohydrate metabolism and ethylene biosynthesis (*Malik* et al., 2011; *Pezzarossa* et al., 2014; *Zhu* et al., 2018). *Zhu* et al. (2018) demonstrated that Se application was important to increase the sugar content, vitamin C, amino acids and bioactive substances in tomato fruits.

The use of Se accumulating species in biofortification programs, such as rocket, ensures a greater capacity to transfer the element to human health. The recommended minimum daily intake of Se in adults according to the *Institute of Medicine* (2000) of USA is 55 μg day^{-1}, with

a maximum tolerance of 400 μg day^{-1}. Considering that the average Se concentration for foliar treatment in the absence and presence of S supplementation was 16.2 and 7.8 μg g^{-1}, respectively, and the dry mass content of 10%, the results showed that the consumption of 34 g of rocket leaves provide approximately 55 and 26 μg Se day^{-1}, respectively. Thus, the daily intake of up to 34 g of fresh leaves of rocket Se-biofortified would be within the recommended intake standards, contributing to a safe intake of Se in human health. It is noteworthy that, S fertilization induces a lower Se accumulation in the leaves, the consumption of rocket can be higher in this case, without risk of toxicity.

Finally, the results indicate that although regulatory effect that S imposes on the Se uptake, reduced shoot Se concentration, S fertilization showed to be essential for productive maintenance and nutritional quality, as well as to improve antioxidant defenses and photosynthetic activity of rocket plants enriched with Se. Therefore, adequate levels of S and Se in the plant should be sought as a key factor for the success and efficiency in crops biofortification programs with Se. In this sense, future studies are needed to better understand the relationships between S and Se on the qualitative and nutraceutical properties of foods.

5 Conclusions

The application of Se via foliar was the most efficient method to increase Se concentration to food safety levels in rocket. On the other hand, there was a significant regulatory effect on the Se concentration with S fertilization, emphasizing the importance of maintaining adequate levels of S in Se-biofortified plants.

The application of S and Se enhanced the nutritional quality of rocket plants by increasing vitamin C, soluble solids and decreasing the titratable acidity concentration.

Improvements in the antioxidant and photosynthetic systems were achieved with application of S and Se, especially by the increased activity of antioxidant enzymes (SOD and APX) that provided protective effect against oxidative damages.

Acknowledgments

This work was partial financially supported by a grant from the National Council for Scientific and Technological Development (CNPq). ARR thanks the CNPq for the research fellowship (grant 309380/2017-0).

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Table 1: Accumulation of nutrients in rocket as a function of the application of Se in the presence and absence of S.

Treatments	N	P	K	B	Fe	Zn
	mg kg^{-1}			µg kg^{-1}		
control	67.31±3.75b	5.58±0.53a	64.74±3.51d	61.05±2.92b	2285.65±93.03a	141.12±7.24a
soil S	83.13±2.66a	7.26±0.13a	132.78±3.86 a	75.28±3.06a	1789.76±114.78b	149.27±2.44a
soil Se	83.74±3.73a	2.74±0.55b	86.87±1.07c	26.55±1.76c	306.35±10.76c	128.13±4.63b
foliar Se	82.87±1.32a	3.85±0.32b	96.76±2.64b	65.11±0.99b	364.26±75.12c	135.30±1.68a
soil S + soil Se	75.31±3.88b	5.90±0.15a	110.98±5.11b	33.48±6.12c	412.12±46.07c	122.51±5.40b
soil S + foliar Se	89.65±7.57a	6.87±0.91a	101.06±5.82b	64.46±4.25b	521.37±136.72c	117.13±9.49b

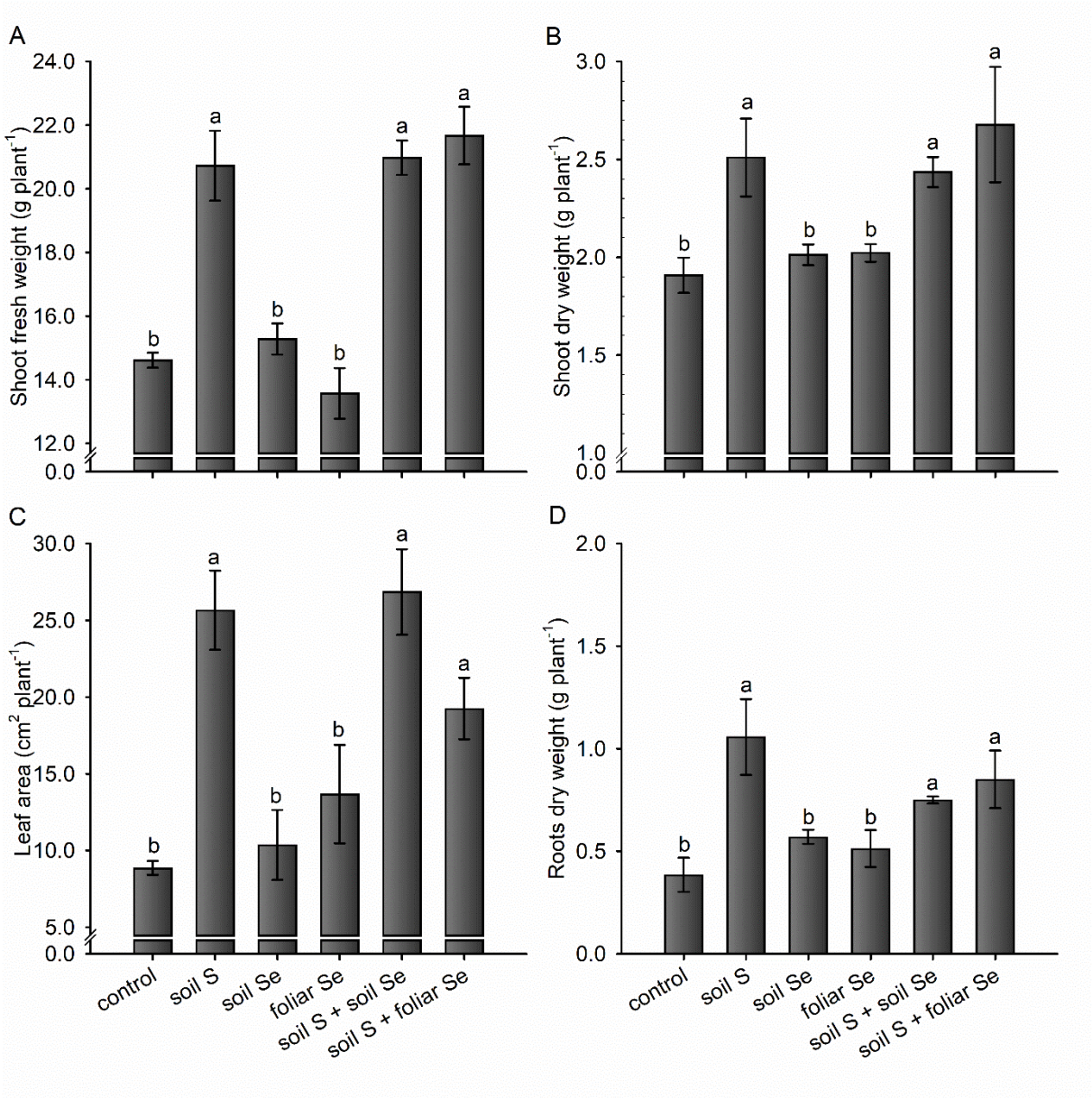

Figure 1: Shoot fresh (A) and dry weight (B), leaf area (C) and root dry weight (D) of rocket as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

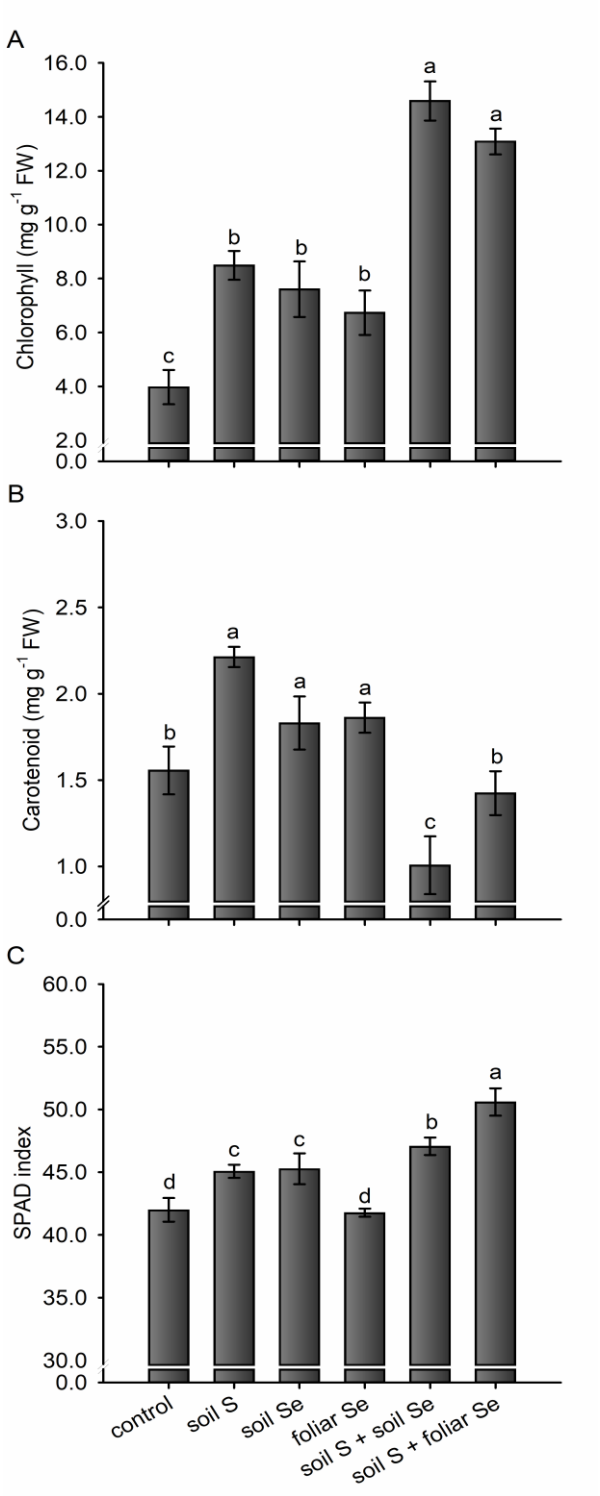

Figure 2: Chlorophyll (A), carotenoid concentration (B) and SPAD index (C) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

