FABRÍCIO WILLIAM DE ÁVILA

SELÊNIO E GLUCOSINOLATOS EM BRÁSSICAS E FOSFITO NA NUTRIÇÃO FOSFATADA EM FEIJOEIRO

LAVRAS – MG
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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.

Orientador
Dr. Valdemar Faquin

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APROVADA em 22 de Abril de 2013.

Dr. André Rodrigues dos Reis	Waseda University
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LAVRAS – MG

2013

A Deus, por me dar forças
para vencer mais essa etapa,

DEDICO.

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e à minha irmã, Andressa, pelo
apoio, incentivo e carinho,

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RESUMO

Algumas formas orgânicas de selênio (Se) e alguns glucosinolatos (ß-tioglicosídio-N-hidroxissulfatos) são conhecidos por apresentarem excelente atividade anticancerígena, enquanto fosfito (P^{+3}) tem sido comercializado como fungicida e, mais recentemente, também como uma complementar fonte de fósforo (P) para as culturas. Objetivou-se neste estudo (i) investigar se é possível simultaneamente aumentar o acúmulo do composto anticancerígeno *Se*-metil-seleno-cisteína (SeMSCis) sem necessariamente afetar o acúmulo de glucosinolatos quimiopreventivos em brotos de brássicas (*Brassica* spp.), e (ii) avaliar se fosfito pode ser usado como uma fonte de P para produção de grãos em feijoeiro (*Phaseolus vulgaris* L.). Nos experimentos com brotos de brássicas, cultivares de seis brássicas amplamente consumidas em todo o mundo (brócolis, couve-flor, repolho, couve-chinesa, couve e couve-de-Bruxelas) foram usadas. Como resultado, foi encontrado que todos os brotos de brássicas biofortificados com Se foram aptos a sintetizar e acumular significantes quantidades de SeMSCis sem, negativamente, afetar as concentrações de glucosinolatos quimiopreventivos. Os tipos e quantidades de glucosinolatos variaram grandemente entre as brássicas estudadas. Em geral, 14 diferentes glucosinolatos foram extraídos dos brotos de brássicas e identificados pelo UPLC-MS/MS (cromatografia líquida de ultra-eficiência acoplada à espectrometria de massas em série), os quais foram classificados como glucosinolatos alifáticos, indólicos e aromáticos. Os brotos de couve-flor apresentaram maior concentração de glucosinolatos totais e os brotos de brócolis contiveram elevada concentração de glucorafanina, um glucosinolato alifático que apresenta potente atividade anticancerígena. Em um experimento de comparação com cinco cultivares de brócolis, foi verificado que brotos e inflorescências ("cabeça", principal parte comestível da cultura de brócolis) apresentaram similares tipos de glucosinolatos, mas, houve considerável diferença nas quantidades acumuladas de cada glucosinolato identificado. Os brotos de brócolis apresentaram uma concentração, aproximadamente, seis vezes maior de glucorafanina que as inflorescências de plantas de brócolis. É interessante destacar que uma relação antagônica entre acúmulo de Se e glucosinolatos foi verificado nas inflorescências, mas não nos brotos. Assim, a biofortificação de brotos de brócolis com Se poderia aumentar sua atividade anticancerígena, em virtude da síntese de SeMSCis, sem afetar, negativamente, o acúmulo de glucosinolatos quimiopreventivos, em particular a glucorafanina. Nos experimentos com feijoeiro, o fornecimento de ânion fosfito via foliar e radicular (ensaios realizados com plantas cultivadas em vasos com solução nutritiva e em vasos com um Latossolo de textura média) não substituiu o ânion

fosfato (P^{+5}) na nutrição de P das plantas. As concentrações e acúmulos de P na parte aérea e raízes das plantas crescidas, sob insuficiente disponibilidade de fosfato (plantas deficientes em fosfato), foram maiores com o aumento das doses de fosfito no meio de crescimento, mas essa concentração de P adicional não foi convertida em maior crescimento ou produção de grãos das plantas. Em adição, o fosfito demonstrou ser altamente tóxico para a produção de grãos em plantas de feijoeiro deficiente em fosfato. A atividade de fosfatases ácidas *in vivo* das plantas deficientes em fosfato foi consideravelmente elevada, mas o fornecimento de pequenas quantidades de fosfito no meio de crescimento dessas plantas já foi suficiente para reduzir, significativamente, a atividade dessas enzimas.

Palavras-chave: Alimentos funcionais. *Brassica spp*. Brotos. Biofortificação. Agentes anticancerígenos. *Phaseolus vulgaris* L. Fósforo. Nutrientes minerais.

ABSTRACT

Some organic forms of selenium (Se) and glucosinolates (β-thioglucoside N-hydroxysulphates) are cancer chemopreventive agents, while phosphite (P^{+3}) has been marketed as a fungicide and recently also as a complement phosphorus (P) source for crops. Aim of this study was to (i) investigate whether it is feasible to simultaneously increase the accumulation of the anticancer compound *Se*-methylselenocysteine (SeMSCys) without necessarily affecting accumulation of chemopreventive glucosinolates in Brassica (*Brassica* spp.) sprouts, and (ii) evaluate whether phosphite may be used as a P source to yield of bean common (*Phaseolus vulgaris* L.) plants. In the experiments with Brassica sprouts, cultivars from the six most extensively consumed Brassica vegetables (broccoli, cauliflower, green cabbage, Chinese cabbage, kale, and Brussels sprouts) were used. It was found that all Se-biofortified Brassica sprouts were able to synthesize and accumulate significant amounts of SeMSCys without negatively affecting contents of chemopreventive glucosinolates. Analysis of glucosinolate profiles revealed that sprouts of each Brassica vegetable accumulated different types and amounts of glucosinolates. In general 14 main types of individual glucosinolates were extracted of the sprouts and distinguished by UPLC-MS/MS, which were classified as aliphatic, indole or aromatic glucosinolates. Cauliflower sprouts had higher total glucosinolate content and broccoli sprouts contained high levels of the potent anticancer glucoraphanin, an aliphatic glucosinolate. In a comparison experiment with five broccoli cultivars, it was showed that broccoli sprouts and florets of mature broccoli plants present similar glucosinolate types, but with substantial differences in accumulated amount of each individual glucosinolate. Broccoli sprouts contained approximately 6-fold higher content of glucoraphanin than florets of mature broccoli plants. Interesting, antagonistic relationship between Se and glucosinolate accumulation was observed in broccoli florets but not in broccoli sprouts. This shows that biofortification of broccoli sprouts with Se could increase their anticancer activity due to the synthesis of SeMSCys without negatively affecting accumulation of chemopreventive glucosinolates, particularly glucoraphanin. In the experiments with common bean plants, supply of phosphite via roots (plants grown in pots with either nutrient solution or weathered tropical soil) and foliar spray did not replace phosphate (P^{+5}) in plant P nutrition. Concentration and accumulation of P in shoot and roots of plants exposed to low phosphate availability (phosphate-starved plant) was increased with increasing phosphite levels in growth medium, but this additional P concentration did not convert into increased growth or grain yield. Furthermore, phosphite also proved to be highly toxic to

grain yield of phosphate-starved common bean plants. *In vivo* acid phosphatase activity of phosphate-starved plants was considerably higher than that of plants exposed to adequate phosphate supply, but small amounts of phosphite in growth medium was already sufficient to significantly decrease the activity of these enzymes in Pi-starved plants.

Keywords: Function foods. *Brassica spp*. Sprouts. Biofortification. Anticancer agents. *Phaseolus vulgaris* L. Phosphorus. Mineral nutrients.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A atividade anticancerígena apresentada por algumas formas orgânicas de selênio (Se) e, também, por alguns glucosinolatos tem sido amplamente explorada em *Brassica* spp., enquanto produtos baseados em fosfito são atualmente comercializados em várias partes do mundo como fungicidas e fertilizantes para as culturas.

O selênio (Se) é um elemento traço essencial para mamíferos, sendo constituinte de selenoproteínas, dentre as quais várias são tidas como indispensáveis ao metabolismo humano. A deficiência de Se afeta centenas de milhões de pessoas por todo o mundo (COMBS, 2001), fato que, também, parece ser relevante em várias regiões do Brasil (FERREIRA et al., 2002; MAIHARA et al., 2004). Estudos têm apresentado conclusivamente o efeito benéfico da adequada ingestão de Se sobre o metabolismo do organismo humano (RAYMAN, 2012). Em adição às selenoproteínas, outras formas orgânicas de Se não proteicas, também, podem exercer importantes benefícios à saúde. Recentemente, grande interesse tem sido centrado no aminoácido *Se*-metil-seleno-cisteína (SeMSCis), o qual possui, comprovadamente, potente atividade anticarcinogênica (ABDULAH et al., 2005; MEDINA et al., 2001).

As plantas desempenham um papel fundamental na introdução de Se para a cadeia alimentar. Nesse contexto, a biofortificação das culturas agrícolas com Se tem mostrado ser uma técnica útil para aumentar a ingestão desse elemento na população. Essa técnica já vem sendo amplamente explorada em

vários países (BROADLEY et al., 2006). Porém, o Se não é essencial para as plantas e a maioria destas apresenta baixa tolerância a esse elemento.

As brássicas (*Brassica* spp.), quando biofortificadas com Se, fazem parte de um seleto grupo de culturas que podem apresentar este elemento em concentrações de várias centenas de microgramas por grama de massa seca e, ainda, sintetizam consideráveis quantidades do composto anticancerígeno SeMSCis. Além das tradicionais partes comestíveis dessas plantas, recentemente, os brotos de brássicas têm sido consumidos em diversos países por serem ricas fontes de compostos bioativos, particularmente glucosinolatos (ß-tioglicosídio-N-hidroxissulfatos). Estes últimos são um grupo de compostos fitoquímicos, encontrados em plantas pertencentes à ordem Brassicales, que têm atraído atenção de pesquisadores por causa de seus efeitos benéficos à saúde. Como exemplo, a glucorafanina é um glucosinolato encontrado em consideráveis quantidades nos brotos de brócolis e que apresenta excelentes propriedades anticancerígenas. Estudos têm relatado que há uma interação antagônica entre acúmulo de Se e glucosinolatos em plantas de brássicas (CHARRON et al., 2001; RAMOS et al., 2011), mas ainda há poucas informações em relação aos brotos.

O ânion fosfito (HPO_3^{2-} e $H_2PO_3^-$) é uma forma reduzida do ânion fosfato (HPO_4^{2-}, $H_2PO_4^-$ e PO_4^{3-}) que vem sendo amplamente utilizado na agricultura moderna como fungicida e bioestimulante. Estudos têm mostrado que o fosfito pode estimular o metabolismo secundário de plantas, resultando em maior capacidade antioxidante, síntese de diversos metabólitos secundários e alterações benéficas na parede celular (AVILA et al., 2011; MOOR et al., 2009; OLIVIERI et al., 2012). Contudo, recentemente, produtos à base de fosfito, também, têm sido comercializados em todo o mundo como fertilizantes.

Sais de fosfito são recomendados como um fertilizante, porque contêm um cátion que pode ser nutriente de planta, tais como K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+} ou Zn^{2+} e, especialmente, porque fosfito é uma forma de fósforo (P). Considerando que o P é um dos mais limitantes nutrientes de planta, em solos de regiões tropicais e subtropicais do mundo, a divulgação de produtos baseados em fosfito, também, como uma possível fonte de P para a nutrição das culturas é particularmente interessante para propostas de comercialização. Essa suposição tem sido suportada pelos relatos de alguns prévios trabalhos apresentando que suprimento de fosfito foi benéfico para culturas deficientes em P (RICKARD, 2000). Por outro lado, recentes pesquisas têm indicado que os vegetais não utilizam o fosfito como um nutriente de P, embora ele seja bem absorvido pelas folhas e raízes. Ainda, fosfito tem apresentado ser nocivo para plantas deficientes em fosfato (THAO; YAMAKAWA; SHIBATA, 2009; VARADARAJAN et al., 2002).

O feijoeiro (*Phaseolus vulgaris* L.) é uma das mais importantes leguminosas de grão para consumo humano e, também, considerado a principal fonte de proteína em muitos países de Terceiro Mundo (GRAHAM; RANALLI, 1997). No entanto, em geral, a sua produtividade é considerada baixa, em razão das doenças e da predominância de solos de baixa fertilidade natural em regiões tropicais e subtropicais do mundo, as quais se localizam, em grande parte, em países em desenvolvimento.

No presente trabalho objetivou-se investigar se é possível, simultaneamente, aumentar o acúmulo de Se total e SeMSCis sem necessariamente afetar o acúmulo de glucosinolatos quimiopreventivos, em brotos de brássicas e avaliar se fosfito pode ser usado como uma fonte de P para a nutrição e produção de grãos em feijoeiro.

2 REFERECIAL TEÓRICO

2.1 Selênio e a saúde humana

Estudos com alimentos enriquecidos e funcionais, ou seja, que fornecem benefícios específicos à saúde, além da nutrição básica, têm tido destaque na literatura internacional. Nesse sentido, pesquisas recentes nas áreas da medicina nutricional e alimentação de humanos têm dado ao selênio (Se) um lugar de destaque.

O Se desempenha uma importante função biológica como constituinte do aminoácido seleno-cisteína, o qual está diretamente relacionado com a síntese de, aproximadamente, 25 proteínas, as quais são denominadas de selenoproteínas (DRISCOLL; COPELAND, 2003; RAYMAN, 2012; STADTMAN, 1996). Várias selenoproteínas são essenciais para o metabolismo do organismo humano, tais como as peroxidases da glutationa (possui funções antioxidantes), redutases da tiorredoxina (importante para a síntese de DNA) e desiodases da iodotironina (responsáveis pela regulação dos hormônios tireoidianos em tecidos periféricos) (LARSEN; BERRY, 1995; RAYMAN, 2012; RIAZ; MEHMOOD, 2012; ROTRUCK et al., 1973; TAMURA; STADTMAN, 1996).

A deficiência de Se no organismo resulta em uma dramática redução nas atividades de selenoproteínas, causando várias doenças e desordens, e.g., disfunções na glândula tireoide, lesão cerebral irreversível, doenças vasculares periféricas, doenças osteoarticulares degenerativas (doença de Kashin–Beck), redução drástica da resposta imunológica às infecções virais (tais como sarampo, hepatite, gripe e HIV-AIDS), infertilidade em homens, pré-eclâmpsia

em mulheres e maior susceptibilidade a vários tipos de câncer (BARRINGTON et al., 1997; BECK et al., 2001; BECK; LEVANDER; HANDY, 2003; COPPINGER; DIAMOND, 2001; IWANIER; ZACHARA, 1995; RAYMAN, 2012; RIAZ; MEHMOOD, 2012; SAPPEY et al., 1994; WILLETT et al., 1983; WU et al., 1973). Em conformidade com o Instituto de Medicina da Academia Nacional de Ciência dos Estados Unidos, a quantidade mínima recomendada e a máxima tolerável de Se é 55 e 400 $\mu g\ dia^{-1}$ para adultos (BENDICH, 2001), e essa faixa de valores tidos como adequados é considerada estreita (TAN et al., 2002). Apesar da carência de Se afetar parte da população mundial, enfatiza-se que o excesso de Se no plasma sanguíneo, também, pode causar sérias doenças ou desordens no organismo (ALDOSARY et al., 2012; GOLDHABER, 2003; RIAZ; MEHMOOD, 2012; YANG et al., 1983).