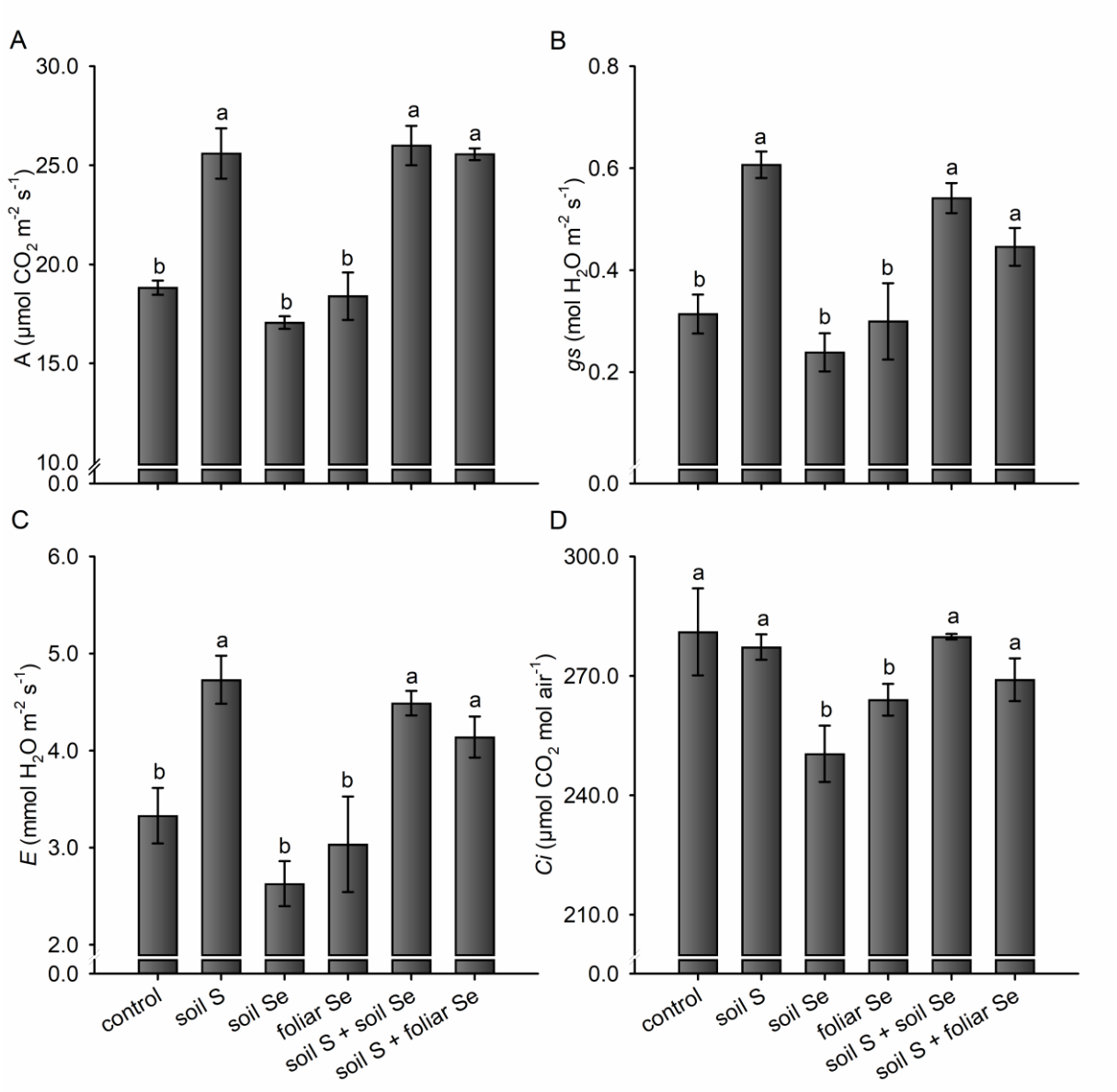

Figure 3: Net photosynthetic rate - A (A), stomatal conductance - *gs* (B), transpiration rate - *E* (C) e substomatal CO_2 concentration – *Ci* (D) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

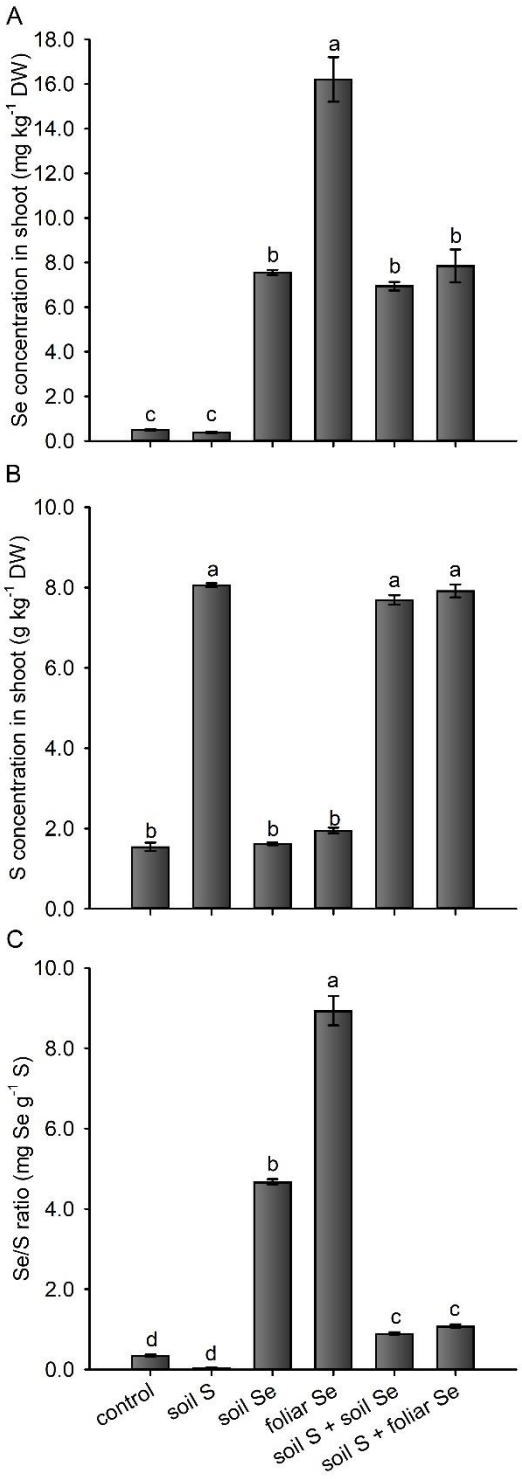

Figure 4: Shoot Se concentration (A), shoot S concentration (B) and Se/S ratio (C) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

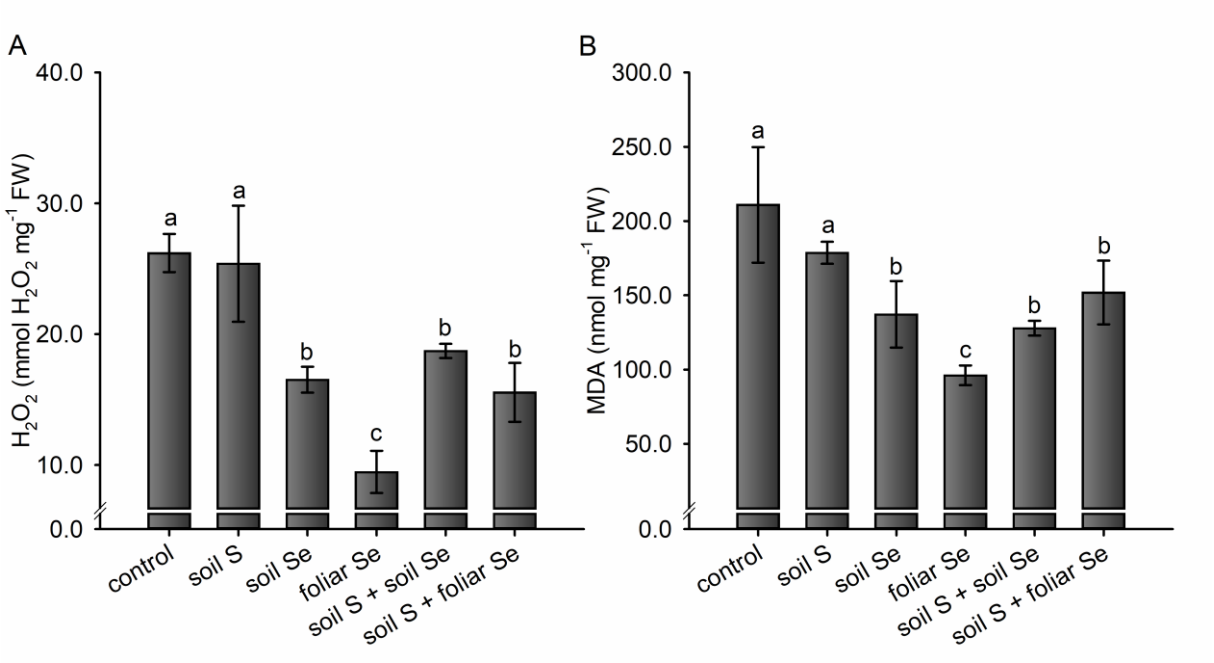

Figure 5: Hydrogen peroxide - H_2O_2 (A) and lipid peroxidation concentration - MDA (B) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