No mundo, é estimado que entre 0,5 a 1 bilhão de pessoas são deficientes em Se (COMBS, 2001). No Brasil, também, são grandes as evidências de insuficiência desse elemento na população em várias regiões do país (FERREIRA et al., 2002; MAIHARA et al., 2004) em função, em parte, do baixo teor de Se apresentado na maioria dos solos agricultáveis (CARVALHO, 2011; FARIA, 2009; MORAES et al., 2009; SILLANPÄÄ; JANSSON, 1992). Recentemente, o IBGE (Instituto Brasileiro de Geografia e Estatística), em parceria com o MS (Ministério da Saúde) e o MPOG (Ministério do Planejamento, Orçamento e Gestão), publicou a POF 2008-2009 (Pesquisa de Orçamentos Familiares). Nessa publicação consta de forma detalhada a concentração de Se presente nos alimentos consumidos no Brasil (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE, 2011b) e, por meio dessa, foi estimada a ingestão média desse elemento (assim como dos demais nutrientes para humanos) pela população do país em função dos hábitos

alimentares (IBGE, 2011a). Contudo, conforme notificado na própria POF 2008–2009, as informações da composição de Se nos alimentos foram obtidas somente na base de dados da Nutrition Data System for Research (UNIVERSITY OF MINNESOTA, 2008), já que a Tabela Brasileira de Composição de Alimentos (UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP, 2006) não apresenta nenhum tipo de informação para esse elemento. Ressalta-se que o teor e acúmulo de Se nas culturas agrícolas e, consequentemente, nos alimentos estão diretamente relacionados com a disponibilidade de Se no solo (ARTHUR, 2003; COMBS, 2001; GAMMELGAARD; JACKSON; GABEL-JENSEN, 2011; GIBSON et al., 2011; HAWKESFORD; ZHAO, 2007; HURST et al., 2013; KESKINEN; TURAKAINEN; HARTIKAINEN, 2010; NYE, 1975). Assim, no caso específico de Se, os dados de consumo desse elemento pela população do Brasil (que constam na POF 2008–2009) devem ser analisados com cautela, em virtude do fato de os solos predominantes no Brasil e nos Estados Unidos exibirem consideráveis diferenças em seus atributos químico, físico, mineralógico e microbiológico (PRADO, 2003; SANTOS et al., 2006; UNITED STATES DEPARTMENT OF AGRICULTURE - USDA, 2013). Provavelmente, a disponibilidade de Se no solo para as plantas e, consequentemente, a concentração de Se nos alimentos pode variar entre o Brasil e os Estados Unidos. Conforme supracitado, a grande parte dos solos cultiváveis no Brasil são deficientes em Se.

Além das selenoproteínas, outros compostos orgânicos de Se não proteicos, também, têm mostrado ter importantes benefícios à saúde humana. Mais recentemente, interesse tem sido focado sobre as propriedades anticarcinogênicas de alguns compostos orgânicos metilados de Se. Embora

existam pesquisas reportando efeitos quimiopreventivos de formas orgânicas de Se não metiladas (RAYMAN, 2012; RIAZ; MEHMOOD, 2012; RYAN-HARSHMAN; ALDOORI, 2005), vários estudos têm mostrado que as formas orgânicas metiladas de Se, especialmente os aminoácidos monometilados *Se*-metil-seleno-cisteína e γ-glutamil-*Se*-metil-seleno-cisteína, apresentam atividade anticancerígena comprovadamente bem maior (DONG et al., 2001; IP et al., 2000; IP; GANTHER, 1990; LEE et al., 2006; MEDINA et al., 2001; WANG et al., 2009).

Os alimentos são as principais fontes de Se para os humanos. Nesse sentido, os alimentos de origem animal contêm principalmente Se na forma do aminoácido seleno-cisteína (forma análoga ao aminoácido cisteína). A maior parte das culturas agrícolas (e.g. cereais), as quais não são acumuladoras de Se, apresentam este elemento na forma do aminoácido seleno-metionina (forma análoga ao aminoácido metionina). Um restrito grupo de plantas cultivadas (tais como as brássicas, cebola e alho), também, contém Se na forma dos aminoácidos *Se*-metil-seleno-cisteína e γ-glutamil-*Se*-metil-seleno-cisteína, estes são conhecidos por seus excelentes efeitos anticarcinogênicos. A forma inorgânica selenato pode ser encontrada em certos casos em plantas e peixes e, ocasionalmente, também, na água (FINLEY, 2006; RAYMAN, 2012; SATHE et al., 1992; WHANGER, 2002).

Em adição aos alimentos naturais, outra forma alternativa é a ingestão de Se, por meio de suplementos nutricionais (cápsulas ou produtos enriquecidos, durante seu processamento nas indústrias), embora haja vários casos de intoxicação de pessoas com Se, em razão da ingestão desses suplementos, conforme recentemente relatados por Aldosary et al. (2012) e Morris e Crane (2013). O Se, em suplementos alimentares para o consumo humano, é

comumente comercializado na forma mineral (Se inorgânico, notadamente selenato ou selenito) ou por meio de leveduras enriquecidas (apresentam predominantemente Se na forma orgânica seleno-metionina). Em comparação às formas orgânicas (particularmente seleno-metionina e seleno-cisteína), estudos têm reportado que as formas inorgânicas de Se possuem menor biodisponibilidade para o organismo humano e, ainda, apresentam maior risco de toxidez por ingestão excessiva (BARCELOUX, 1999; FINLEY, 2006; THOMSON, 2004; VEATCH et al., 2005).

Nesse contexto, as plantas desempenham um papel fundamental na introdução de Se para a cadeia alimentar (ARO; ALFTHAN; VARO, 1995; BROADLEY et al., 2006; COMBS, 2001). Estas absorvem o Se em suas formas inorgânicas predominantes no ambiente e o converte para formas orgânicas funcionais (ARO; ALFTHAN; VARO, 1995; PILON-SMITS; QUINN, 2010; SORS; ELLIS; SALT, 2005). Assim, dentre as estratégias atualmente utilizadas para incrementar o Se na população, a biofortificação, ou seja, a adubação de plantas cultivadas com sais de Se está sendo difundida, tanto para alimentação humana quanto animal. Este fato vem sendo amplamente abordado nos países desenvolvidos que apresentam casos de deficiência de Se, com destaque para a Finlândia (ARO; ALFTHAN; VARO, 1995; EUROLA et al., 1991; HARTIKAINEN, 2005; VARO et al., 1988).

O selenato (Se^{+6}, ânion SeO_4^{2-}) e o selenito (Se^{+4}, ânions SeO_3^{2-} e $HSeO_3^-$) são as principais formas inorgânicas de Se encontradas no ambiente e, também, utilizadas nos programas de biofortificação de culturas. Naturalmente, o selenato é a forma predominante de Se biodisponível em solos que apresentam altos valores de potencial redox (pe + pH > 15), enquanto o selenito é a forma de Se biodisponível que predomina em condições de médios valores

de potencial redox (pe + pH 7.5–15) (ELRASHIDI et al., 1987). Quanto à fertilização do solo, o selenito pode apresentar forte interação com as argilas de grande parte dos solos cultivados das regiões tropicais e subtropicais do mundo (GOH; LIM, 2004; GONZALEZ et al., 2010; HARTIKAINEN, 2005; HAYES et al., 1987; MOUTA et al., 2008; SAEKI; MATSUMOTO; TATSUKAWA, 1995); portanto, apresentando menor biodisponibilidade que o selenato. Entretanto, este pode ser mais facilmente lixiviado no perfil do solo, podendo ficar fora do alcance das raízes após certo tempo.

Na planta, o selenato é absorvido ativamente via transportadores do sulfato (S^{+6}, ânion SO_4^{2-}). Esses ânions também compartilham, em grande parte, os mesmos processos de assimilação (CHANG et al., 2008; LI; MCGRATH; ZHAO, 2008; SORS; ELLIS; SALT, 2005). Consequentemente, a adubação sulfatada interfere diretamente sobre a absorção e acúmulo de Se (quando fornecido como selenato) pelas culturas (LYI et al., 2005; ULRICH; SHRIFT, 1968). Durante a assimilação de selenato, que ocorre, predominantemente, nos cloroplastos das folhas, a redução desse selenito parece ser um passo limitante na via da assimilação de Se, uma vez que a maioria das plantas tratadas com selenato acumula relativa quantidade dessa forma inorgânica de Se, enquanto a maior parte das plantas tratadas com selenito acumula predominantemente apenas Se na forma orgânica (PILON-SMITS; QUINN, 2010; SOUZA et al., 1998). Esse processo de redução de selenato a selenito nos vegetais é mediado por duas enzimas chaves, a sulfurilase do ATP (EC 2.7.7.4) e a redutase do APS (EC 1.8.4.9) (SORS; ELLIS; SALT, 2005). Já o ânion selenito é absorvido do solo pelas plantas, por meio de difusão passiva e, também, há relatos de que o selenito pode ser absorvido de forma ativa por intermédio de transportadores de fosfato (P^{+5}, ânions $H_2PO_4^-$ e HPO_4^{2-}). Diferentemente do selenato, assim que

absorvido, o selenito é quase totalmente assimilado nas raízes e, posteriormente, os produtos dessa assimilação (formas orgânicas de Se) são transportados para a parte aérea (LI; MCGRATH; ZHAO, 2008; SORS; ELLIS; SALT, 2005).

2.2 Selênio e glucosinolatos quimiopreventivos em brássicas

A grande maioria das culturas agrícolas (e.g. cereais) é classificada como não acumuladora de Se por não apresentar mais que 100 $\mu g\ g^{-1}$ de Se em sua massa seca quando cultivada em solos seleníferos (TERRY et al., 2000). Essas culturas, quando biofortificadas com Se, apresentam esse elemento, predominantemente, na forma do aminoácido seleno-metionina, que exerce uma função biológica essencial. Assim que ingerida pelo organismo humano ou animal, a seleno-metionina é desmetilada e metabolizada ao aminoácido seleno-cisteína, a partir do qual são formadas as selenoproteínas (FINLEY, 2006; RAYMAN, 2012; SATHE et al., 1992; SCHRAUZER, 2000).

Por outro lado, um seleto grupo de plantas cultivadas pode exibir a partir de centenas até alguns milhares de microgramas de Se por grama de massa seca, sendo essas culturas classificadas como acumuladoras de Se (TERRY et al., 2000). Embora espécies de plantas acumuladoras de Se, também, possam conter relativas quantidades de seleno-metionina, elas apresentam Se, predominantemente, na forma de aminoácidos metilados (SORS; ELLIS; SALT, 2005). Quando comparado com a seleno-metionina, os aminoácidos metilados de Se possuem menor biodisponibilidade para síntese de selenoproteínas, mas, vários deles têm recebido atenção especial por atuar

efetivamente contra células cancerígenas (ABDULAH et al., 2005; FINLEY, 2006).

As brássicas (*Brassica* spp.), em geral, são boas acumuladoras de Se (PILON-SMITS; QUINN, 2010; TERRY et al., 2000), apresentando considerável quantidade desse elemento na forma do aminoácido monometilado *Se*-metil-seleno-cisteína (ROBERGE; BORGERDING; FINLEY, 2003; WHANGER, 2002), o qual, comprovadamente, possui potente atividade anticancerígena (ABDULAH et al., 2005; MEDINA et al., 2001). Essa estável forma monometilada de Se é diretamente metabolizada à metil-senenol por intermédio das enzimas β-liases e, diferentemente de seleno-metionina, *Se*-metil-seleno-cisteína não pode ser incorporada em proteínas de forma não específica (ABDULAH et al., 2005; FOSTER; KRAUS; GANTHER, 1986). O metil-senenol é um composto instável que age diretamente sobre os metabolitos carcinogênicos.

Partes comestíveis de várias brássicas (e.g. brócolis, couve-flor, repolho, couve-chinesa, couve e couve-de-Bruxelas) são amplamente consumidas em todo o mundo. Recentemente, os brotos de brássicas (notadamente brotos de brócolis) têm sido comumente utilizados em países desenvolvidos para preparo de saladas frescas, por serem ricas fontes de compostos bioativos, particularmente glucosinolatos (GU; GUO; GU, 2012; MARTINEZ-VILLALUENGA et al., 2010; NICOLA et al., 2013; WEI; MIAO; WANG, 2011; YUAN et al., 2010).

Glucosinolatos (ß-tioglicosídio-N-hidroxissulfatos) fazem parte de um grupo de compostos fitoquímicos encontrados, na grande maioria, em plantas da ordem Brassicales, podendo exercer notáveis benefícios à saúde humana (TRAKA; MITHEN, 2009). Isotiocianatos, fenetil-isotiocianatos e indole-3-

carbinol são exemplos de produtos de hidrólises de glucosinolatos que agem no organismo como agentes quimiopreventivos (BREW et al., 2009; CARTEA et al., 2008; KRAJKA-KUZNIAK et al., 2011; LI et al., 2010; TAROZZI et al., 2012). Entretanto, nem todos os glucosinolatos são benéficos e, ainda, produtos do metabolismo de alguns glucosinolatos podem causar sérios danos à saúde. Por exemplo, o excesso de goitrina (produto da hidrólise do glucosinolato progoitrina) no organismo causa hipertrofia na glândula tireoide (bócio) (LIU et al., 2012; VERMOREL; HEANEY; FENWICK, 1988). Consequentemente, uma adequada nutrição com glucosinolatos requer não apenas o conhecimento da quantidade total presente no alimento, mas, também de cada glucosinolato individualmente.

Atualmente são conhecidas mais de 120 diferentes estruturas de glucosinolatos, os quais são classificados em três grandes grupos: alifáticos, indólicos e aromáticos (BELLOSTAS et al., 2007; SONDERBY; GEU-FLORES; HALKIER, 2010). As brássicas são umas das poucas culturas agrícolas que sintetizam e acumulam glucosinolatos em suas partes comestíveis. Diferentes tipos e concentrações de glucosinolatos são encontrados entre as brássicas (CARTEA et al., 2008; KUSHAD et al., 1999). Entretanto, atualmente ainda há poucas informações a respeito de brotos. Alguns estudos têm relatado que brotos de brócolis apresentam elevadas concentrações de glucorafanina, um glucosinolato alifático com potente atividade anticancerígena (FAHEY; ZHANG; TALALAY, 1997; KENSLER et al., 2012). A glucorafanina é precursora direta de sulforafano, um isotiocianato que atua na indução de enzimas da fase II (GU; GUO; GU, 2012; LI et al., 2010). As enzimas da fase II, conhecidas, também, como enzimas de desintoxicação, convertem compostos altamente nocivos (e.g., metabólitos carcinogênicos)

oriundos do metabolismo em formas hidrofílicas e bem menos reativas, facilitando, assim, a excreção desses compostos por meio da urina ou bílis (CHEN, 2012).

2.3 Fosfito e sua utilização na agricultura

A baixa disponibilidade de fósforo (P) no solo para as plantas é um dos fatores que mais limita a produtividade das culturas agrícolas em regiões tropicais e subtropicais do mundo. Nessas regiões, a maior parte do ânion fosfato (P^{+5}, ânions $H_2PO_4^-$ e HPO_4^{2-}; principais formas de P usadas para nutrição de plantas) fornecido ao solo é fixada nas argilas de forma não disponível para as plantas, ou formam complexos insolúveis com os íons alumínio e ferro sob condições ácidas (NOVAIS; SMITH, 1999; WISSUWA, 2003).

Na década de 1970, foi verificado que o efeito fungicida da molécula etil-fosfonato (comercializado na época como Aliette® ou Fosetil-Al) era resultante do ânion fosfito liberado na planta (FENN, 1989; FENN; COFFEY, 1984; GUEST; GRANT, 1991; MCDONALD; GRANT; PLAXTON, 2001). Assim, o P na forma de fosfito (P^{+3}; ânions $H_2PO_3^-$ e HPO_3^{2-}), também, passou a ser utilizado na agricultura para propostas fitopatológicas. A partir de então vários trabalhos têm demonstrado que o fosfito é eficiente na prevenção e controle de algumas importantes doenças de plantas causadas por organismos classificados taxonomicamente no filo Oomycota, e.g. *Bremia* spp., *Peronospora* spp., *Phytophthora* spp., *Plasmopara* spp. e *Pythium* spp. (BERTRAND et al., 1977; COOK; LANDSCHOOT; SCHLOSSBERG, 2009; DERCKS; BUCHENAUER; CRUGER, 1986; FENN; COFFEY, 1984; ORBOVIC et al., 2008; REUVENI; SHEGLOV; COHEN, 2003; SILVA et al., 2011; SMILLIE;

GRANT; GUEST, 1989). Esse efeito do fosfito baseia-se em dois diferentes mecanismos de atuação. O primeiro é uma ação tóxica direta sobre o patógeno (COOK; LANDSCHOOT; SCHLOSSBERG, 2009; NIERE; DEANGELIS; GRANT, 1994; SMILLIE; GRANT; GUEST, 1989) e o segundo é de um modo indireto pelo qual o fosfito estimula positivamente o metabolismo secundário da planta, aumentando as respostas de defesa durante situações de estresse biótico e abiótico (DANIEL; GUEST, 2005; DERCKS; BUCHENAUER; CRUGER, 1986; JACKSON et al., 2000). Em acordo com o modo de ação indireto, fosfito foi reportado por ter promovido alterações bioquímicas na periderme e córtex em cultura da batata (*Solanum tuberosum* L.) (OLIVIERI et al., 2012), estímulos na atividade de fenilalanina amônia-liase e na biossíntese de fitoalexinas (NEMESTOTHY; GUEST, 1990), excitação sobre a atividade de guaiacol peroxidases em plantas de milho (*Zea mays* L.) (AVILA et al., 2011) e incremento na concentração de ácido ascórbico em plantas de morangueiro (*Fragaria* x *ananassa* Duch.) (MOOR et al., 2009).

Em adição à aplicação na área fitopatológica, produtos fundamentados em fosfito vêm sendo divulgados como um insumo de função mista, sendo também usado como fertilizante e bioestimulante de planta (THAO; YAMAKAWA, 2009). Sais de fosfito são recomendados como fertilizante porque eles contêm em sua composição um cátion que pode ser um nutriente de planta (e.g., K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+} e Zn^{2+}) e, particularmente, o P. Considerando que a adubação fosfatada é um dos principais empecilhos para a agricultura dos países localizados em regiões tropicais e subtropicais do mundo, a divulgação de produtos baseados em fosfito, como também uma fonte nutricional de P para as culturas agrícolas é interessante para a sua comercialização. Ademais, em geral, há maior dificuldade em registrar um

insumo agrícola como fungicida que como fertilizante.

O respaldo científico para a consideração de fosfito como um fertilizante de P e, ao mesmo tempo, também, como um bioestimulante de crescimento de planta foi dado pelos resultados de pesquisas publicados na década de 1990 (THAO; YAMAKAWA, 2009). Lovatt (1990b) relatou que a deficiência de P em citrus (*Citrus* spp.) causou alterações no metabolismo do nitrogênio, mas a aplicação de fosfito de potássio restabeleceu o metabolismo normal das plantas, assim como o seu crescimento. Na mesma época, essa autora, também, reportou que a frutificação e produção de abacateiro (*Persea americana* L.) foram melhoradas com a aplicação foliar de fosfito (LOVATT, 1990a). Lovatt e Mikkelsen (2006) relataram que o fosfito pode estimular a rota metabólica do ácido chiquímico, promover alterações hormonais e influenciar o metabolismo de açúcar, resultando em maior intensidade floral, produção e melhor qualidade dos frutos (e.g., maior concentração de sólidos solúveis). Albrigo (1999), Rickard (2000) e Watanabe (2005) também mencionaram efeitos positivos de fosfito sobre a nutrição de P e produção de algumas culturas.

Por outro lado, recentes trabalhos questionam a utilização de fosfito como uma fonte de P para as culturas, apesar de ele ser bem absorvido pelas raízes e folhas. Em vários desses estudos têm sido relatado que as plantas não se beneficiam do P fornecido na forma de fosfito para o seu desenvolvimento e crescimento e que, ainda, o ânion fosfito não pode ser oxidado a ânion fosfato em tempo hábil para propostas de nutrição, nem no solo (por intermédio da atividade de algumas bactérias) e muito menos na planta (THAO; YAMAKAWA, 2009). Além do mais, em condições de deficiência de P, o fornecimento de fosfito pode ser nocivo para o desenvolvimento e o crescimento

vegetal, o mesmo não ocorrendo em plantas supridas adequadamente em fosfato (FORSTER et al., 1998; THAO et al., 2008; THAO; YAMAKAWA, 2010; THAO; YAMAKAWA; SHIBATA, 2009; ZAMBROSI; MATTOS; SYVERTSEN, 2011). Nesse caso, parece que o fosfito pode inibir a resposta da planta à deficiência de fosfato, tal como a atividade de fosfatases ácidas (LEE et al., 2005; TICCONI; DELATORRE; ABEL, 2001; VARADARAJAN et al., 2002). Nesse contexto, McDonald, Grant e Plaxton (2001) reportaram que os efeitos benéficos de fosfito sobre as culturas podem estar relacionados somente com o controle de patógenos, dentre os quais, a presença desses em reduzidas proporções (sem exibir sintomas visuais) são suficientes para reduzir a produção e a qualidade dos produtos agrícolas.

Segundo a literatura, entre os seres vivos, apenas algumas estirpes de bactérias são capazes de oxidar fosfito a fosfato (WHITE; METCALF, 2007). Em *Pseudomonas stutzeri* WM88, essa oxidação é oriunda da expressão do gene *ptx*D que codifica uma enzima (oxidorredutase) específica para fosfito (COSTAS; WHITE; METCALF, 2001). Recentemente, por meio de técnicas modernas de biologia molecular, foi produzido linhagens de *Arabidopsis* sp. que expressam o gene *ptx*D e, consequentemente, que apresentam a capacidade de converter fosfito a fosfato dentro de seus tecidos (LOPEZ-ARREDONDO; HERRERA-ESTRELLA, 2012). Esses autores relataram que as novas linhagens transgênicas de *Arabidopsis* sp. requerem 30 a 50% menor fertilização de P, quando este nutriente é fornecido como fosfito que como fosfato.

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SEGUNDA PARTE

ARTIGO 1

Impact of selenium supply on *Se*-methylselenocysteine and glucosinolate accumulation in selenium-biofortified Brassica sprouts

Artigo redigido conforme normas do periódico ao qual foi aprovado para publicação. Essa é uma versão preliminar, considerando que o conselho editorial do periódico poderá sugerir alterações para adequá-lo ao seu próprio estilo. Todavia, a presente tese será disponibilizada somente após a publicação da versão final desse artigo pelo periódico.

Currently (July 2013) this article is approved for publication in a peer-reviewed scientific journal. This is a preliminary version and it may be revised before publication. The present thesis will be made publicly available only after the publication of the final version of this article by the scientific journal.

Impact of selenium supply on *Se*-methylselenocysteine and glucosinolate accumulation in selenium-biofortified Brassica sprouts

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Abstract

Brassica sprouts are widely marketed as functional foods. Here we examined the effects of Se treatment on the accumulation of anticancer compound *Se*-methylselenocysteine (SeMSCys) and glucosinolates in Brassica sprouts. Cultivars from the six most extensively consumed Brassica vegetables (broccoli, cauliflower, green cabbage, Chinese cabbage, kale, and Brussels sprouts) were used. We found that Se-biofortified Brassica sprouts all were able to synthesize significant amounts of SeMSCys. Analysis of glucosinolate profiles revealed that each Brassica crop accumulated different types and amounts of glucosinolates. Cauliflower sprouts had high total glucosinolate content and broccoli sprouts contained high levels of glucoraphanin, a precursor for potent anticancer compounds. Although the literature has reported an inverse relationship between accumulation of Se and glucosinolates in mature Brassica plants, Se supply generally did not affect the glucosinolate accumulation in Brassica sprouts. We conclude that Brassica vegetable sprouts can be biofortified with Se for the accumulation of SeMSCys without negative effects on chemopreventive glucosinolate contents.

Keywords: Brassica sprouts; Selenium; *Se*-methylselenocysteine; Glucosinolates; Function food

1. Introduction

Selenium (Se) is an essential trace element for mammals and its deficiency affects 500–1000 million people worldwide. As a constituent of 25 selenoproteins, Se plays critical roles in a variety of physiological processes,

such as in immune function, antioxidant defense system, and thyroid hormone metabolism (Rayman, 2012). Se is associated with reduced incidence of viral infections (e.g. measles, hepatitis, influenza, and HIV-AIDS), cardiovascular diseases (e.g. Keshan disease), and degenerative osteoarticular disorders (e.g. Kashin–Beck disease) (Rayman, 2012). In addition, Se has been implicated to act as an anticarcinogenic agent. Although the recent SELECT trial (the Selenium and Vitamin E Cancer Preventive Trial) shows that Se in the form of selenomethionine (SeMet) does not prevent prostate cancer in healthy men (Lippman et al., 2009), a large number of animal studies have clearly demonstrated the anticancer property of monomethylated selenoamino acids (Dong, Lisk, Block, & Ip, 2001; Medina, Thompson, Ganther, & Ip, 2001). Monomethylated forms of Se, especially *Se*-methylselenocysteine (SeMSCys) and γ-glutamyl-*Se*-methylselenocysteine, are precursors to methylselenol, which has potent cancer chemopreventive activity (Dong et al., 2001; Medina et al., 2001).

Glucosinolates (β-thioglucoside N-hydroxysulphates) are a group of phytochemical compounds that have also received special attention due to their effects on human health. Several aliphatic and indole glucosinolate breakdown products, e.g. isothiocyanates, phenethyl isothiocyanate, and indole-3-carbinol, are known as excellent cancer-preventing agents (Brew, Aronchik, Kosco, McCammon, Bjeldanes, & Firestone, 2009; Krajka-Kuzniak, Szaefer, Bartoszek, & Baer-Dubowska, 2011; Li et al., 2010). In contrast, other glucosinolate hydrolytic produces, e.g. oxazolidine-2-thione, exhibit adverse effects on health to cause goiter (Liu, Hirani, McVetty, Daayf, Quiros, & Li, 2012; Vermorel, Heaney, & Fenwick, 1988). Consequently, not only the total

glucosinolate content but also the knowledge of the glucosinolate profile that presents in the food is important for healthy dietary habits.

Brassica (*Brassica* spp.) vegetable crops are known as chemopreventive food. They contain many bioactive compounds, such as carotenoids, vitamin C, folate, soluble fibre, and glucosinolates, with protective roles against cancer (Kim & Park, 2009). While most Se enriched staple crops (such as cereals) accumulate the proteogenic selenoamino acid SeMet, Brassica plants are a special group of crops that have high ability to synthesize and accumulate monomethylated forms of Se, such as SeMSCys (Terry, Zayed, de Souza, & Tarun, 2000). Consequently, it is proposed that biofortification of Brassica crops with Se further increases their chemopreventive activities besides being a dietary source of a number of other bioactive compounds (Abdulah et al., 2009; Finley, 2005).

Brassica vegetables are consumed worldwide. Recently, Brassica sprouts (notably broccoli sprouts) have also been marketed as excellent functional foods. While edible parts of mature Brassica vegetables have been studied extensively (Kushad et al., 1999; Lyi et al., 2005), comparative less researches have investigated chemopreventive compounds in Brassica sprouts, and most of these studies have focused on glucosinolates in broccoli sprouts. Thus, the aims of this work were to evaluate the bioactive forms of Se and the different types of glucosinolates present in sprouts of various Brassica crops, and to investigate whether it is feasible to simultaneously increase of Se and glucosinolate compounds given the antagonistic relationship between Se and S metabolism in plants. To this end, we selected sprouts of Brassica vegetable crops extensively consumed throughout the world, which include three cultivars of broccoli (*Brassica oleracea* L. var. *italica*), three cultivars of cauliflower

(*Brassica oleracea* L. var. *botrytis*), three cultivars of cabbage (*Brassica oleracea* L. var. *capitata*), three cultivars of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), three cultivars of kale (*Brassica oleracea* L. var. *acephala*), and one cultivar of Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*), and examined the effects of Se treatment on total Se content and synthesis of the anticancer compound SeMSCys, as well as on the levels of total and individual glucosinolates, total sulphur (S), and total free amino acids in these Brassica sprouts.

2. Materials and methods

2.1 Brassica vegetable crop seeds

Brassica seeds were obtained either commercially (Harris Seeds, Rochester, NY) or as gifts. A total of 16 different cultivars were used in this study, which included three cultivars of broccoli (*Brassica oleracea* L. var. *italic*, cultivars Packman, Diplomat and GYPSY), three cultivars of cauliflower (*Brassica oleracea* L. var. *botrytis*, cultivars Graffiti, Apex and Absolute), three cultivars of green cabbage (*Brassica oleracea* L. var. *capitata*, cultivars Bravo, Blue Lagoon and Early Thunder), three cultivars of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*, cultivars Tender Gold, Blues, and Autumn 56), three cultivars of kale (*Brassica oleracea* L. var. *acephala*, cultivars Vates, Winterbor and Blue Ridge), and one cultivar of Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*, cultivar Oliver).

2.2 Sprout growth and treatments

Seeds were sown on two sheets of filter paper (3 mm, Whatman®) soaked either with MilliQ water (control) or 50 µM Na_2SeO_4 (Sigma-Aldrich) solution in Magenta boxes (Bio-World, Dublin, OH) and grown in a growth chamber with photoperiod of 16/8 h light/dark and temperature at 22/18 ^oC day/night. Treatments consisted of 16 different Brassica cultivars at two growth solutions (water or 50 µM Na_2SeO_4) with three replicates. During sprout growth, an additional 1mL of either water or 50 µM Na_2SeO_4 was added every 24 h. After 7 days, sprouts were harvested, washed with MilliQ water, and frozen in liquid nitrogen. The samples were ground with mortar and pestle to fine powder in liquid nitrogen and dried for 48 h in a freeze-dry system (Labconco FreeZone®, 6 litre Benchtop Freeze Dry System Model 77520). The lyophilized samples were stored in a desiccator at 4 °C until used.