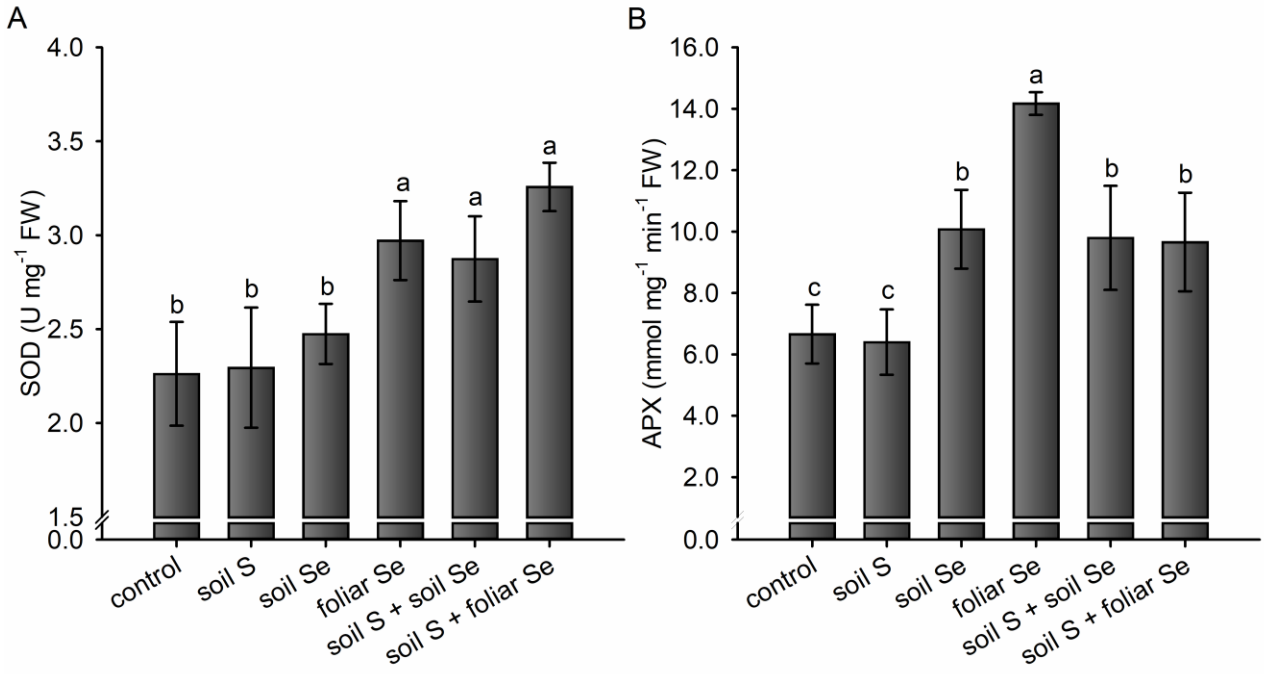

Figure 6: Superoxide dismutase - SOD (A) and ascorbate peroxidase activity - APX (B) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

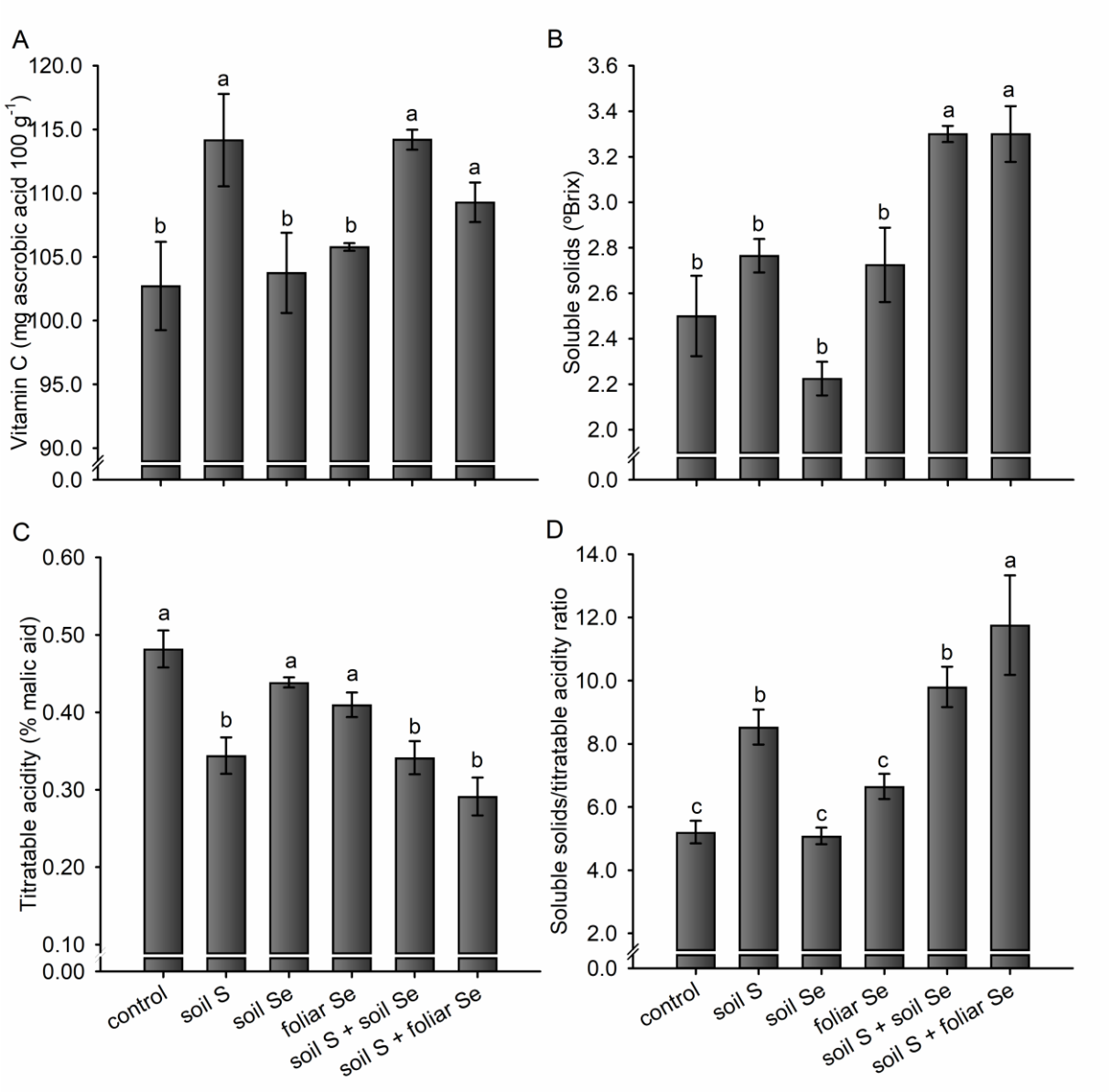

Figure 7: Vitamin C (A), soluble solids (B), titratable acidity concentration (C) and soluble solids/titratable acidity (D) in rocket plants as a function of the application of Se in the presence and absence of S. Means followed by the same letter do not differ (P<0.05) by Scott-Knott test. Bars indicate the standard error of the mean (n = 4).

ccc

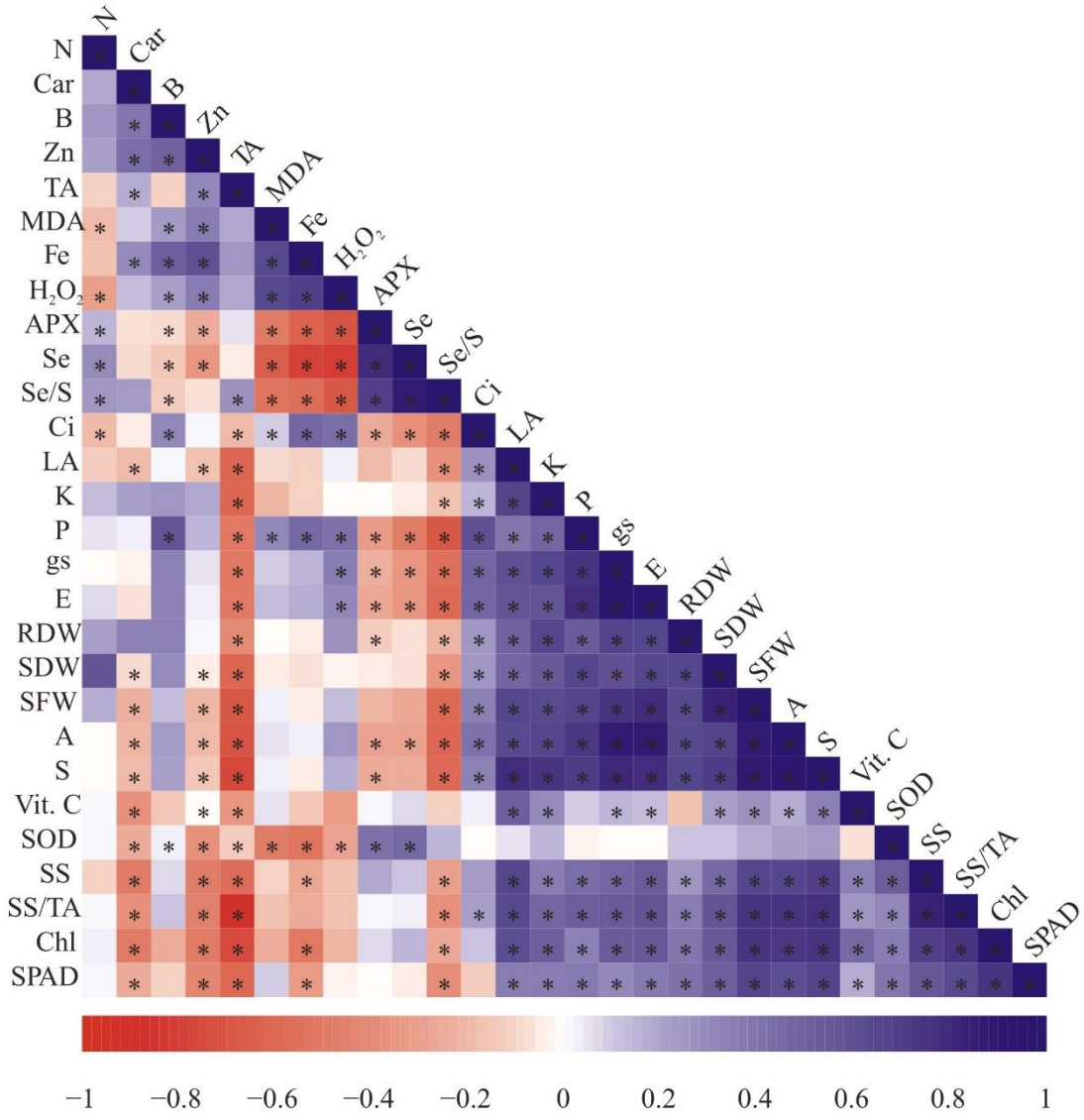

Figure 8: Heatmap showing the Pearson correlations among the growth and biochemistry parameters analyzed in this study in response the S and Se application. * indicates significant relationship (P<0.05). Abbreviations: shoot fresh weight (SFW), shoot dry weight (SDW), root dry weight (RDW), leaf area (LA), chlorophyll (Chl), carotenoid (Car), SPAD index (SPAD), net photosynthetic rate (A), stomatal conductance (gs), transpiration rate (E), intercellular CO_2 concentration (Ci), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), superoxide dismutase (SOD), ascorbate peroxidase (APX), vitamin C (Vit. C), soluble solids (SS), titratable acidity

(TA), selenium (Se), sulfur (S), nitrogen (N), phosphorus (P), potassium (K), boron (B), iron (Fe) and zinc (Zn).

ARTIGO 2

Selenium biofortification differentially affects sulfur metabolism in rocket (*Eruca Sativa* Mill) and lettuce (*Lactuca sativa*)

(Formatado de acordo com as normas do periódico *Frontiers in Plant Science*)

Selenium biofortification differentially affects sulfur metabolism in rocket (*Eruca sativa* Mill) and lettuce (*Lactuca sativa*)

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Abstract

Selenium (Se) is a micronutrient for human health. Plants biofortification is an important strategy for increasing Se intake. However, Se concentration in plants is affected both by the presence of other elements in the growth medium, such as sulfur (S), and by the different Se accumulative capacities. Here, we compared the effect of Se and S treatments on growth and biochemical responses between rocket and lettuce plants seeking to understand the basic mechanisms of greater tolerance to Se of accumulators (rocket) versus non-accumulators (lettuce) plants. We found that under the same Se treatment, rocket obtained higher biomass production, accumulated three-times more Se and S and presented higher antioxidant activity than lettuce plants. We discovery that the higher Se tolerance of rocket compared to lettuce plants is related to higher redox capacity, demonstrated by higher ascorbate peroxidase (APX) activity and higher glutathione (GSH) and non-protein thiols (NPT) content. In addition, our

data also showed that much higher levels of Se in proteins were observed in rocket than lettuce, indicating that Se accumulators might have higher tolerance to selenoamino acids in proteins, which presumably affect the normal function of enzymes and proteins.

Keywords: *Eruca sativa* Mill, *Lactuca sativa*, selenium tolerance, redox capacity, sulfur compounds.

1. Introduction

Worldwide more than 2 billion people suffer from low daily intake of vitamins and minerals like iron, zinc, iodine, selenium and vitamin A (Lyons and Cakmak, 2012). Selenium (Se) performs essential functions in the immune and antioxidant system of humans and animals. Selenium daily intakes below 50 µg may cause numerous Se deficiency-related health problems such as hyperthyroidism, increased viral infection and cancer development (Jones et al., 2017; Rayman, 2012).

Plants are the primary dietary source for Se intake. However, most crops have low Se concentration in the edible portion due to low and non-uniform Se levels in the soil (Reis et al., 2017). Thus Se biofortification in crops is an important and efficient strategy to mitigate Se deficiency as well as provide bioactive Se compounds with beneficial properties for human health (Lyons, 2018).

Although not essential to plant metabolism, Se is recognized for its beneficial effects on plant growth and quality (Malagoli et al., 2015). Plants have different capacities to accumulate and metabolize Se. Most crops are non-accumulative and do not tolerate Se tissue concentrations higher than 100 µg g^{-1} dry weight, such as rice, wheat and lettuce. However, species belonging to Brassicaceae (Cruciferae) are typically good Se accumulators and able to

tolerate Se concentrations up to 1000 μg g^{-1} dry weight, such as broccoli and rocket (Gupta and Gupta, 2017).

Selenium uptake by plants is preferably in selenate form. Selenium as a sulfur (S) analogue shares the sulfate uptake, translocation and assimilation pathways (Liu et al., 2017; Schiavon et al., 2015; White et al., 2004). Thus selenate competes with sulfate for transporters as well as replacing in amino acids synthesis, using a number of key enzymes in S assimilation pathway, such as ATP-sulfurylase (ATPS), APS reductase (APR), serine acetyltransferase (SAT) and O-acetylserine (OAS) (White, 2018).

Crop selection is crucial to the success of Se biofortification programs. In addition to opting for high consumption crops, it should be taken into consideration the nutraceutical properties that can be enhanced with Se enrichment. In this sense, vegetables have great potential for functional foods production when biofortified with Se (Puccinelli et al., 2017). Rocket and lettuce are vegetables widely consumed in many parts of the world. Besides their distinct ability to accumulate S/Se, they are foods rich in minerals such as iron and potassium, flavonoids and vitamins A, C and E (Chun et al., 2017; Ramos et al., 2011; Wiesner-Reinhold et al., 2017).

In this study, we compared the effect of Se and S treatments on growth and biochemical responses between rocket and lettuce plants seeking to understand the basic mechanisms of greater tolerance to Se of accumulators (rocket) versus non-accumulators (lettuce) plants.

2. Material and Methods

2.1. *Plant materials and experiment designs*

Seeds of two commercial varieties of rocket (Rocket I and II) and lettuce (Bibb and Parris) were obtained from Harris Seeds (Rochester, NY, United States) and used in this study. Seeds were germinated in trays with soil mix (Metro-Mix 360, Sun Gro Horticulture) for 10 days in a greenhouse at 23–25ºC with a 14-h light and 10-h dark photoperiod. The young

seedlings were then transferred into a container with Hoagland solution (Hoagland and Arnon, 1938) for conditioning under constant aeration. After conditioning for 7 days, uniform seedlings were transferred into 2.2 L black pots containing the Hoagland solution with five treatments containing increased levels of selenate (Na_2SeO_4) and sulfate ($MgSO_4$) in nutrient solution (0, 10/2, 20/2, 0/5 and 20/5 (µM Se/ mM S) and grown in the same greenhouse. The nutrient solutions were changed twice every week. After 2 weeks of treatments, 60 plants (2 species x 2 varieties × 5 treatments × 3 biological repeats) were harvested individually and separated into shoots and roots. After recording the fresh weight, the samples were either dried for mineral analysis or immediately frozen in liquid nitrogen and stored at −80◦C for RNA extraction and enzyme activity analysis.

2.2. *Total Se and S level analysis*

The ionomics of rocket and lettuce leaf tissues containing a total 26 elements including Se and S were determined using an inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA, United States) essentially as described previously (Tian et al., 2017). Briefly, the dried tissues (approximately 100 mg) were weighed into borosilicate glass tubes, acid- digested in 2.0 mL of H_3NO_3 with 2.0 mL of $HClO_4$ at 120ºC for 1 h, and then at 220ºC until $HClO_4$ fumes were observed. The digested samples were solubilized with 20 mL of 18 MΩ water before analysis. Three biological replicates were analyzed.

2.3. *ATPS and OASTL activity assays*

ATP sulfurylase (ATPS, EC 2.7.7.4) activity was measured using molybdate dependent formation of pyrophosphate as described by Lappartient and Touraine (2016). Samples (100 mg) were ground in a mortar at 4 °C with 20 mM Tris-HCl (pH8.0) containing 10 mM EDTA,

2 mM DTT, and 0.01g/mL polyvinylpyrrolidone (PVP).The homogenate was centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was used for ATPS assays. The reaction was initiated by adding 0.1 mL of crude extract to 0.5 mL of the reaction mixture containing 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 U/mL of sulfate-free inorganic pyrophosphatase (Sigma) in 80 mM Tris-HC1 buffer (pH8.0). Another aliquot from the same extract was added to the same reaction mixture without Na_2MoO_4. The mixture was incubated side by side at 37°C for 15 min, and phosphate was then determined colorimetrically. The ATPS-dependent formation of pyrophosphate was determined from the difference between the two measurements.

O-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8) activity was measured according to the method by Riemenschneider et al. (2005). Samples (100 mg) were ground in 1 mL of ice-cold 20 mM Tris-HCl (pH8.0). The extract was then centrifuged at 13,000 g for 10 min at 4°C. The reaction mixture contained a total volume of 1mL of 5 mM OAS, 5 mM Na_2S, 33.4 mM DTT, 100 mM Tris-HCl (pH7.5) and 50 mL of the enzyme extract. The reaction was initiated by adding Na_2S and incubated for 30 min at 37°C. The reaction was then terminated by adding 1 mL of acidic ninhydrin reagent (0.8% ninhydrin [w/v] in 1:4 concentrated HCl:HOAc) to determine Cys concentration. The samples were heated at 100°C for 10 min for color development and then cooled on ice. The color complex was stabilized by adding 900 mL of 70% ethanol to 100 mL of the samples. Absorbance was determined at 560 nm.

2.4. *Analysis of free amino acids*

Levels of free amino acids were analyzed according to a method described previously with some modifications Ramos et al. (2011). Free amino acids were extracted overnight from freeze-dried tissues (25 mg) at 4°C in 50 mM HCl (20:1, v/w). The mixtures were centrifuged at 12,000g for 15 min, and the extracted amino acids in the supernatants were tagged using the

AccQ·Tag Ultra UPLC derivatization kit according to the manufacturer's instruction (Waters). Derivatized amino acids were analyzed on an Acquity UPLC system (Waters) using an AccQ·Tag Ultra column (100 × 2.1 mm). Free amino acids were identified on the basis of coelution with Pierce Amino Acid Standard H (Thermo Scientific). The total contents were calculated on the basis of peak areas and calibration curves generated with commercial standards.