2.3 Total Se and S Analysis by an Inductively Coupled Plasma (ICP) Trace Analyzer

Freeze-dried samples of 100 mg were weighed into glass digestion tubes and acid-digested in 1.0 mL HNO_3 with 1.0 mL $HClO_4$ at 120 °C for 1 h and then at 220 °C until $HClO_4$ fumes were observed. Total Se and S contents were determined using an ICP trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) as described previously by Lyi, Heller, Rutzke, Welch, Kochian, and Li (2005).

2.4 Determination of SeMSCys and free amino acids

Analysis of SeMSCys and free amino acids were performed according to the method described previously (Ramos, Rutzke, Hayes, Faquin, Guilherme, & Li, 2011a; Ramos, Yuan, Faquin, Guilherme, & Li, 2011b) with some modifications. Freeze-dried tissues (25 mg) were extracted overnight at 4°C in 50 mM HCl (20:1, v/w) and centrifuged at 12,000 g for 12 min three times to remove cell debris. AccQ.Tag derivatives of extracted SeMSCys and free amino acids were obtained using the AccQ•Tag Ultra UPLC derivatization kit according to the manufacturer's protocol (Waters Corporation, Milford, USA). Analysis of the derivatized amino acids was performed on an Acquity UPLC™ system (Waters) using an AccQ.Tag Ultra column (100 mm × 2.1 mm). SeMSCys level was calculated based on peak area and calibration curves generated from commercial standard (Sigma-Aldrich) (Ramos et al., 2011b). Free amino acids were identified and their total content was calculated based on peak area and calibration curves generated with amino acid standard H (Pierce) and commercially available individual amino acids (Sigma) according to the method described previously (Ramos et al., 2011a).

2.5 Analysis of glucosinolates

Extraction of glucosinolates was performed as described (Ramos et al., 2011b) with slight modifications. Freeze-dried tissues (25 mg) were extracted in 1.4 mL of 80% MeOH preheated to 75-80 °C. After incubation in water bath for 15 min at 80 °C, the extracts were centrifuged at 12,000 g for 12 min twice, and 0.8 mL of supernatants were added to DEAE Sephadex A-25 columns. Then, 140 µL of sulfatase (15 U, Sigma) were added to each column and left overnight in dark at room temperature. Desulfo-glucosinolates were eluted with 0.2 mL of

80% MeOH and 0.2 mL of water, Speedvac-dried, and resuspended in 600 µL water. The desulfo-glucosinolates were analyzed in an Acquity UPLC™ system (Waters Corporation, Milford, USA) using an HSS T3 column (1.8 µm, 100 mm × 2.1 mm) following the method described previously (Ramos et al., 2011b). Individual glucosinolate level from samples was calculated based on peak area and calibration curves generated from commercial sinigrin standard (Sigma-Aldrich).

2.6 Identification of individual glucosinolate by LC-MS/MS

Identification of each desulfo-glucosinolate was performed on Acquity UPLC™ system coupled to a Xevo G2 QTof mass spectrometer equipped with a LockSpray source (Waters Corporation, Milford, USA). Briefly, the reconstituted desulfo-glucosinolate sample (5 µL) was injected and separated on HSS T3 column employing a 3-min gradient of 7% to 30% acetonitrile in 0.1% formic acid at flow rate of 500 µL/min (Kushad et al., 1999). The separated desulfo-glucosinolates were detected by UV absorbance at 229 nm and the Xevo G2 QTof using a standard ion source. The Xevo G2 QTof was operated in positive ion, data-dependent acquisition (DDA) mode with a capillary voltage of 2.5 kV, a sample cone voltage of 10 V, and source temperature at 120 ^{o}C. The QTof was externally calibrated using the sodium formate ions from *m/z* 50 - 1200. The MS and MS/MS data were post-acquisition lock mass corrected using the monoisotopic mass at *m/z* 566.2771 Da of the single charged ion of Leucine Enkephalin.

Identification of desulfo-glucosinolates was based on the protonated precursor ion masses $(M+H)^+$ and the glucosinolate-specific fragment ions such

as an ion generated through the neutral loss of the sugar group ($C_5H_{10}O_5$) resulting in the fragment ion with the general formula $(M+H-C_6H_{10}O_5)^+$ and the metal ion adducts: $(M+Na)^+$ and $(M+K)^+$ (Mellon, Bennett, Holst, & Williamson, 2002; Zimmermann, Gerendas, & Krumbein, 2007). The characteristic loss represented by the general formula $(M+H-C_6H_{10}O_5)^+$ was found to be the most dominant peak in each of the relevant MS/MS spectra, which is particularly useful for identification of any new precursor ions belonging to new types of glucosinolates.

2.7 Statistical analysis

All the data were submitted to variance analysis (ANOVA, $p < 0.05$). Standard errors for all means were calculated.

3. Results

3.1 Total Se and S content in Se-biofortified sprouts

Selenium was not detectable in sprouts of all Brassica vegetable crops germinated in water (control treatment). However, sprouts of all the Brassica crops studied accumulated Se when they were treated with 50 µM Na_2SeO_4 (Fig. 1a).

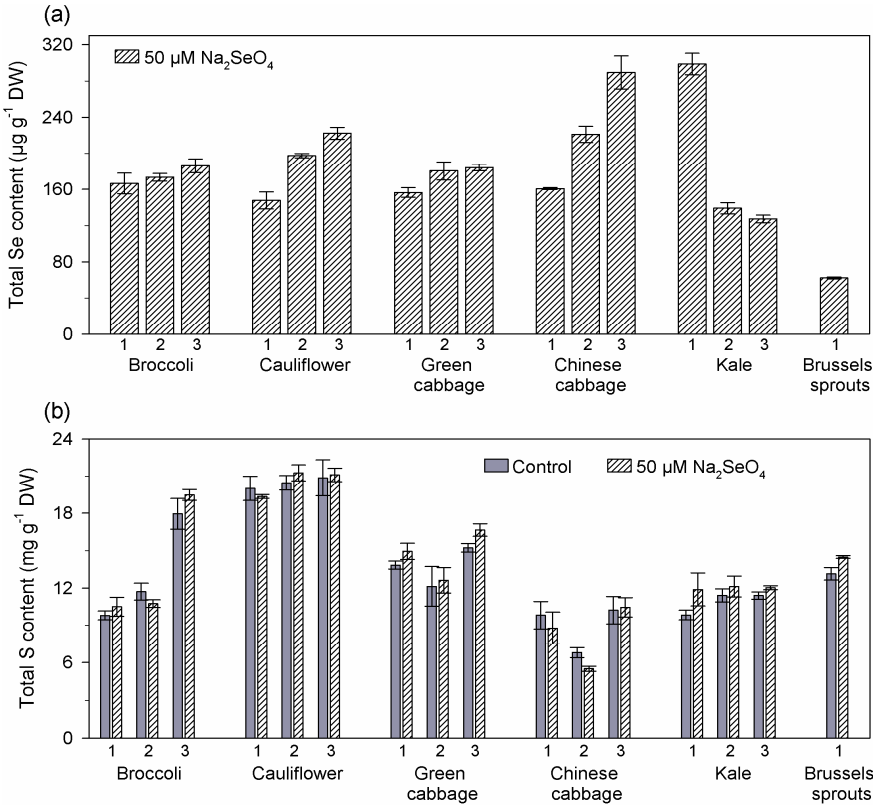

Fig. 1. Content of total Se (a) and total S (b) in 7-day-old sprouts of Brassica crops exposed to 50 µM Na_2SeO_4. Error bars indicate standard deviation (± SD) (*n* = 3).

Broccoli: 1 = cv. Packman, 2 = cv. Diplomat, and 3 = cv. GYPSY;

Cauliflower: 1 = cv. Graffiti, 2 = cv. Apex, and 3 = cv. Absolute;

Green cabbage: 1 = cv. Bravo, 2 = cv. Blue Lagoon, and 3 = cv. Early Thunder;

Chinese cabbage: 1 = cv. Tender Gold, 2 = cv. Blues, and 3 = cv. Beijing Autumn 56;

Kale: 1 = cv. Vates, 2 = cv. Winterbor, and 3 = cv. Blue Ridge;

Brussels sprouts: 1 = cv. Oliver.

This Se level was used as it caused no harmful effects of Se treatment on seed germination and sprout growth based on our pilot experiments. Na_2SeO_4 was chosen as Se source because selenate is the most effective form to stimulate SeMSCys synthesis in Brassica crops (Lyi et al., 2005) and the major inorganic Se form used to biofortify crops (Hawkesford & Zhao, 2007).

In general, the levels of Se accumulation among sprouts of different Brassica crops were similar, although 2-fold variation between cultivars was observed (Fig. 1a). Brussels sprouts appeared to have less capacity to accumulate Se than all the other sprouts studied. Variation of total Se content among sprouts of the three selected broccoli cultivars as well as among the sprouts of the three green cabbage cultivars were not significantly different (ANOVA, $p > 0.05$). However, significant variation (ANOVA, $p < 0.05$) among sprouts of cauliflower cultivars, Chinese cabbage cultivars, and kale cultivars was observed. The average total Se content in cauliflower cv. Graffiti was 25% and 34% lower than that in cauliflower cv. Apex and cv. Absolute, respectively. Similarly, the average total Se content in Chinese cabbage cv. Tender Gold was 28% and 44% lower than that in cv. Blues and cv. Beijing Autumn 56, respectively. In kale, cv. Vates exhibited 2.1 and 2.3-fold higher total Se content than cv. Winterbor and cv. Blue Ridge, respectively.

As an S analog, Se supply affects S uptake and assimilation (Broadley, Brown, Cakmak, Ma, Rengel, & Zhao, 2012; Pilon-Smits & Quinn, 2010). Thus, total S content in sprouts of Brassica crops was measured. In the absence of Se, sprouts of the three cauliflower cultivars all contained high level of total S content, while sprouts of Chinese cabbage had relatively low total S content (Fig. 1b). Up to 2-fold variation of total S content was observed between broccoli cultivars and between Chinese cabbage cultivars. The cultivars of the

other Brassica crops showed similar levels of total S content. In all Brassica cultivars studied, supplementation of Se at 50 µM Na_2SeO_4 did not significantly affect total S content in sprouts when compared with the controls (without Se supply).

3.2 Sprouts of Brassica crops accumulate various levels of SeMSCys

While sprouts grown in water (control treatment) contained undetectable amounts of SeMSCys, all the sprouts supplied with 50 µM Na_2SeO_4 accumulated substantial levels of SeMSCys (Fig. 2).

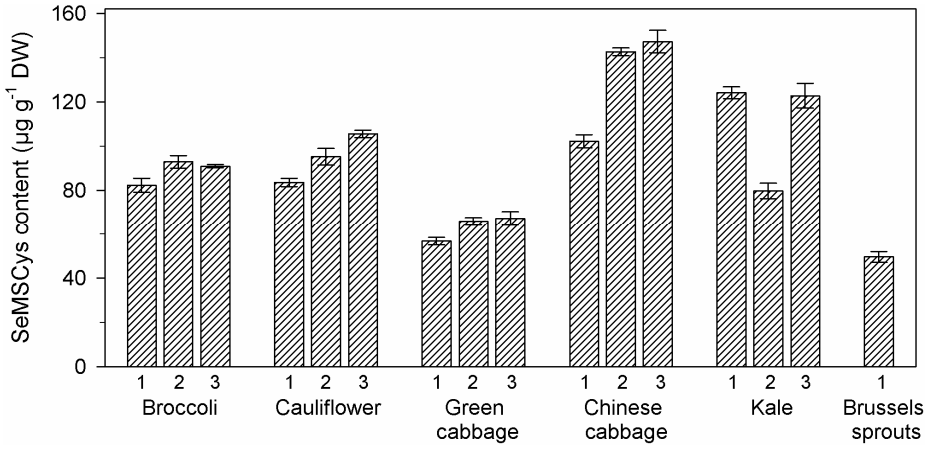

Fig. 2. SeMSCys content in 7-day-old sprouts of Brassica crops treated with 50 µM Na_2SeO_4. Error bars indicate standard deviation (± SD) (*n* = 3). The cultivars used are as described in Figure 1 legend.

Variation of SeMSCys content among sprouts of the Brassica crop cultivars was in general correlated with total Se content, with some notable

exceptions. Sprouts of both green cabbage cultivars and Brussels sprouts had low SeMSCys content in comparison with sprouts of the other Brassica crops. Chinese cabbage accumulated relatively high SeMSCys content. No significant difference in the SeMSCys content between sprouts of broccoli cultivars and green cabbage cultivars was observed. On the other hand, sprouts of cauliflower cultivars exhibited significant difference in SeMSCys content with 26% higher in cv. Absolute than cv. Graffiti. Large and significant variation of SeMSCys content was found between sprouts of Chinese cabbage cultivars and kale cultivars. The average SeMSCys content in Chinese cabbage cv. Tender Gold was approximately 30% lower than that in either cv. Blues or cv. Beijing Autumn 56. In kale crop, cv. Winterbor exhibited around 35% lower average SeMSCys content than either cv. Vates or cv. Blue Ridge.

3.3 Sprouts of Brassica crops contain different individual glucosinolates with various abundances

Glucosinolates in sprouts of the Brassica crops studied were separated by UPLC and identified by MS/MS. A total of 14 glucosinolates were identified from sprouts of these Brassica crops, which included nine aliphatic glucosinolates, four indole glucosinolates, and one aromatic glucosinolate (Fig. 3).

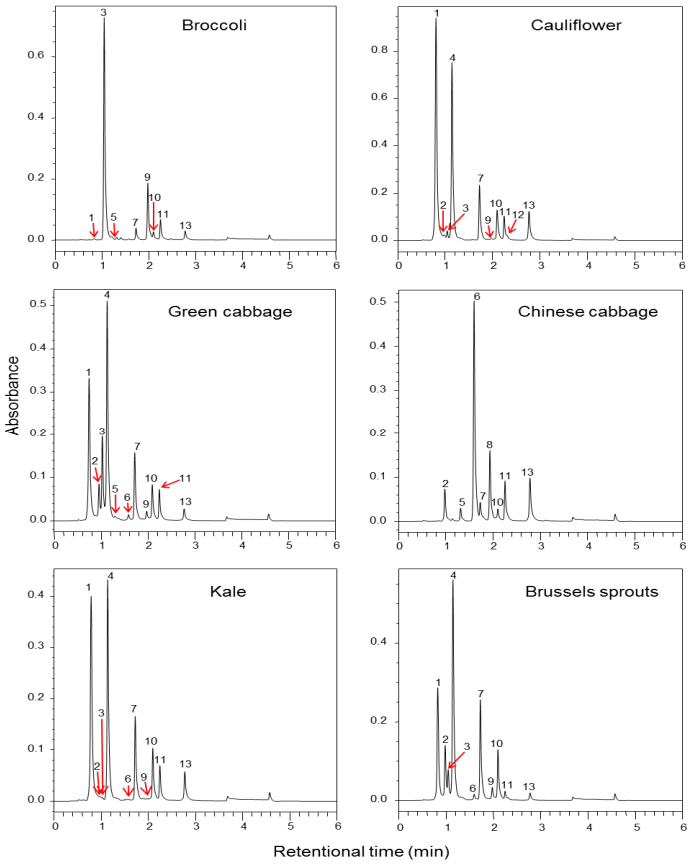

Fig. 3. UPLC elution chromatograms of individual glucosinolate (as desulpho-glucosinolate) in 7-day-old sprouts of Brassica crops. Individual glucosinolate: glucoiberin (1), progoitrin (2), glucoraphanin (3), sinigrin (4), glucoalyssin (5), gluconapin (6), glucoiberverin/4-hydroxyglucobrassicin (7), glucobrassicanapin (8), glucoerucin (9), glucobrassicin (10), 4-methoxyglucobrassicin (11), gluconasturtiin (12), and neoglucobrassicin (13). Numbers 1-6, 7 (glucoiberverin), 8 and 9 are aliphatic glucosinolates; numbers 7 (4-hydroxyglucobrassicin), 10, 11 and 13 are indole glucosinolates; and number 12 is aromatic glucosinolate.