2.5. *Analysis of Se levels in proteins*

Selenium in proteins was extracted and analyzed following the method as described by Wu et al. (2014). Frozen fresh tissues (0.1 g) were ground into powder and extracted using 0.5 mL of cold (−20ºC) trichloroacetic acid (TCA)/acetone (0.1 g mL^{-1}). The homogenized samples were centrifuged at 12,000 g for 5 min at 4ºC to collect the precipitated proteins. The supernatant was discarded and the pellets were resuspended in 0.1 mL of cold TCA/acetone (−20ºC) followed by centrifugation again. This step was repeated one more time. The final pellets were resuspended in 1.5 mL 1% SDS, and incubated between 60 and 70ºC for 1–2 h. Se levels in proteins were analyzed by ICP. The experiment was repeated with three biological replicates for all samples.

2.6. *Analysis of antioxidant enzyme activities*

To quantify the activity of the antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) in the leaves, the extract was obtained from liquid N_2 maceration of 0.2 g of leaves and addition of the extraction buffer containing 100 mM KH_2PO_4 (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 M ascorbic acid and 10% polyvinylpyrrolidone (PVPP) (Biemelt et al., 1998). The mixture was kept on ice for 10 min,

followed by centrifugation at 12,000 g for 10 min at 4ºC. The supernatant was used immediately for enzyme activity assays.

SOD (EC.1.15.1.1) activity was determined by the ability of the enzyme to inhibit the photoreduction of nitrobluetetrazolium (NBT) (Giannopolitis and Ries, 1977). Thus, an aliquot of the supernatant was added to the incubation medium composed of 50 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin. The tubes with the reaction medium and samples were illuminated for 7 minutes with a 20 W fluorescent lamp. For the control, the same reaction medium without illumination was performed. The readings were carried out at 560 nm, and a unit of SOD corresponded to the ability to inhibit 50% the NBT photoreduction.

APX (EC 1.11.1.11) activity was determined by monitoring the oxidation rate of ascorbate. First, an aliquot of the enzyme extract was added to the incubation buffer, composed of 100 mM potassium phosphate (pH 7.0) and 0.5 mM ascorbic acid, previously incubated at 30 °C (Nakano and Asada, 1981). The reaction was initiated by adding 0.1 mM hydrogen peroxide (H_2O_2), and the decrease in absorbance at 290 nm for 3 minutes was recorded. The enzyme activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

2.7. *Determination of non-enzymatic antioxidants*

Glutathione (GSH) was determined by the method described previously Rahman et al. (2007). Briefly, leaves tissues (150 mg) were homogenized in 5 ml 5% (v/v) TCA and immediately centrifuged at 11,000 rpm for 20 min at 4°C. Then 0.1 ml supernatant was mixed with 0.9 ml 150 mM phosphate buffer (pH 7.8), 2 ml 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DNTB) thoroughly. The reaction was terminated after 10 min and the absorbance was analyzed at 412 nm. Standard curve was used to calculate the GSH concentration.

Non-protein thilols (NPT) content was measured following the method of Ellman (1959). Shoot material (700 mg) was homogenized in 3 ml of 6.67% 50-sulfosalicylic acid and centrifuged at 13,000 g for 10 min at 4°C. NPT content was measured in the supernatant by diluting it with Ellman reagent (1:9) (containing 5 mM EDTA, 0.6 mM DTNB in 120 mM phosphate buffer; pH 7.5). After 15 min, absorbance was recorded at 412 nm. NPT content was calculated using GSH as standard.

2.8. *Statistical analysis*

The dates were submitted to variance analysis ($p<0.05$) and the mean values were compared by the Scott-Knott test, using the statistical software R 3.2.3 (*R Core Team*, 2018).

3. Results

3.1. *Effect of Se and S treatments on plant growth*

Se treatments affect biomass production in plants but this response is quite different between accumulators and non-accumulators. To examine rocket and lettuce growth response to different Se and S treatments, two cultivars for each species were grown in Hoagland nutrient solution and treated with increased levels of Na_2SeO_4 and $MgSO_4$. As shown in Figure 1A and 1B, the biomass production of both species varied in response to Se and S treatments. Rocket plants increased the shoot biomass production when treated with up to 20 µM Se. However, there was great reduction in growth for the same Se dose (20 µM) when 5 mM S was added in nutrient solution (Figure 1A). Lettuce plants increased biomass production up to 10 µM Se, and higher Se levels in nutrient solution was associated with a decline in shoot biomass (Figure 1B). These results evidenced a higher tolerance of rocket plants to higher Se levels in growth solution than lettuce plants.

3.2. *Se and S concentration in response to Se and S treatments*

Selenium/sulfur accumulators and non-accumulators have different capacities to accumulate Se and S in tissues. As expected, rocket plants showed over 3.5-fold more S concentration compared to lettuce (Figure 2A and 2B). The S concentration in shoots of both species was enhanced with increased level of Se application. Over 2-fold increase in total S concentration was observed with the addition of 20 µM Se for the two species in comparison with the control. Additional S supplement significantly increased Rocket II S contents but caused no significant changes in the other rocket variety and in lettuce, indicating genotypic variation in rocket varieties and a saturated S concentration in the nutrition solution for lettuce.

The total Se concentration in shoots of both species increased with increased Se doses (Figure 2C and 2D). Noticeably, the two species present a great difference in the Se accumulation capacity, with the rocket accumulating 3-fold more Se than lettuce when treated with the same Se doses. However, when S was applied at higher levels in nutrient solution, there were dramatic reductions of the total Se concentration for both species. Furthermore, in presence of high S treatments lettuce reduced the Se concentration by 6-fold while the rocket reduced only 3-fold. The Se/S ratio consequently reflected the same trend for both species (Figure 2E and 2F). The highest Se/S ratio was observed with application of 20 µM Se, with significant reduction when high S levels were added in nutrient solution.

3.3. *Effect of Se and S treatments on key enzyme activities of S assimilation*

Selenium can affect S metabolism in plants since it shares S uptake, translocation and assimilation pathways (White, 2018). To investigate differences in S assimilation between rocket and lettuce plants under Se and S treatments in nutrient solution, the key enzyme activity of S assimilation was examined. As shown in Figure 3A and 3B, ATPS activity showed varied responses to Se treatments between the rocket cultivars. Rocket I showed significantly reduced ATPS activity with increasing Se doses, while Rocket II exhibited increased activity at 20 µM Se (Figure 3A). The enzyme activity was enhanced in Rocket I when higher S levels were added

and when combined with 20 µM Se. The results indicate great genotype variation. For lettuce plants, ATPS activity remained similar when treated with increased doses of Se for both cultivars (Figure 3B). Much higher ATPS activity were observed when treated with only higher S levels, but the presence of Se treatments significantly reduced ATPS activity.

OASTL activity for Rocket I was higher in treatments with addition of 10 µM Se and 5 mM S. On the other side, the Rocket II increased OASTL activity with increasing Se doses and when combined with high S levels in nutrient solution (Figure 3C). For lettuce plants, the application of 10 µM Se and the combination of 20 µM Se and 5 mM S enhanced the enzyme activity for Bibb cultivar. The Parris cultivar was not affected by Se and S treatments (Figure 3D).

3.4. *Effect of Se and S treatments on total free amino acids concentration*

Selenium and S treatments affected the total amino acids concentration more in lettuce than rocket (Figure 4A and 4B). With application of higher S levels and when combined with 20 µM Se, the Rocket I cultivar enhanced the total amino acid content. However, the Rocket II not was affected by Se and S treatments (Figure 4A). The amino acids accumulation in lettuce plants were reduced with addition of 20 µM Se as well as with the combination of high Se and S levels in nutrient solution, except Parris cultivar which increased with 10 µM Se (Figure 4B).

3.5. *Effect of Se and S treatments on Se in proteins*

The non-specific integration of the selenoamino acid selenocysteine (SeCys) and selenomethionine (SeMet) into proteins is believed to be the major contributor of Se toxicity in plants and the Se accumulators have the ability to synthesize non-proteinogenic amino acids to avoid the accumulation of SeCys/SeMet to cause toxicity (Sors et al., 2005). Thus, we examined the capacity of Se accumulation in proteins in rocket and lettuce plants in response to different

Se and S treatments. Under the same Se dose, rocket showed 4.5-fold more Se accumulation in proteins than lettuce (Figure 5A and 5B). This result is unexpected and suggests that rocket plants can tolerate higher levels of selenoamino acids in proteins than lettuce for Se toxicity.

3.6. *Effect of Se and S treatments on antioxidant enzyme activity*

The main benefit of Se in plant growth is believed to be its action on the antioxidant system against oxidative stress (Ríos et al., 2008). We examined the APX and SOD enzymes activity to evaluate the influence of Se and S treatments on antioxidant defense system. Different responses were observed for cultivars of both species when exposed to various levels of Se and S treatments (Figure 6). Noticeably, Rocket showed 6-fold more APX activity than lettuce (Figure 6A and 6B), while no great difference between the two species was observed for SOD activity (Figure 6C and 6D).

3.7. *Effect of Se and S treatments on non-enzymatic antioxidants*

Glutathione (GSH) and non-protein thiols (NPT) are important thiols compounds that can scavenge ROS and alleviate oxidative stress damage (Hasanuzzaman et al., 2017). To further examine the basis underlying higher tolerance of rocket to Se than lettuce, we examined the non-enzymatic antioxidant production. As shown in Figure 7A and 7B, rocket have about 5-fold more GSH than lettuce. The rocket and lettuce cultivar showed different response to Se and S treatments (Figure 7A). While Rocket I decreased GSH content, the Rocket II increased with the addition of 20 µM Se in nutrition solution. However, both rocket cultivar and Bibb lettuce cultivar decreased GSH production when grown under high Se and S levels (Figure 7B). The Parris lettuce cultivar was not significantly affected by Se and S treatments.

Notably the NPT content also differs dramatically between the two species, with rocket having 5-fold more NPT than lettuce (Figure 7C and 7D). The rocket decreased NPT content

with increased Se doses and when combined with high S levels in growth solution (Figure 7C). But in the absence of Se supplementation, high S levels increased the NPT content with values similar to the control. Lettuce cultivars showed differential response to Se and S treatments for NPT content (Figure 7D). The cultivar Bibb tended increased with combination of high S and Se levels, while the Parris reduced. Taken together, these results suggest that higher GSH and NPT content may partly explain the higher Se tolerance of rocket than lettuce.

4. Discussion

Plants biofortification with Se is an important strategy to mitigate the nutritional Se-deficiency in human health. However, plants have different capacities to accumulate Se in tissues, their uptake and assimilation may also be affected by other elements in the growth medium, such as S. In this study, we evaluated the differences in growth, nutrition, and metabolism of two rocket and lettuce cultivars in response to different Se and S treatments in nutrient solution to understand the differentially mechanism to Se tolerance between accumulators and non-accumulators plants.

The benefit effects of Se on plant growth is directly related to its concentration in the growth medium and Se accumulative capacity by plants (Pilon-Smits et al., 2017). Our results showed that both species responded positively to Se supplement (Figure 1A and B). But significant differences in biomass production were observed as a function the Se treatments between the two species. Rocket showed higher tolerance with better growth than lettuce when they were exposed to 20 µM Se treatment.

Total S concentration was increased with the Se treatments. In general, application up to 20 µM Se more than doubled the S content in both species (Figure 2A and 2B). The synergistic interaction between Se and S observed in the present study is consistent with previously reported studies with rocket, *Arabidopsis*, lettuce and wheat (Boldrin et al., 2016;

Dall'Acqua et al., 2019; Ramos et al., 2011; White et al., 2004). As selenate is uptaken through sulfate transporters, it is suggested that low selenate levels increase the expression of high affinity sulfate transporters by mimicking S deficiency to stimulate S uptake (Boldrin et al., 2016).

The large difference in total Se/S content observed between rocket and lettuce plants reflects their distinct ability to accumulate Se/S. Se concentration in rocket was 3-fold higher than lettuce (Figure 2C and 2D). A dramatic reduction in Se content with high S dose in growth solution was observed for both species. We also observed that the magnitude of S inhibition on Se uptake was 2-fold higher in lettuce than rocket. This result is partially explained by antagonistic effect imposed by S on Se uptake as a result of selenate competition by S transporters and assimilation pathway in plants (Cabannes et al., 2011; Schiavon et al., 2015; White et al., 2004).

ATPS is the metabolic entry point into the S assimilation pathway, which catalyzes the formation of adenosine phosphosulfate from ATP and sulfate. The final step of S assimilation is catalyzed by OASTL to form Cys from OAS and H_2S (Gigolashvili and Kopriva, 2014). Rocket and lettuce plants presented different responses between cultivars and to Se and S treatments for ATPS and OASTL activity, but in general no dramatic differences between species and in response to Se and S treatment were observed for the activity of these enzymes (Figure 3A and 3B).

Amino acid content is an important parameter of plant nutritional quality. In the present study, we can see that treatments with Se and S appeared to affect lettuce plants more consistently than rocket (Figure 4A and 4B). In addition, genotype variation in response to Se treatment was observed. The result of the present study is in contrast to that observed by Ramos et al. (2011) who found no dramatic differences in Se-treated lettuce plants.

Non-specific integration of acids SeCys and SeMet in proteins leads to damage structure and function of protein, which is believed as a major cause of Se toxicity in plants (Terry et al., 2000; Tian et al., 2017). Our results showed that rocket have higher Se accumulation capacity in proteins than lettuce, which indicates a great tolerance of rocket to selenoamino acids SeCys and SeMet in proteins than lettuce (Figure 5A and 5B).

To protect against oxidative damage, plants have a sophisticated and complex antioxidant system involving enzymes and non-enzymatic antioxidant. APX and SOD are important antioxidant system enzymes that catalyze the conversion of anion superoxides to H_2O_2 and O_2 (Czarnocka and Karpiński, 2018). Here, we show that both species increased APX and SOD activity at different Se and S levels in the growth solution (Figure 6A and 6B). In addition, rocket showed higher APX activity than lettuce under Se and S treatments, which might provide higher Se tolerance (Figure 6). The increased in APX activity in rocket plants might be associated with higher GSH concentration, since APX is an integral component of the Ascorbate-Glutathione cycle (AsA-GSH) which promotes hydrogen peroxide decomposition (Sharma et al., 2012). Furthermore, previous studies have demonstrated that Se and S can stimulate the antioxidant enzymes activity protecting plants against abiotic stress (Ramos et al., 2011; Ríos et al., 2008; Zhang et al., 2010).

The greater capacity of rocket to tolerate higher Se levels than lettuce plants is most likely associated with an efficient antioxidant defense mechanism. Rocket plants presented GSH and NPT accumulation fivefold more than lettuce plants, suggesting that the non-enzymatic antioxidants play a greater role in the Se tolerance (Figure 7A and 7B). Previous results indicate that non-enzymatic antioxidants action like GSH and NPT are crucial to antioxidant system of plants against various abiotic stresses, especially against metal toxicity (Luo et al. 2011; Zagorchev et al., 2013; Zhang et al., 2013; Zeng et al., 2011). GSH is the major thiol compound responsible for maintenance of cellular redox homeostasis, as well as is

part of the AsA-GSH cycle, which eliminates H_2O_2 (Noctor et al., 2002; Hasanuzzaman et al. 2017). In addition, NPT, such as phytochelatins, act in the sequestration and homeostasis of different metals as the main detoxification strategy in plant cells through chelation and subsequent vacuolar compartmentalization (Zheng et al., 2011, Zagorchev et al., 2013).

5. Conclusions

In conclusion, our data showed that rocket has higher tolerance to Se treatment than lettuce, which might be due to higher redox capacity, demonstrated by the greater APX activity and higher GSH and NPT concentration. In addition, our data also showed that much higher levels of Se in proteins were observed in rocket than lettuce, indicating that Se accumulators might have higher tolerance to selenoamino acids in proteins, which presumably affect the normal function of enzymes and proteins.

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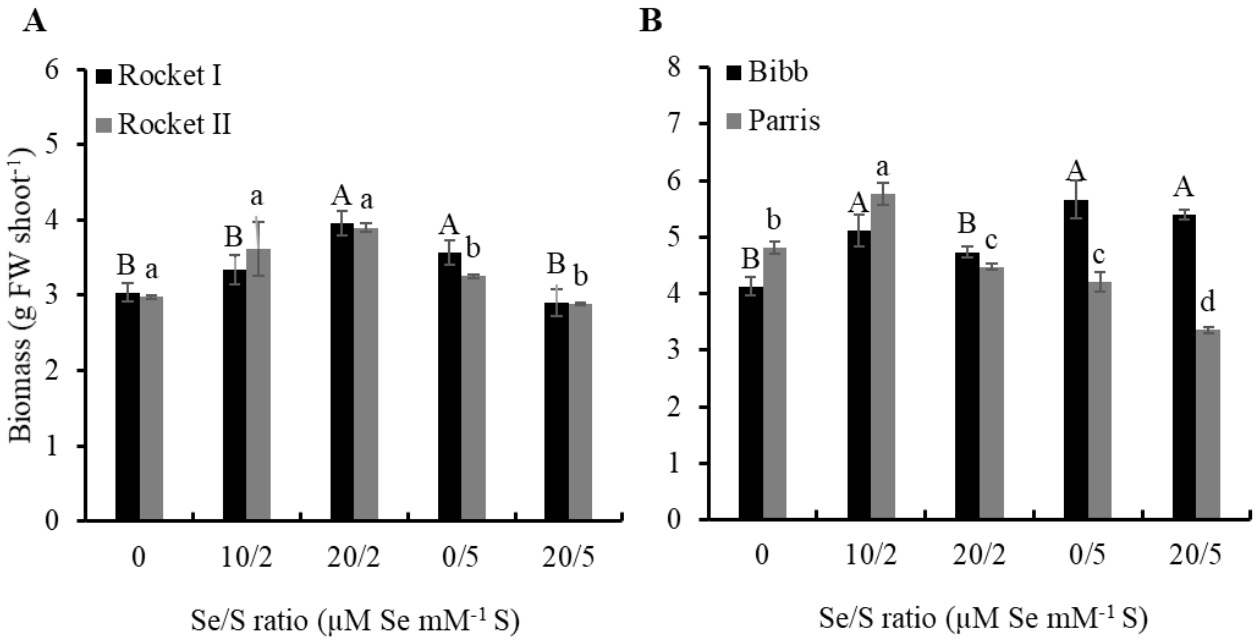

Figure 1. Shoot biomass of two cultivars of rocket (A) and lettuce (B) subjected to different Se/S treatments. Data represent means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