The aliphatic glucosinolates were glucoiberin (3-methylsulphinylpropyl glucosinolate), progoitrin (2-hydroxy-3-butenyl glucosinolate), glucoraphanin (4-methylsulphinylbutyl glucosinolate), sinigrin (2-propenyl glucosinolate), glucoalyssin (5-methylsulphinylpentyl glucosinolate), gluconapin (3-butenyl glucosinolate), glucobrassicanapin (4-pentenyl glucosinolate), glucoiberverin (3-methylthiopropyl), and glucoerucin (4-methylthiobutyl glucosinolate). The indole glucosinolates were 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl glucosinolates), glucobrassicin (indol-3-ylmethyl glucosinolate), 4-methoxyglucobrassicin (4-methoxyindol-3-ylmethyl glucosinolate), and neoglucobrassicin (1-methoxyindol-3-ylmethyl glucosinolate). The aromatic glucosinolate was gluconasturtiin (2-phenylethyl glucosinolate). The peaks of glucoiberverin and 4-hydroxyglucobrassicin were overlapped. Contents of six major glucosinolates identified in sprouts of each Brassica crop are shown in Figure 4.

In sprouts of the three broccoli cultivars, glucoraphanin was the dominant glucosinolate constituting approximately 70% of the total glucosinolate content (Fig. 4a). Glucoerucin was the second most abundant glucosinolate consisting of approximately 14% of the total glucosinolate content. The other four main glucosinolates in broccoli sprouts were glucoiberverin/4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin.

Glucoiberin and sinigrin were the dominant glucosinolates constituting approximately 43% and 35% of the total glucosinolate content, respectively, in sprouts of the three cauliflower cultivars (Fig. 4b). The other main individual glucosinolates in cauliflower sprouts were glucoiberverin/4-

hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin.

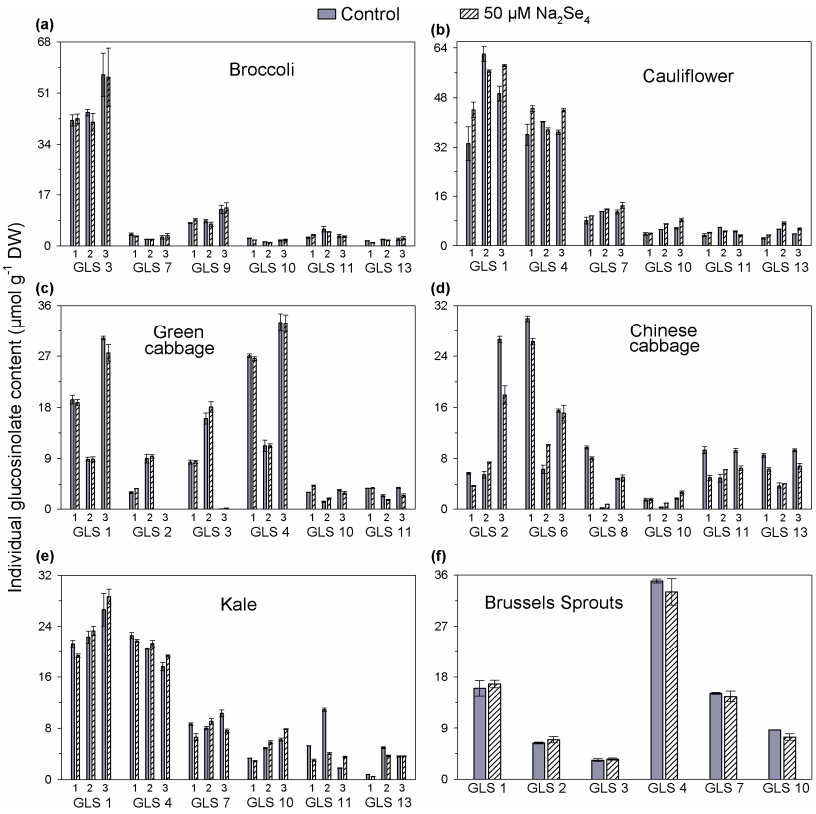

Fig. 4. Contents of 6 main glucosinolates in 7-day-old sprouts of Brassica crops. Error bars indicate standard deviation (± SD) (*n* = 3). Individual glucosinolate: glucoiberin (GLS1), progoitrin (GLS 2), glucoraphanin (GLS 3), sinigrin (GLS 4), gluconapin (GLS 6), glucoiberverin/4-hydroxyglucobrassicin (GLS 7), glucobrassicanapin (GLS 8), glucoerucin (GLS 9), glucobrassicin (GLS 10), 4-methoxyglucobrassicin (GLS 11), and neoglucobrassicin (GLS 13). The cultivars used are as described in Figure 1 legend.

The individual glucosinolate content had large variation among the cultivars in sprouts of green cabbage (Fig. 4c). Glucoiberin and sinigrin were the dominant glucosinolates in cv. Bravo (approximately 25% and 35% of the total glucosinolate content, respectively) and cv. Early Thunder (approximately 36% and 41% of the total glucosinolate content, respectively). In contrast, glucoraphanin was the dominant glucosinolate (approximately 26% of the total glucosinolate content) with considerable amounts of glucoiberin, progoitrin, and sinigrin in cv. Blue Lagoon. The other main individual glucosinolates in green cabbage sprouts were glucobrassicin and 4-methoxyglucobrassicin.

Like sprouts of green cabbage, individual glucosinolate content in sprouts of Chinese cabbage also exhibited large variation between cultivars (Fig. 4d). Gluconapin (approximately 46% of the total glucosinolate content) was the dominant glucosinolate followed by progoitrin, glucobrassicanapin, 4-methoxyglucobrassicin, and neoglucobrassicin in cv. Tender Gold. Progoitrin, gluconapin, and 4-methoxyglucobrassicin were present in higher amounts (approximately 25%, 32% and 22% of the total glucosinolate content, respectively) in cv. Blues. In cv. Beijing Autumn 56, progoitrin and Gluconapin were the dominant glucosinolates with a proportion of approximately 35% and 24% of the total glucosinolate content, respectively.

Glucoiberin and sinigrin were the dominant glucosinolates with a proportion of approximately 36% and 31% of the total glucosinolate content, respectively, in sprouts of the three kale cultivars (Fig. 4e). The other main glucosinolates in kale sprouts are glucoiberverin/4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin.

In sprouts of the Brussels sprouts cv. Oliver, sinigrin was the dominant glucosinolate with a proportion of 39% of the total glucosinolate content (Fig.

4f). Glucoiberin and glucoiberverin/4-hydroxyglucobrassicin also were detected in considerable amount. The other main individual glucosinolates in sprouts of Brussels sprouts cv. Oliver were progoitrin, glucoraphanin, and glucobrassicin.

3.4 Total glucosinolate contents vary among sprouts of Brassica crops and are not affected by Se treatment

The total glucosinolate contents that summarized all individual glucosinolates in sprouts of each studied Brassica crop are shown in Figure 5. Sprouts of cauliflower cultivars contained higher levels of total glucosinolates in comparison with the other Brassica crops, and these cultivars shared similar levels of total glucosinolates (Fig. 5).

No dramatic variations in total glucosinolate contents were observed among cultivars of the same Brassica crop, with the exception of Chinese cabbage sprouts, at which the cv. Blues exhibited much lower total glucosinolate content in comparison to the other two cultivars.

In general, supplementation of 50 µM Na_2SeO_4 did not significantly affect the individual glucosinolate content of the sprouts in comparison to the control treatment, except in Se-enriched sprouts of cauliflower (cv. Graffiti and cv. Absolute) and Chinese cabbage (cv. Tender Gold and cv. Beijing Autumn 56) (Fig. 4). Concomitantly, Se supply did not dramatically affect the total glucosinolate accumulation in sprouts of these Brassica crops when compared with the sprouts germinated in water, except that there was a significant increase in total glucosinolate levels in two cauliflower cultivars (cv. Graffiti and cv. Absolute) and a decrease in two Chinese cabbage cultivars (cv. Tender Gold and cv. Beijing Autumn 56) (Fig. 5).

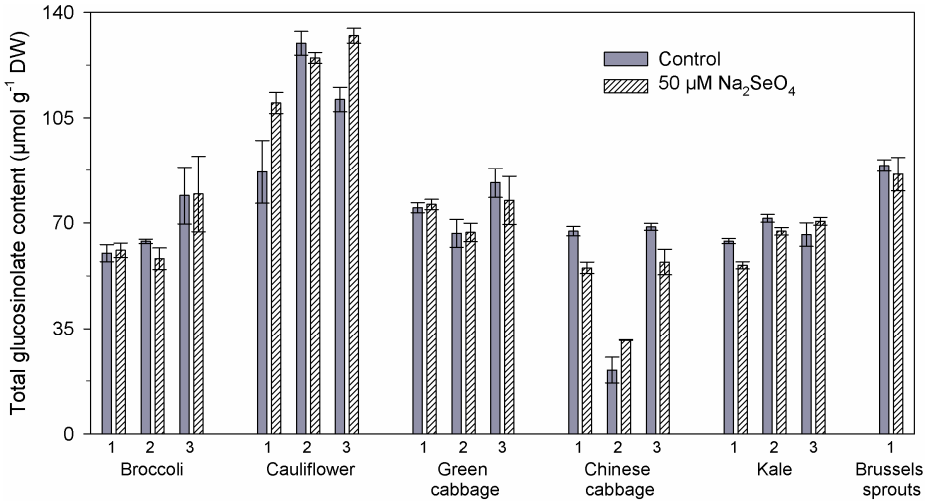

Fig. 5. Total glucosinolate content in 7-day-old sprouts of Brassica crops with and without Na_2SeO_4 supply. Error bars indicate standard deviation (± SD) (*n* = 3). The cultivars used are as described in Figure 1 legend.

3.5 Total free amino acid contents are similar among sprouts of Brassica crops and not affected by Se treatment

The total content of free amino acids from sprouts of various Brassica crops was also measured and shown to be in a similar range although some variation between cultivars of each vegetable crop existed (Fig. 6). There was no significant difference in total free amino acid content in nontreated sprouts among three green cabbage cultivars and three kale cultivars. On the other hand, significant difference in content of total free amino acids was observed in sprouts among cultivars of broccoli, cauliflower and Chinese cabbage.

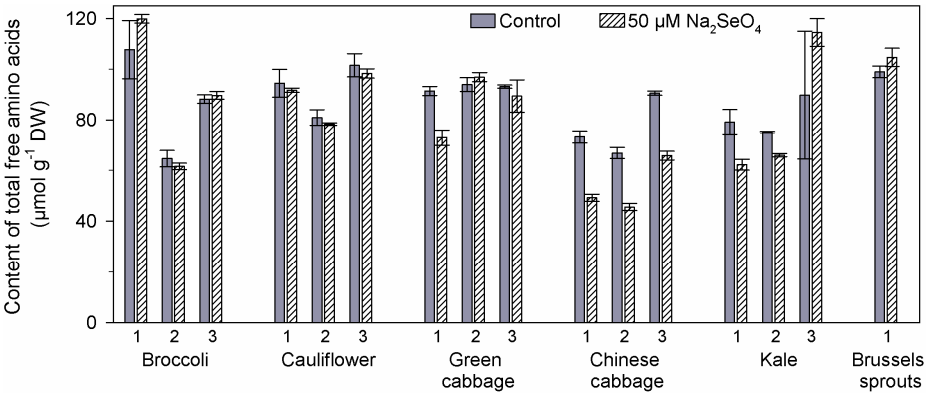

Fig. 6. Total free amino acids in 7-day-old sprouts of Brassica crops with and without Na_2SeO_4 supply. Error bars indicate standard deviation (± SD) (*n* = 3). The cultivars used are as described in Figure 1 legend.

Selenium supplementation at 50 μM Na_2SeO_4 did not dramatically affect total free amino acid content in sprouts of most Brassica crops, except Chinese cabbage (Fig. 6). However, Se supply reduced total content of free amino acids in all three Chinese cabbage cultivars (Fig. 6). A reduced level of total amino acids was also observed in green cabbage cv. Bravo and kale cv. Vates.

4. Discussion

Brassica sprouts have received considerable attention recently due to their capacities in accumulating health beneficial phytochemical compounds, especially those with anti-cancer properties. This study was designed to examine the ability of various Brassica crop sprouts to simultaneously accumulate the anticancer agents, SeMSCys and glucosinolates.

Plants differ widely in their ability to accumulate Se in their tissues and can be classified into Se-accumulators (such as some species of the genera *Astragalus*, *Xylorrhiza* and *Stanleyea*) and non-accumulators (most agricultural plant species) (Broadley et al., 2012). Several Brassica vegetables may accumulate hundreds μg Se g^{-1} DW when grown on soils contained moderate levels of Se, and they are known as Se secondary accumulators (Terry et al., 2000). In our results, sprouts of various Brassica vegetable crops contained approximately 160 μg g^{-1} DW of total Se when germinated in 50 μM Na_2SeO_4, suggesting that the Brassica sprouts accumulated important amounts of Se for human dietary use. However, this total Se level found in broccoli sprouts is lower than that found in other parts of mature broccoli plants when similar concentration of Se was used (Lyi et al., 2005).

The ability of Brassica species to accumulate relatively large amounts of Se is due to its greater ability to accumulate S (Broadley et al., 2012). It is generally believed that selenate is taken up by plants from the soil via sulfate transporters and assimilated via the sulfur assimilation pathway (Pilon-Smits & Quinn, 2010). Thereby, Hawkesford and Zhao (2007) noticed that selenate influx depends primarily upon the level of selenate in the growth medium, on the availability of sulfate which competes for uptake at the transporter active site, on the level of expression of the sulfate transporter genes, and on the discrimination of the transporters for either selenate or sulfate transport.