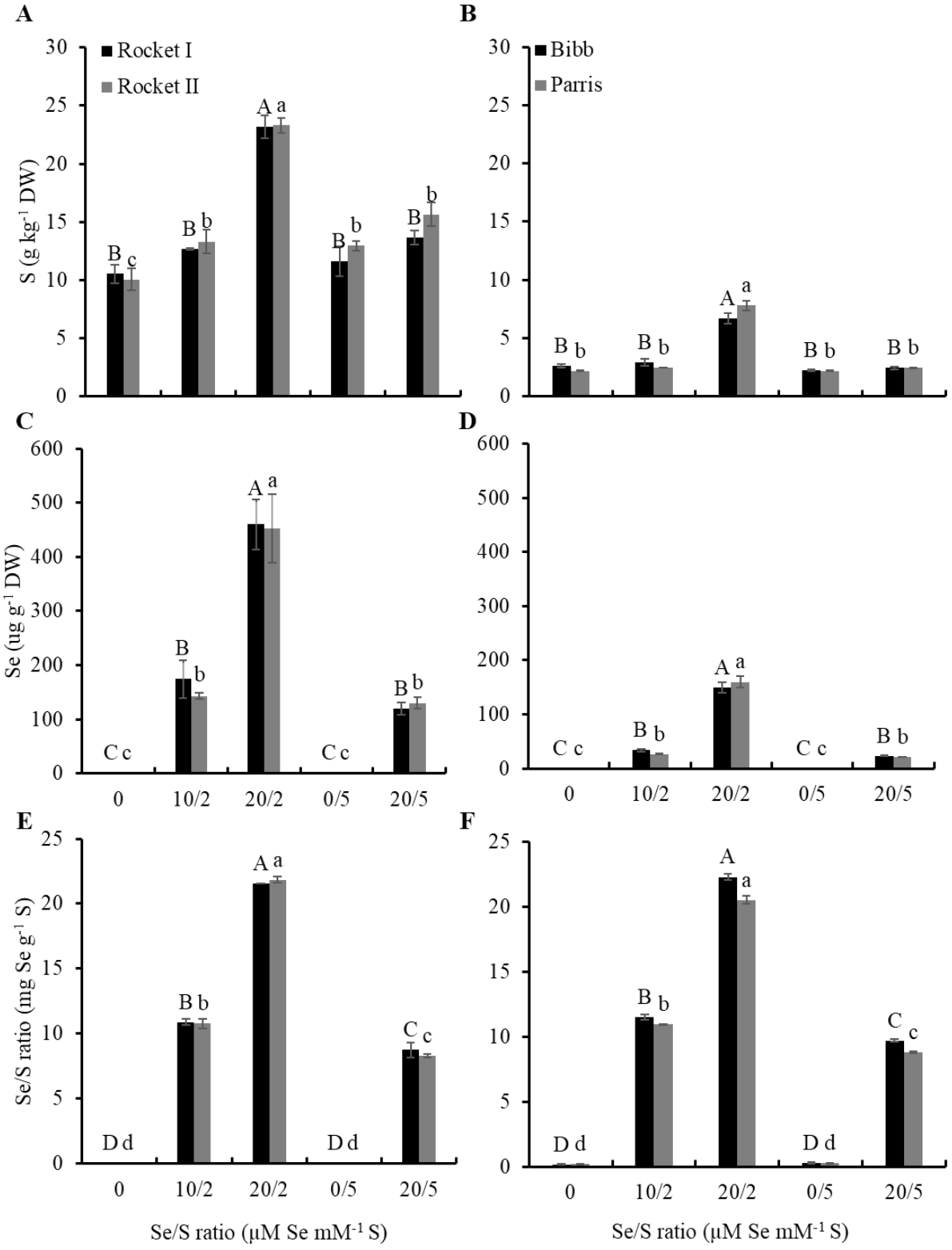

Figure 2. Total S (A and B) and Se (C and D) concentration and Se/S ratio (E and F) of two cultivars of rocket and lettuce subjected to different Se/S treatments. Data represents means three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

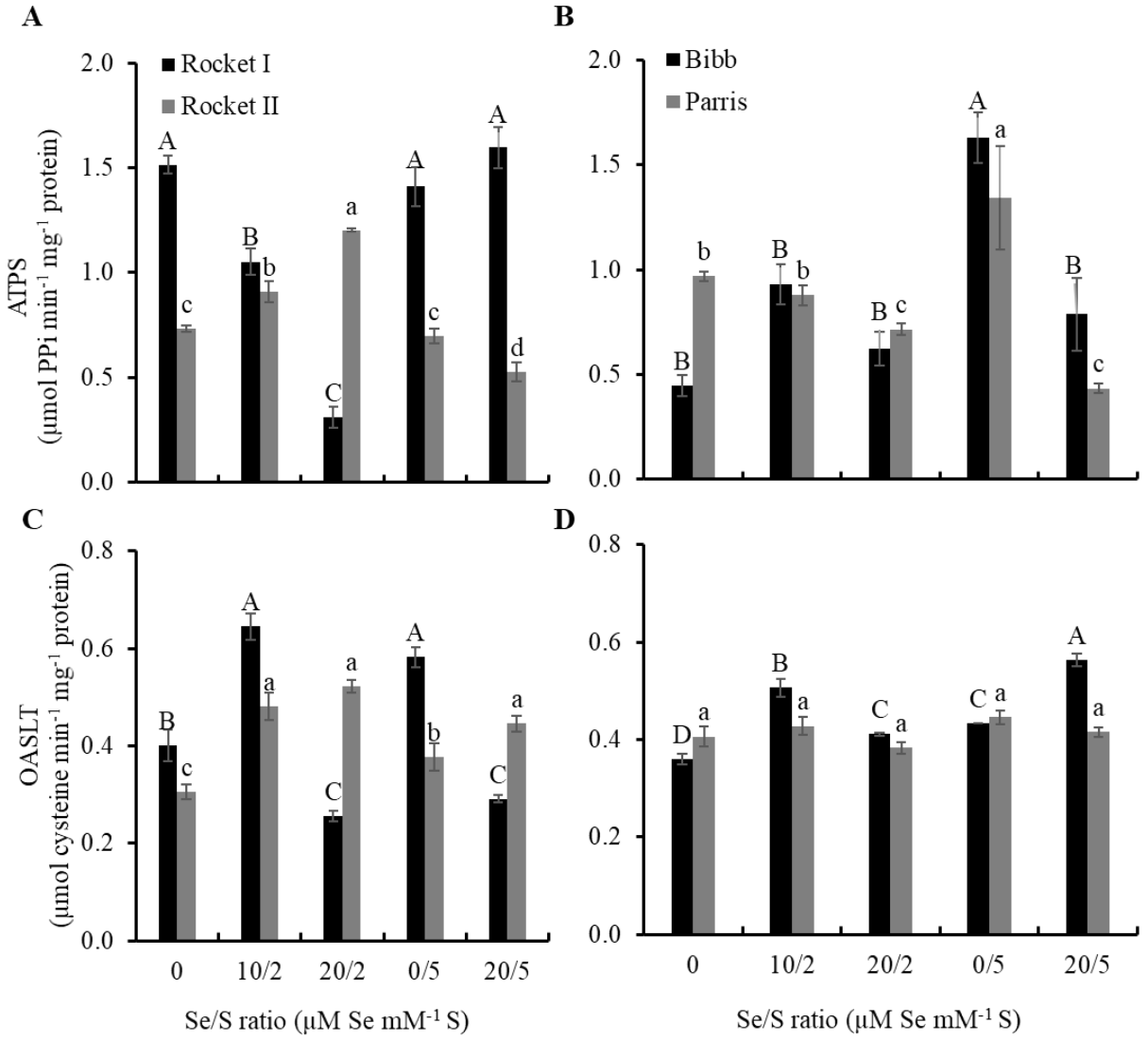

Figure 3. ATP sulfurylase – ATPS (A and B) and O-acetylserine activity - OASTL (C and D) of two cultivars of rocket and lettuce subjected to different Se/S treatments. Data represents means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

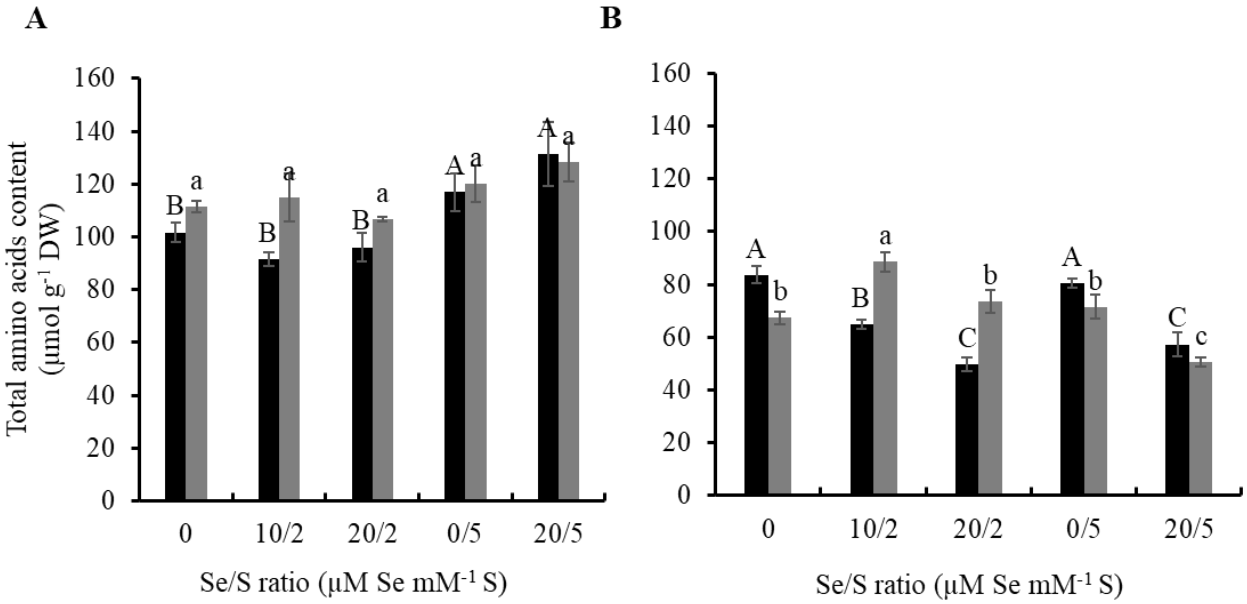

Figure 4. Total amino acids content of two cultivars of rocket (A) and lettuce (B) subjected to different Se/S treatments. Data represents means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