The Se-biofortified sprouts from all studied Brassica crops were able to synthesize and accumulate significant amounts of SeMSCys, with approximately 50% of total Se accumulated in sprouts being SeMSCys. While there are little data available regarding the accumulation of SeMSCys in sprouts of most Brassica crops, Sugihara, Kondo, Chihara, Yuji, Hattori, and Yoshida

(2004) showed that SeMSCys counts about 75% of total Se in both Se-enriched broccoli and Chinese cabbage sprouts. It is important to mention that in our study the total Se and SeMSCys were extracted and measured by different methods, which may count for the difference in SeMSCys to total Se ratio between our work and that of (Sugihara et al., 2004). On the other hand, the total amount of SeMSCys observed in the sprouts of all Brassica crops may be considered “expressive” since most agricultural species (e.g. cereal crops) are not able to accumulate significant amounts of SeMSCys in their edible parts (Broadley et al., 2012). Brassica vegetables synthesize and accumulate SeMSCys when grown in Se-enriched soils due to their ability to convert the selenoamino acid selenocysteine into various non-protein selenoamio acids, and this conversion is catalyzed by enzyme selenocysteine methyltransferase (Broadley et al., 2012). This pathway is restricted only to a small group of Se-accumulator crops. (Lyi et al., 2005) and (Ramos et al., 2011b) found that SeMCys accumulation in shoot and floret tissues of broccoli correlates closely with the expression of the gene for selenocysteine methyltransferase.

The fact that consumption of Brassica crops decreases the incidence of certain cancer in humans is linked in part to the presence of glucosinolates (Bellostas, Kachlicki, Sorensen, & Sorensen, 2007). Our results demonstrate that sprouts of the Brassica crops studied accumulate considerable amounts of glucosinolates. Cauliflower sprouts had about twice amounts of total glucosinolate content in comparison with sprouts from other Brassica crops.

Analysis of glucosinolate profiles from sprouts of the individual Brassica crops revealed that each crop accumulated different types and amounts of glucosinolates in sprouts. Not all glucosinolates are transformed into biologically active products. Glucoraphanin (an aliphatic glucosinolate) is a

direct precursor of the isothiocyanate sulforaphane that acts as potent monoinducer of phase II enzymes in inactivating carcinogenic metabolites (Li et al., 2010). Due to its important role in human health, glucoraphanin has been investigated in Brassica plants (Gu, Guo, & Gu, 2012). Sprouts of various broccoli cultivars had substantial levels of glucoraphanin (GLS3), which consisted of approximately 70% of the total glucosinolate content. Moderate amounts of glucoraphanin were also found in sprouts of green cabbage (except cv. Early Thunder) and Brussels sprouts cv. Oliver, but undetectable in sprouts of cauliflower, Chinese cabbage and kale cultivars examined. Glucoerucin (another aliphatic glucosinolate, GLS9) was the second most dominant glucosinolate in sprouts of broccoli cultivars, but in a much smaller proportion than glucoraphanin. Glucoerucin is metabolized to the isothiocyanate erucin which also exhibits excellent beneficial effects on human health (Barillari et al., 2005), including as an anticancer agent (Abbaoui et al., 2012). It was present at negligible amount in sprouts of other Brassica crops (Fig. 4). Therefore broccoli sprouts are the best source for providing isothiocyanates for cancer-preventing.

Interestingly, the sprouts of cauliflower and kale cultivars had similar types and proportion of individual glucosinolates, although the total glucosinolate content was much higher in sprouts of cauliflower cultivars (Fig. 5). Glucoiberin (GLS1) and sinigrin (GLS4) were the major glucosinolates in sprouts of cauliflower and kale. They also accumulated abundantly in sprouts of green cabbage and Brussels sprouts cv. Oliver. The breakdown products of glucoiberin and sinigrin have also been reported to be effective in suppressing carcinogenesis, among other benefits to health (Cabello-Hurtado, Gicquel, & Esnault, 2012).

In sprouts of Chinese cabbage, the dominant aliphatic glucosinolates were progoitrin and gluconapin, but their levels varied greatly among cultivars. We also found relative high level of progoitrin (GLS2) in sprouts of green cabbage (except cv. Early Thunder) and Brussels sprouts cv. Oliver. Gluconapin is hydrolyzed to isothiocyanates (Kim et al., 2010). In contrast, progoitrin hydrolysis produces oxazolidine-2-thione (goitrin) which in excess may cause adverse effects on health such as goiter (Vermorel et al., 1988). Consequently progoitrin is less desirable than the other glucosinolates (Liu et al., 2012). Spouts of these Brassica crops also contained low levels of indole glucosinolates, notably 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, glucobrassicin and neoglucobrassicin. Both 4-methoxyindole-3-carbinol and especially indole-3-carbinol, breakdown products of 4-methoxyglucobrassicin and glucobrassicin, respectively, were proven to be efficient anticancer agents (Brew et al., 2009). On the other hand, it was demonstrated that breakdown products of neoglucobrassicin may negatively impact the activation of sulforaphane-induced Nrf2 (Haack et al., 2010), which induces phase II enzymes in inactivating carcinogenic metabolites.

In general, we have showed a large variation of types and amounts of individual glucosinolate among sprouts of the studied Brassica vegetables, although broccoli, cauliflower, green cabbage, kale and Brussels are belonging to the same specie (*Brassica oleracea* L.). This may be justified in part by wide and complex both gene regulatory network and metabolic pathway that regulate the metabolism of indole and aliphatic glucosinolates in plants, as shown by Sonderby, Geu-Flores, and Halkier (2010).

Se biofortification in Brassica sprouts generally did not affect the accumulation of total glucosinolates or the profile of individual glucosinolates.

In contrast with our results for Brassica sprouts, mature Brassica plants treated with Se have been reported to be accompanied by a reduction in glucosinolate contents, and the magnitude of this decrease may vary depending on cultivars (Charron, Kopsell, Randle, & Sams, 2001; Kim & Juvik, 2011; Ramos et al., 2011b; Toler, Charron, Sams, & Randle, 2007). Further, Se supplementation did not affect total amino acid content in sprout of most Brassica crops except Chinese cabbage, which may have low tolerance to Se treatment. Ramos et al. (2011a) evaluated 30 diverse accessions of lettuce (*Lactuca sativa* L.), and reported that Se supply did not significantly alter total amino acid content in most cultivars too.

In conclusion, our data show that various Brassica sprouts exhibit different capacity in accumulating the health beneficial SeMSCys and glucosinolate compounds. Brassica vegetable sprouts can be biofortified with selenium for the accumulation of bioactive selenium without negative effects on anticance glucosinolate content and profiles among the sprouts of the Brassica crops studied.. Hence the Se-biofortified Brassica sprouts further enhance their chemopreventive activities. In accordance with this concept, Abdulah et al. (2009) shows that Se-enriched broccoli sprouts exhibit increased chemosensitivity and apoptosis of LNCaP prostate cancer than normal broccoli. This study will be useful for development of nutraceutical functional foods for better human health.

Acknowledgement

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ARTIGO 2

Assessment of the anticancer compounds *Se*-methylselenocysteine and glucosinolates in Se-biofortified broccoli (*Brassica oleracea* L. var. *italica*) sprouts and florets

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Assessment of the Anticancer Compounds *Se*-Methylselenocysteine and Glucosinolates in Se-Biofortified Broccoli (*Brassica oleracea* L. var. *italica*) Sprouts and Florets

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ABSTRACT:

Broccoli (*Brassica oleracea* L. var. *italica*) is a rich source of chemopreventive compounds. Here, we evaluated and compared the effect of selenium (Se) treatment on the accumulation of anticancer compounds *Se*-methylselenocysteine (SeMSCys) and glucosinolates in broccoli sprouts and florets. Total Se and SeMSCys content in sprouts increased concomitantly with increasing Se doses. Selenate was superior to selenite in inducing total Se accumulation, but selenite is equally effective as selenate in promoting SeMSCys synthesis in sprouts. Increasing sulfur doses reduced total Se and SeMSCys content in sprouts treated with selenate, but not in those with selenite. Examination of five broccoli cultivars reveals that sprouts generally have better fractional ability than florets to convert inorganic Se into SeMSCys. Distinctive glucosinolate profiles between sprouts and florets were observed, and sprouts contained approximately 6-fold more glucoraphanin than florets. In contrast to florets, glucosinolate content was not affected by Se treatment in sprouts. Thus, Se-enriched broccoli sprouts are excellent for simultaneous accumulation of chemopreventive compounds SeMSCys and glucoraphanin.

Keywords: Selenium; *Se*-methylselenocysteine, glucoraphanin, glucosinolates, broccoli sprout, broccoli floret, chemopreventive agent

INTRODUCTION

Selenium (Se) is an essential trace element for mammals. It is estimated that between 500–1000 million people worldwide are deficient in Se (*1*). Se in the form of selenocysteine constitutes 25 selenoproteins in mammals and plays both structural and enzymatic roles such as glutathione peroxidases in

antioxidant functions, thioredoxin reductases in thioredoxin system, and iodothyronine deiodinases in thyroid hormone metabolism (*2*). According to the Institute of Medicine of the National Academy, the RDA (Recommended Dietary Allowance) values for Se is 55 µg day^{-1} for adults (*3*). Deficiency in Se results in a dramatic reduction in activity of selenoproteins in humans, causing various diseases and disorders such as thyroid gland dysfunction, irreversible brain injury, Keshan disease (cardiomyopathy), decreased immune responses to viral infections, and increased risk to various cancers (*2*). Apart from selenoproteins, other (non-protein) selenocompounds have also been implicated to have important health benefits. Interest has been focused on the anticancer property of monomethylated selenoamino acids (*4-7*). Although some selenoproteins may also act as cancer preventive agents (*2, 4*), studies have shown that monomethylated Se forms, especially *Se*-methylselenocysteine (SeMSCys) that serves as precursor to methylselenol, possess potent anticancer activity (*4, 6*).

Ingestion of Se-biofortified plant foods has been assumed to be important to decrease Se deficiency in the population (*1*). In general, plants take up inorganic forms of Se (selenate, Se^{+6} or selenite, Se^{+4}) applied in soil, nutrient solution (in hydroponic culture system) or leaves (through foliar spray) and metabolize them. Given that Se is an analogue of sulfur (S), Se uptake and assimilation in plants follow S metabolic pathways (*8, 9*).

Glucosinolates are a group of S-containing phytochemicals largely found in plants of the order Brassicales. More than 120 different types of glucosinolates are known. They are classified as aliphatic (derived from methionine, alanine, leucine, isoleucine or valine), indole (derived from tryptophan) or aromatic (derived from phenylalanine or tyrosine) glucosinolates

(*10*). Many of these phytochemical compounds have attracted great attention because of their beneficial effects on human health (*11, 12*). Glucoraphanin (4-methylsulphinylbutyl glucosinolate) is an aliphatic glucosinolate that is being widely studied due to its superior anticancer activity. This important glucosinolate is metabolized to isothiocyanate sulforaphane, which acts as potent monoinducer of phase II enzymes in inactivating carcinogenic metabolites (*13, 14*).

Broccoli (*Brassica oleracea* L. var. *italica*) vegetable is known to contain large amount of glucoraphanin besides other chemopreventive glucosinolates (*14-17*). Furthermore, broccoli is part of a small group of agricultural crops that accumulate considerable amounts of SeMSCys when grown in Se containing soils (*18, 19*), thereby increasing its anticancer property (*4, 20*). Florets of mature broccoli plants constitute the main edible part of this crop consumed worldwide. Broccoli sprouts have also been consumed because it is a rich source of bioactive compounds, in particular glucoraphanin (*15, 21*). Early works have shown that there is an inverse relationship between Se and glucosinolate accumulation in mature broccoli plants (*19, 22-25*), but little information is available on broccoli sprouts. In this work we investigated the possibility to simultaneously enrich SeMSCys and glucoraphanin in broccoli sprout and floret, the two edible tissues. For this, we evaluated the total Se accumulation, SeMSCys synthesis and glucosinolate profiles in sprouts and floret of broccoli exposed to different forms of inorganic Se (selenate and selenite), as well as the interaction between Se and S. To obtain a general trend of changes in broccoli, five broccoli cultivars were used in this study.

MATERIALS AND METHODS

Plant Materials and Treatments. Seeds of broccoli (*Brassica oleracea* L. var. *italica*) cv. GYPSY were obtained commercially (Harris Seeds, Rochester, NY). For experiments with sprouts, the seeds were sown on two sheets of filter paper (3 mm, Whatman®) soaked with treatment solutions in Magenta boxes (Bio-World, Dublin, OH), and grown in a growth chamber with photoperiod of 16/8 h light/dark period at temperature of 22/18°C day/night. The treatments consisted of one control (MilliQ water), five Se doses (10, 25, 50, 75 and 100 µM) of either selenate (Na_2SeO_4) or selenite (Na_2SeO_3), and one treatment with 25 µM of each selenate and selenite. A sulfur dosage treatment consisted of three doses (0.1, 1 and 10 mM) of sulfate (Na_2SO_4) in the presence and absence of 50 µM Se (either selenate or selenite). The experiment was conducted in a completely randomized design, and all treatments were performed with three replicates. During sprout growth, an additional 1 mL of either water or solution corresponding to each treatment was added every 24 h. After 7 days, sprouts were harvested, washed with MilliQ water, dried with paper towels, and frozen in liquid nitrogen. The samples were ground with mortar and pestle to fine powder in liquid nitrogen and dried for 48 h in a freeze-dry system (Labconco FreeZone®, 6 litre Benchtop Freeze Dry System Model 77520). The lyophilized samples were stored in a desiccator at 4°C until used.

In addition, two independent experiments (an experiment with broccoli sprouts and an experiment with mature broccoli plants) also were simultaneously carried out in a completely randomized design. Seeds of five broccoli cultivars (Packman, Diplomat, GYPSY, Marathon and De Cicco) were obtained either commercially (Harris Seeds, Rochester, NY) or as gifts. The experiment with sprouts was conducted as described above, and treatments

consisted of five broccoli cultivars exposed to either MilliQ water (control) or 25 µM Se (supplied as selenate, Na_2SeO_4). The experiment with mature broccoli plants was conducted as following. Seeds were sown into 23-cm-diameter pots filled with 6 dm^3 of soil mix (Metro-Mix 360, Sun Gro Horticulture) in a greenhouse with photoperiod of 14/10 h light/dark period at temperature of 24°C. During the plant growth period, soil mix from each pot was fertilized with Osmocote® according to nutritional needs of the broccoli crop. Treatments consisted of five broccoli cultivars grown without (control) or with selenate (Na_2SeO_4) supply. When plants just initiated floral primordia, six applications (twice per week for three weeks) of 100 mL of 1.5 mM Na_2SeO_4 solution were performed in each pot, resulting in a final dosage of 25 µM Se in each application (considering 6 dm^3 of soil mix per pot). The application of 25 µM Na_2SeO_4 was chosen based on previous studies (*7, 22, 26*) and our preliminary tests for its ability to stimulate SeMSCy synthesis without negative effect on plant growth. Plants were harvested individually when heads were fully formed and at market harvest maturity, and the fresh weights of stem, leaves and florets were evaluated. Floret samples were collected from each plant at harvesting time, washed with MilliQ water, dried with paper towels, and immediately frozen in liquid nitrogen. Later, these samples were ground with mortar and pestle to fine powder in liquid nitrogen, freeze-dried and stored as described above.

Analysis of Total Se and S Contents by ICP. Total Se and S contents were determined using an ICP trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA). Freeze-dried samples of 200 mg were weighed into glass digestion tubes, acid-digested, and

measured for their total Se and S levels as described previously (*7*). Each sample was analyzed in triplicate.

Quantification of SeMSCys by UPLC. SeMSCys content in broccoli samples was analyzed following the method essentially as described previously (*19*, *27*). SeMSCys from 25 mg of freeze-dried tissues was extracted in 50 mM HCl (20:1, v/w) at 4°C overnight with continuous shaking. Following centrifugation at 12,000 g for 12 min for three times to remove cell debris, the extracted SeMSCys was tagged with AccQ.Tag using the AccQ•Tag Ultra UPLC derivatization kit according to the manufacturer's protocol (Waters Corporation, Milford, USA). The AccQ.Tag derivatives were analyzed on an Acquity UPLC™ system (Waters Corporation, Milford, USA) using an AccQ.Tag Ultra column (100 mm × 2.1 mm). The concentration of SeMSCys in the samples was calculated based on peak areas and a calibration curve generated from the commercial standard (Sigma-Aldrich).

Analysis of Glucosinolates by UPLC. Extraction and analysis of glucosinolates were carried out following the protocol described by Ramos et al. (*19*). Approximately 25 mg of freeze-dried tissues were mixed in 1.4 mL of 80% MeOH preheated to 75-80°C and vortexed for 10 sec. The mixtures were incubated in water bath for 15 min at 80°C and centrifuged at 12,000 g for 12 min twice. The supernatants (0.8 mL) were transferred to DEAE Sephadex A-25 columns. To each column, 140 µL of sulfatase (15 U, Sigma) were added and incubated at room temperature overnight in dark. Desulfo-glucosinolates were then eluted with 0.2 mL of 80% MeOH followed by 0.2 mL of water. The eluents were combined, speedvac-dried, and dissolved in 600 µL water. The samples were analyzed on an Acquity UPLC™ system (Waters) using an HSS T3 column (1.8 µm, 100 mm × 2.1 mm) and eluted with a mobile phase

consisting of solvent A (water) and solvent B (100% acetonitrile) at a flow rate of 0.65 mL/min for a total of 6 min. Quantification of individual glucosinolate from samples was achieved based on peak areas and a calibration curve constructed from commercial sinigrin standard (Sigma-Aldrich).

Identification of Individual Glucosinolate by LC-MS/MS. LC-MS/MS was performed on Acquity UPLC™ system coupled to a Xevo G2 QTof mass spectrometer with a LockSpray source (Waters Corporation, Milford, USA). The desulfo-glucosinolates from both sprouts and florets of broccoli were separated on HSS T3 column (1.8 µm, 2.1 mm × 100 mm, waters) and then detected by PDA at UV absorbance of 229 nm and the Xevo G2 QTof using a standard ion source. The Xevo G2 QTof was operated in positive ion, data-dependent acquisition (DDA) mode. The MS and MS/MS data was post-acquisition lock mass corrected using the monoisotopic mass at *m/z* 566.2771 Da of the single charged ion of Leucine Enkephalin. Identification of individual glucosinolate was carried out following the methods as reported (*28, 29*). Each desulfo-glucosinolate was identified based on the protonated precursor ion masses $(M+H)^+$ and its group-specific fragment ions including the ion with the loss of a sugar group $(M+H-C_6H_{10}O_5)^+$ and the observed metal ion adducts: $(M+Na)^+$ and $(M+K)^+$.

Statistical Analyses. Statistical analyses of the data were performed using variance analysis (ANOVA) at 5% probability level to test for significant difference between treatment means. The values obtained were expressed as means of three replicates with corresponding standard deviations (± SD).

RESULTS AND DISCUSSION

Se Level in Broccoli Sprout Increases with Increasing Se Dosage. Broccoli is a Se and S accumulator. To examine meticulously the ability of broccoli to accumulate Se, we first performed the dosage analysis in broccoli sprouts. Sprouts of broccoli cv. GYPSY were germinated and grown under different doses of either selenate or selenite. Se was not detectable in broccoli sprouts germinated in MilliQ water. In contrast, the total Se content in the broccoli sprouts increased concomitantly with increasing doses of both selenate and selenite from 10 to 100 µM Se in the growth solution (Table 1). Significantly higher levels of total Se content were observed in those sprouts treated with selenate than selenite ($p \leq 0.0001$). For example, the total Se content in 50 µM selenate and selenite treated sprouts was 179 and 98 µg g^{-1} DW, respectively, showing an over 1.8-fold difference in total Se content. This result is consistent with those reported in leaf and floret of mature broccoli plants (*7*) and others crops (*5*, *27*, *30-32*) showing that selenate is much more effective than selenite in promoting Se accumulation. Noticeably, while the Se content is much lower in selenite treated leaves and florets than selenate treated tissues (*7*), the total Se contents on average were only around 35% lower in selenite treated sprouts than selenate treated ones (Table 1).

The total Se content in broccoli sprouts simultaneously treated with 25 µM of each selenate and selenite was about intermediary to the values obtained for treatments with 50 µM of selenate and 50 µM of selenite (Table 1). These data suggest that there was no interaction between selenate and selenite in affecting total Se content in the broccoli sprouts. In contrast, previous studies in leaf tissue of broccoli show that supplementation of selenite to selenate treatments inhibits the selenate promoted Se accumulation (*7*).

Table 1. Total Se and SeMSCys content in 7-day-old sprouts of broccoli cv. GYPSY exposed to different forms (selenate and selenite) and various dosages of Se.

Se form	Se dose (µM)	Total Se (µg g^{-1} DW)[a]	SeMSCys (µg g^{-1} DW)[a]	Conversion (%)[b]
Selenate (Na_2SeO_4)	10	31.6 ± 1.4	29.2 ± 3.4	40.1
	25	80.0 ± 0.1	62.1 ± 2.7	33.7
	50	178.9 ± 0.9	105.8 ± 2.3	25.7
	75	214.5 ± 0.9	149.2 ± 5.5	30.2
	100	263.2 ± 6.0	157.3 ± 2.2	25.9
Selenite (Na_2SeO_3)	10	19.9 ± 6.3	19.2 ± 0.5	41.8
	25	50.0 ± 0.2	68.9 ± 4.5	59.9
	50	97.8 ± 1.3	112.7 ± 5.9	50.0
	75	146.1 ± 2.0	149.8 ± 3.3	44.5
	100	185.3 ± 2.1	167.4 ± 10.6	39.2
ANOVA[c]	Se form	****	*NS*	
	Se dose	****	****	
	Se form x Se dose	****	*NS*	
25 µM selenate + 25 µM selenite	50	124.7 ± 2.5[d]	103.9 ± 3.4	36.2

[a]Values are averages of three replicates ± SD (standard deviation). [b]Calculated using only the Se (atomic weight = 79) from SeMSCys (molecular weight = 182). [c]*NS* and **** indicate non-significance and significance at $p \leq 0.0001$, respectively. [d]Significant difference ($p \leq 0.05$) between 25 µM selenate + 25 µM selenite treatment and 50 µM Se (either selenate or selenite) treatment.

It is noteworthy that 7-day-old broccoli sprouts exposed to higher dose of selenate at 100 µM and selenite at 75 µM and 100 µM exhibited toxicity symptoms with decreased root growth and purple cotyledons (data not shown). These symptoms were more evident in treatment with 100 µM selenite, showing that selenite was more toxic than selenate to broccoli sprouts.

Selenate and Selenite Are Equally Effective in Promoting SeMSCys Synthesis in Broccoli Sprouts. SeMSCys is a monomethylated form of Se that has been demonstrated to have strong anticarcinogenic activity (*4, 6*). Among many crops, broccoli plants are known to have the ability to accumulate high levels of SeMSCys when grown in Se containing environments (*18, 19*). SeMSCys was not detectable in broccoli sprouts exposed to water (control treatment). Its content increased concomitantly with increasing doses of Se up to 75 µM in growth solution, and it did not differ greatly between treatments at 75 and 100 µM of Se (Table 1). SeMSCys content correlated well with total Se content (Table 1) at dosage up to 75 µM of Se in 7-day-old broccoli sprouts.

Interestingly, unlike the case for total Se content, SeMSCys content in broccoli sprouts was not influenced by the Se form supplied. Both selenate and selenite promote same levels of SeMSCys synthesis in broccoli sprouts (Table 1). Thus, although selenate treated broccoli sprouts accumulated higher amounts of total Se when exposed to the same doses of selenate and selenite, this additional Se accumulation did not convert into SeMSCys. This result disagrees with that reported for mature broccoli plants in previous studies, where selenate is found to be much effective in stimulating SeMSCys accumulation in leaves and florets than selenite (*7*). The mechanisms involved in plant Se metabolism are not similar between the two Se forms. Selenate is taken up by roots and largely transported to shoot where it is assimilated predominantly in leaf chloroplasts, while selenite is largely and rapidly assimilated in roots (*8, 9, 33*). The different localization of metabolism plus that SeMSCys is synthesized in young tissues may explain the equal capacity of broccoli sprouts to synthesize SeMSCys when treated with selenate or selenite as sprouts are young tissues that contain both shoot and root tissues.

Broccoli sprouts exposed to 25 µM of each selenate and selenite synthesized SeMSCys at the similar level as that obtained in either 50 µM of selenate or 50 µM of selenite treatment (Table 1), indicating that there was no interaction between selenate and selenite in promoting SeMSCys accumulation in the broccoli sprouts.

Although both selenate and selenite promoted similar levels of SeMSCys accumulation when sprouts were exposed to same dosage of Se treatment, much high rate of conversion of total Se into SeMSCys was observed in selenite-treated sprouts than selenate-treated sprouts (Table 1). The conversion rates in 50 µM selenite and selenate treated sprouts were 50% and 25.7%, respectively, showing that selenite is more efficient than selenate in converting total Se into SeMSCys in broccoli sprouts.

Total Se Levels Are Reduced by High Dosage of Sulfate in Sprouts Treated with Selenate but Unaffected with Selenite. As an analog of selenate (SeO_4^{2-}), sulfate (SO_4^{2-}) affects Se accumulation in plants (*8*, *9*). To gain a better understanding of the interaction between S and Se, we investigated whether supply of S as sulfate influenced the responses of the sprouts of broccoli cv. GYPSY to selenate and selenite treatments. Supply of S in growth solution improved root development of the 7-day-old broccoli sprouts, which could be verified visually. Increasing S doses affected total Se content of the broccoli sprouts differently depending on the Se form applied (Table 2). A low S dose (0.1 mM) did not dramatically altered total Se content in sprouts treated with 50 µM selenate, but medium (1 mM) and high (10 mM) S dose significantly decreased total Se contents in these sprouts by 20% and 67%, respectively, ($p \leq 0.0001$) when compared to sprouts treated only with 50 µM selenate (without S supply) (Table 2).

Table 2 Total Se and SeMSCys content in 7-day-old sprouts of broccoli cv. GYPSY exposed to different Se forms (selenate and selenite) and various S dosages

Se form	S dose (mM)	Total Se (µg g^{-1} DW)[a]	SeMSCys (µg g^{-1} DW)[a]	Conversion (%)[b]
	0.0	178.9 ± 0.9	105.8 ± 2.3	25.7
Selenate	0.1	168.6 ± 2.4	99.5 ± 1.8	25.6
(50 µM)	1.0	142.5 ± 0.3	64.3 ± 3.8	19.6
	10.0	59.9 ± 0.4	30.3 ± 1.8	21.9
	0.0	97.8 ± 1.3	112.7 ± 5.9	50.0
Selenite	0.1	112.7 ± 3.1	110.8 ± 3.1	42.7
(50 µM)	1.0	116.7 ± 2.0	114.9 ± 8.6	42.8
	10.0	129.1 ± 2.2	114.3 ± 5.3	38.4
ANOVA[c]	Se form	***	****	
	S dose	****	****	
	Se form x S dose	****	****	

[a]Values are averages of three replicates ± SD (standard deviation). [b]Calculated using only the Se (atomic weight = 79) from SeMSCys (molecular weight = 182). [c] *** and **** indicate significance at $p \leq 0.001$ and 0.0001, respectively. Data of treatment without S (0 µM S dose) was copied from the Table 1 (50 µM Se dose; selenate or selenite) for comparison, since both Tables 1 and 2 are part of the same experiment.

The inhibitory effect of S on Se accumulation is also reported in mature broccoli plants treated with selenate (*7*). This antagonistic interaction between sulfate and selenate seems to be mostly due to competition of uptake and assimilation system, which has been well documented (*8, 9, 34-36*). On the other hand, no inhibition of total Se accumulation in sprouts grown in 50 µM selenite was observed with increased levels of S supply (Table 2). Increasing S doses slightly increased total Se content. This increase might be due to better root development of sprouts grown under S supplement, which enabled a slight increase in selenite uptake from growth solution. Furthermore, because sulfate transporters do not mediate selenite uptake in plants, S did not compete for

transport to interfere with Se uptake. Selenite is taken up by plants through passive diffusion and may use phosphate transporters (*9*, *35*).

SeMSCys Levels Are Reduced by High Dosage of Sulfate in Sprouts Treated with Selenate but Unaffected with Selenite. Sprouts treated with 50 μM of selenate contained 35% and 70% lower SeMSCys content when exposed to medium (1 mM) and high (10 mM) S dose, respectively (Table 2), showing an inhibition of SeMSCys synthesis in selenate-treated sprout. In contrast, SeMSCys content in selenite-treated sprouts was not influenced by increasing S dosage from 0.1 to 10 mM and remained similar between control and 10 mM S supply. The effect of S dosage on SeMSCys accumulation in the 7-day-old broccoli sprouts was linked with that of total Se accumulation. Consistently, a general much high rate of conversion of total Se into SeMSCys was observed in selenite-treated sprouts than selenate-treated sprouts (Table 2).

Increasing S dosage was also reported to decrease SeMSCys content in leaves of selenate-treated broccoli plant (*7*). The decreased SeMSCys synthesis of selenate-treated sprouts in response to high S dose treatments was most likely to be linked with the reduction of Se uptake, which diminished the Se availability in the cells.

Different Capacity of Total Se and SeMSCys Accumulation in Sprouts and Florets of Broccoli. To investigate the general response of broccoli to Se, we examined the total Se content in sprouts and florets of five broccoli cultivars exposed to Se supply. In this experiment only selenate was used as Se source, since this form increased total Se and SeMSCys content in both broccoli sprouts (Table 1) and mature broccoli plants (*7*) more effectively than selenite. Both broccoli sprouts and mature broccoli plants were exposed to 25 μM of selenate as this concentration of selenate supplement did not show

any toxicity symptom in comparison to control, which was verified by visual inspection of the sprouts and plants, and also by fresh weight of root, shoot and floret of the mature plants (data not shown). Slight variable values of total Se content in sprouts and florets were found among these broccoli cultivars studied (Table 3).

Table 3 Total Se and SeMSCys content in 7-day-old sprouts and florets of five broccoli cultivars exposed to 25 µM of selenate

Edible part	Cultivars	Total Se (µg g^{-1} DW)[a]	SeMSCys (µg g^{-1} DW)[a]	Conversion (%)[b]
Sprout	Packman	79.1 ± 5.3	67.5 ± 1.8	37.0
	Diplomat	71.7 ± 8.3	73.2 ± 2.9	44.3
	GYPSY	87.5 ± 5.2	72.3 ± 3.0	35.9
	Marathon	85.6 ± 6.7	70.8 ± 4.7	35.9
	De Cicco	58.4 ± 4.6	64.6 ± 5.1	48.0
	Average	76.5	69.7	40.2
	ANOVA [c]	*	*NS*	
Floret	Packman	472.4 ± 64.3	102.6 ± 16.0	9.4
	Diplomat	531.1 ± 58.5	126.8 ± 20.2	10.4
	GYPSY	401.1 ± 50.8	87.8 ± 5.4	9.5
	Marathon	483.2 ± 80.0	88.5 ± 11.3	8.0
	De Cicco	557.6 ± 82.6	137.1 ± 24.7	10.7
	Average	489.1	108.6	9.6
	ANOVA	*NS*	*	

[a]Values are averages of three replicates ± SD (standard deviation). [b]Calculated using only the Se (atomic weight = 79) from SeMSCys (molecular weight = 182). [c]*NS* and * indicate non-significance and significance at $p \leq 0.05$, respectively.