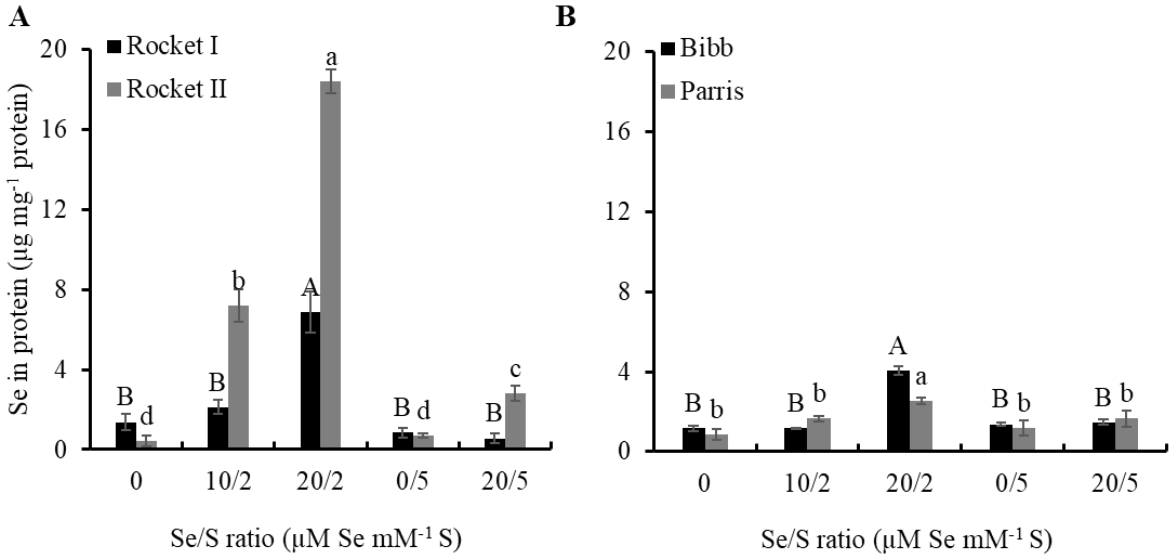

Figure 5. Se in protein levels of two cultivars of rocket (A) and lettuce (B) subjected to different Se/S treatments. Data represents means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

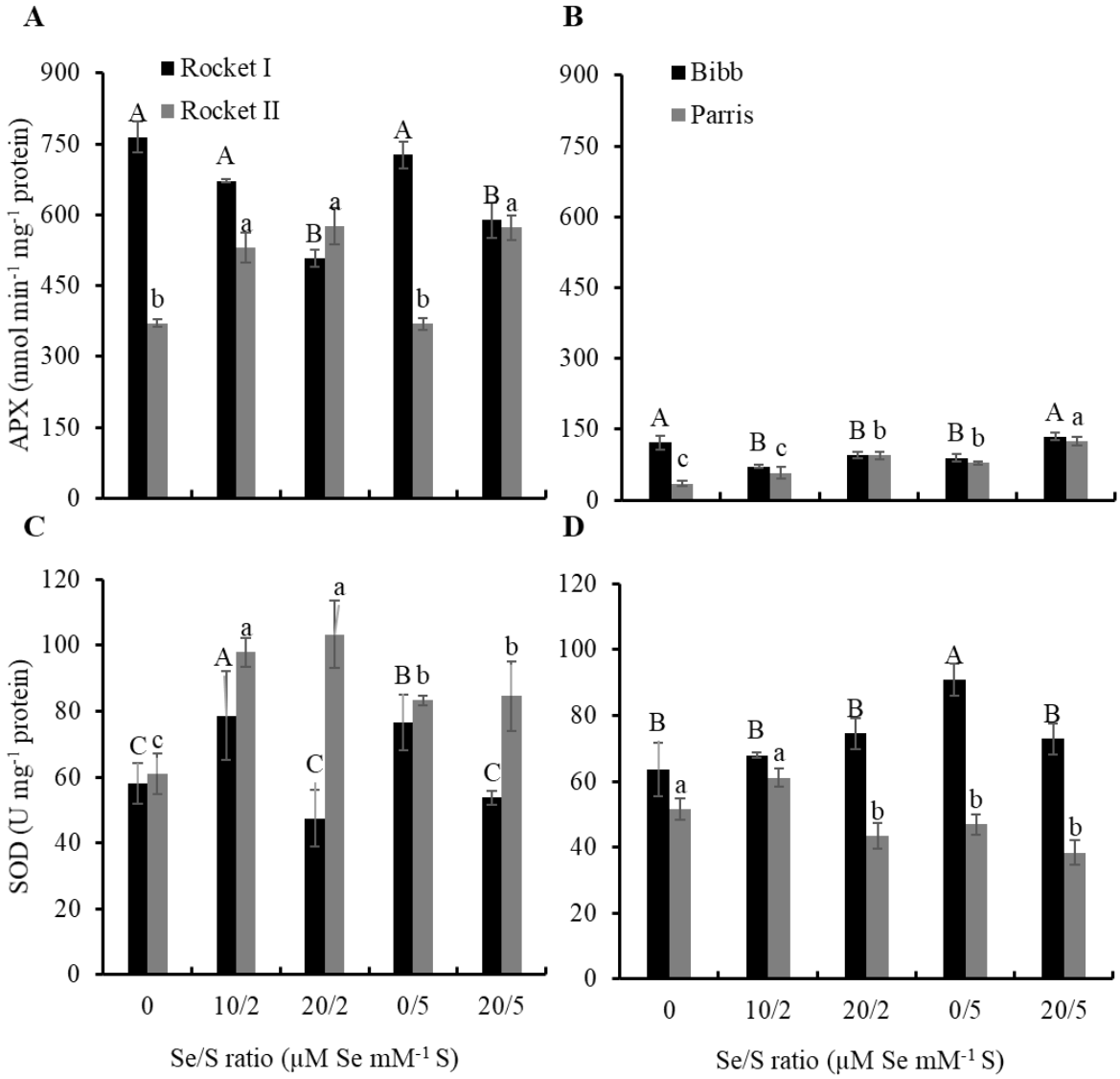

Figure 6. Ascorbate peroxidase – APX (A and B) and superoxide dismutase – SOD (C and D) of two cultivars of rocket and lettuce subjected to different Se/S treatments. Data represents means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.

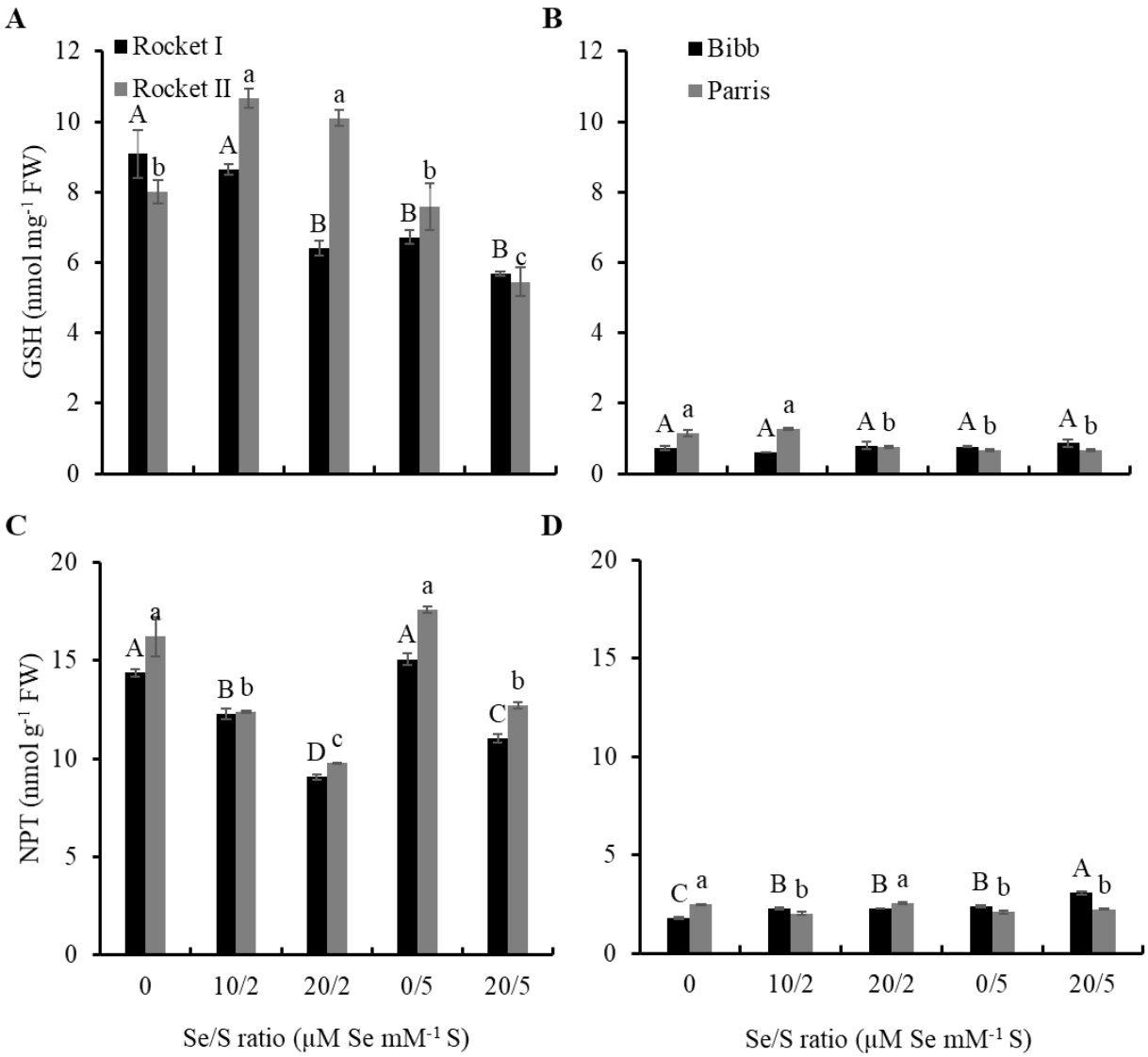

Figure 7. Glutathione – GSH (A and B) and non-protein thiols – NPT (C and D) of two cultivars of rocket and lettuce subjected to different Se/S treatments. Data represents means from three independent biological replicates. Bars indicate standard error. Different letters above the columns indicate significant differences at $p < 0.05$ by Scott-Knot test.