Variation in total Se content among accessions of mature broccoli plants was reported in previous study (*19*). In general, florets (average value of 489 µg g^{-1}) of mature broccoli plants had approximately 6.5-fold higher total Se content than 7-day-old broccoli sprouts (average value of 76 µg g^{-1}) under the treatment conditions. Exposure time, architecture of the root system, and other factors

may have contributed to the difference noted in the Se accumulation between broccoli sprouts and florets. Broccoli plants, as well as other brassica crops, are classified as Se secondary accumulators because they can accumulate hundreds µg Se g^{-1} DW when exposed to Se-enriched growth media (*19, 37*).

SeMSCys content in sprouts and florets of various broccoli cultivars was examined. SeMSCys level was linked with that of total Se content, with some exceptions. SeMSCys content of the sprouts (average value of 70 µg g^{-1} DW) did not vary greatly among cultivars (Table 3). However, florets of both cv. GYPSY and cv. Marathon (average value of 88 µg g^{-1} DW) had lower SeMSCys content than florets of cv. De Cicco (average value of 137 µg g^{-1} DW), while florets of cv. Packman and cv. Diplomat exhibited intermediary SeMSCys content (Table 3). In general, much high conversion rate of total Se into SeMSCys was observed in sprouts than florets under the current treatment conditions.

Variation in SeMSCys accumulation among accessions of mature broccoli plants was also reported previously (*19*). The ability of broccoli specie to synthesize and accumulate considerable amounts of the potent anticancer SeMSCys is related to the activity of several enzymes from the Se transport and assimilation pathway (*5, 7-9, 19*). In this study, florets of mature broccoli plants contained around 1.6-fold higher SeMSCys content than 7-day-old broccoli sprouts. On the other hand, total Se content of florets was 6.5-fold higher than that of sprouts, indicating a better fractional ability of sprouts to convert inorganic Se into SeMSCys.

Glucosinolate Profile in Sprouts. The fact that ingestion of broccoli reduces the incidence of certain cancer in humans is most likely to be linked to presence of glucosinolates (*38*). In this study, glucosinolates from the broccoli

were separated by UPLC and identified by MS/MS. A total of 9 individual glucosinolates were identified in the 7-day-old sprouts of broccoli cv. GYPSY, which included five aliphatic glucosinolates and four indole glucosinolates. The aliphatic glucosinolates were glucoiberin (3-methylsulphinylpropyl), glucoraphanin (4-methylsulphinylbutyl), glucoalyssin (5-methylsulphinylpentyl), glucoiberverin (3-methylthiopropyl), and glucoerucin (4-methylthiobutyl). The indole glucosinolates were 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl), glucobrassicin (indol-3-ylmethyl), 4-methoxyglucobrassicin (4-methoxyindol-3-ylmethyl), and neoglucobrassicin (1-methoxyindol-3-ylmethyl) (Figure 1).

The aliphatic glucosinolates glucoraphanin and glucoerucin were the first and second most abundant, constituting approximately 72% and 13% of the total glucosinolate content, respectively, in broccoli sprouts. The sum of the other 7 glucosinolates consisted of approximately only 15% of the total glucosinolates. A previous study also reported that glucoraphanin and glucoerucin represented more than 70% and 20% of the glucosinolates found in 3-day-old broccoli sprouts, respectively (*21*). Glucoraphanin is a direct precursor of the isothiocyanate sulforaphane, a very potent monoinducer of phase II enzymes that metabolically inactivate carcinogens (*13*). Consequently glucoraphanin as well as sulforaphane has been recently largely investigated in broccoli specie (*14*, *16*, *39*). Glucoerucin also plays an important role for human health (*11*), and it may also exhibit chemopreventive properties (*14*).

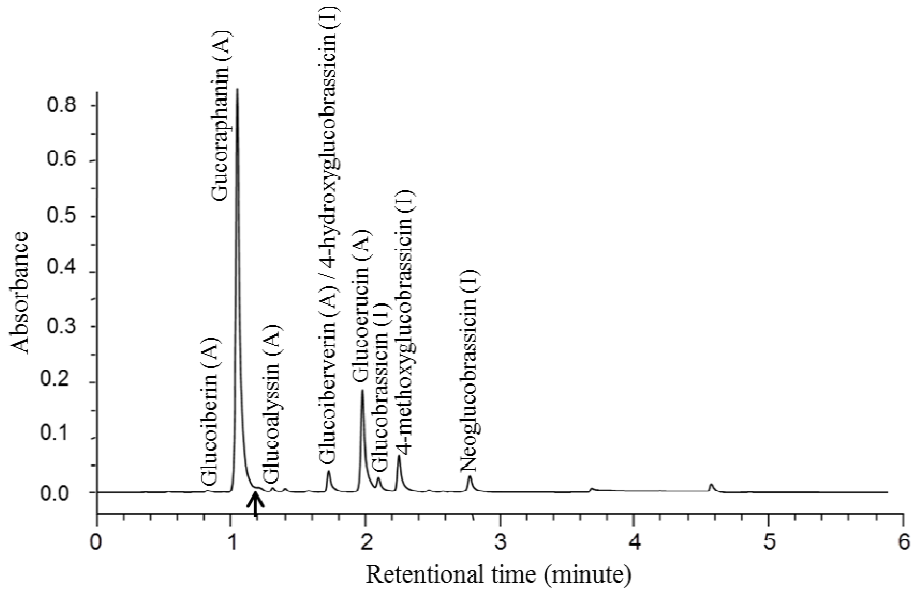

Figure 1. Typical UPLC elution chromatogram of individual glucosinolates (as desulpho-glucosinolates) that were found in the 7-day-old sprouts of broccoli cv. GYPSY. The arrow indicates elution position of the internal standard (sinigrin). Glucoiberverin was eluted slightly earlier than 4-hydroxyglucobrassicin and their peaks were overlapped. Letters (A) or (I) indicate aliphatic or indole glucosinolates, respectively.

Glucoraphanin Content in Sprouts is not Affected by Se and S Treatment. Considering that glucoraphanin is the dominant and most important glucosinolate in broccoli sprouts, we examined the effects of Se and S dosage on the glucoraphanin content in 7-day-old broccoli sprouts. In general, glucoraphanin content was not considerably affected by increasing Se dosage in growth solutions (Figure 2A). Similar tendencies were observed for the content of the other eight glucosinolates (data not shown), with a few minor exceptions. Increasing S dosage caused slight (not significant) variations in glucoraphanin content (Figure 2B). The literature has shown that S fertilization may favor

glucoraphanin accumulation (*16*), although an early study reports negative effects in broccoli sprouts (*15*). Glucoerucin content also had similar variation as that of glucoraphanin, while content of glucobrassicin and neoglucobrassicin (both are indole glucosinolates) was significantly ($p \leq 0.05$) increased under high S dose (10 mM) as compared to control (data not shown).

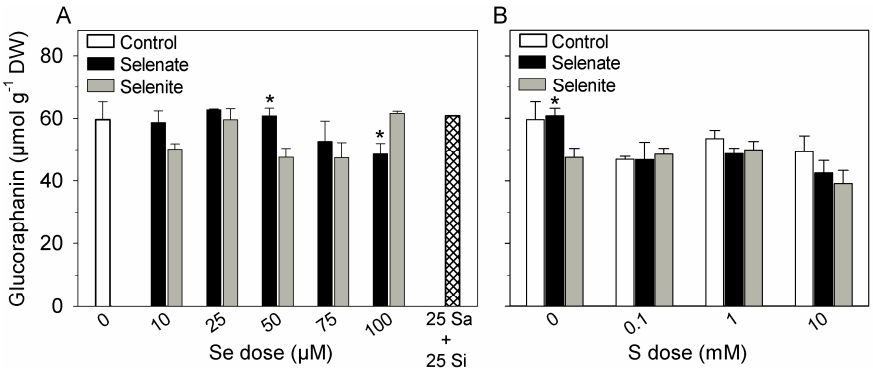

Figure 2. Glucoraphanin content in 7-day-old broccoli sprouts exposed to treatments with various Se dosage (A) and S dosage in the absence (control) and presence of 50 μM Na_2SeO_4 (selenate) or Na_2SeO_3 (selenite) (B). Error bars indicate standard deviation (n = 3). Values marked by asterisks indicate significant difference between selenate and selenite treatments ($p \leq 0.05$). In Figure 2A, 25 Sa + 25 Si represents treatment with 25 μM selenate + 25 μM selenite.

Distinctive Glucosinolate Profiles in Sprouts and Florets of Broccoli. The glucosinolate profiles in sprouts and florets of various cultivars were examined. Similar pattern of individual glucosinolate composition was found among these five broccoli cultivars, and the glucosinolate profiles were substantially different between sprouts and florets (Figure 3).

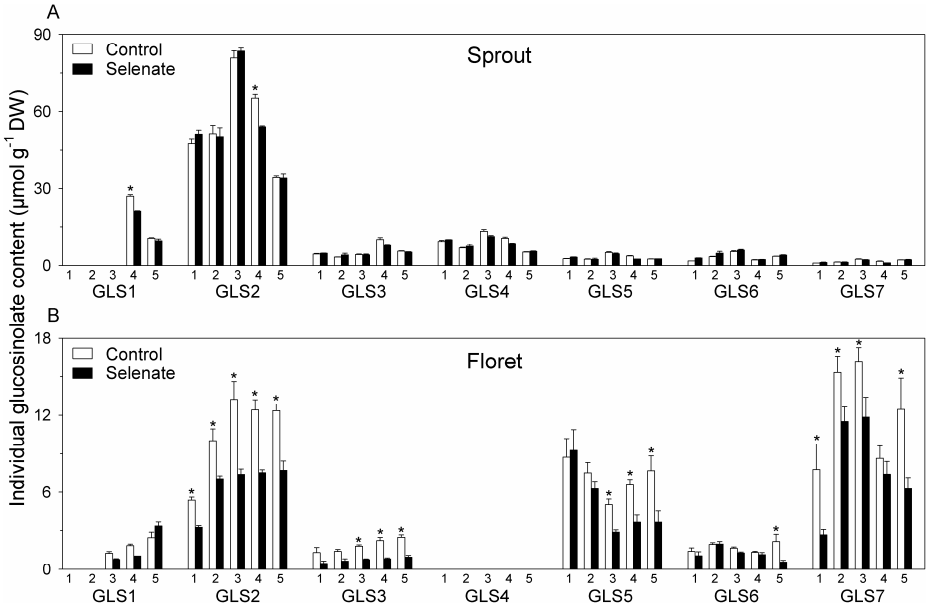

Figure 3. Contents of seven main individual glucosinolates quantified in 7-day-old broccoli sprouts (A) and broccoli floret (B) exposed to 25 μM of selenate. Error bars indicate standard deviation (n = 3). Values marked by asterisks indicate significant difference between control and selenate treatments ($p \leq 0.05$). In the selenate treatments, average Se contents in tissues were approximately 75 μg g^{-1} DW in sprouts and 490 μg g^{-1} DW in florets as detailed in Table 3. Individual glucosinolate: glucoiberin (GLS 1), glucoraphanin (GLS 2), glucoiberverin/4-hydroxyglucobrassicin (GLS 3), glucoerucin (GLS 4), glucobrassicin (GLS 5), 4-methoxyglucobrassicin (GLS 6), and neoglucobrassicin (GLS 7). Numbers 1-5 represent the five broccoli cultivars (Packman, Diplomat, GYPSY, Marathon, and De Cicco, respectively).

In 7-day-old sprouts of the studied broccoli cultivars, glucoraphanin was the most abundant glucosinolate, accounting an average over 65% of the total glucosinolates accumulated (Figure 3A). On the other hand, florets of mature broccoli plants contained glucoraphanin, glucobrassicin and neoglucobrassicin as major glucosinolates, accounting an average approximately 30%, 22% and

34% of the total glucosinolates (Figure 3B). Although glucobrassicin is metabolized to indole-3-carbinol that is proven to be a chemopreventive agent (*40*), breakdown products of neoglucobrassicin seem to inhibit the anticancer activity of the glucoraphanin hydrolysis products (*41*). Thus, broccoli sprouts appear to be a better source for accumulating glucosinolates with high chemopreventive properties. Further, total glucosinolates accumulated (value that summarizes the accumulation of all individual glucosinolates) were much higher in sprouts than in florets (data not shown).

Selenate Exerts Minimal Effect on Glucosinolate Level in Sprouts but Suppresses Glucosinolate Accumulation in Florets. The effect of Se on glucosinolate accumulation was examined in sprouts and florets of these broccoli cultivars. In general, the individual glucosinolates in the 7-day-old sprouts of the five broccoli cultivars were not affected by 25 μM selenate treatment (Figure 3A). The fact that Se exerts no effect on glucosinolate content in sprouts could be due to the fact that those glucosinolates are pre-existed and not newly synthesized. Indeed, glucosinolates are rich in broccoli seeds and sprouts (*16, 38*).

In contrast, florets of selenate-treated plants exhibited approximately 36% (an average value among the five cultivars) lower total glucosinolate content than florets of non-treated plants (Figure 3B). This floret result is consistent with previous studies showing suppression of total glucosinolate levels by Se treatment in broccoli and rapid-cycling *Brassica oleracea* plants (*19, 22-25*). Thus, it appears that Se exerts a considerable effect in reducing the general accumulation of glucoraphanin in florets (Figure 3B). The molecular mechanism that underlies the inhibition of the glucosinolate accumulation in response to Se treatment is not clarified because the metabolic pathways that

regulate the biosynthesis of indole and aliphatic glucosinolates in plants are complex (*10*). In florets, Se competes with or affects sulfur metabolism that influenced glucosinolate synthesis and accumulation although supplementation of 25 µM of selenate was found to slightly enhance total S accumulation in florets of these broccoli cultivars (data not show).

In conclusion, the present results show that Se-enriched broccoli sprouts might be expected to have greater anticancer activity due to synthesis of SeMSCys without negatively affecting chemopreventive glucosinolate accumulation. Distinctive glucosinolate profiles between broccoli sprouts and florets were observed and sprouts contained approximately 6-fold higher content of the potent anticancer glucoraphanin than floret of mature broccoli plants. Noticeably, a recent work indicates that Brassica crops supplied with selenate have the potential to incorporate Se into selenoglucosinolates (*42*). The synthetic Se-containing isothiocyanates are reported to be more potent anticancer inhibitors than their sulfur counterparts (*43*). Under the conditions of our experiments, when broccoli sprouts and plants were treated with 25 µM selenate, a serving size of 7.20 g fresh sprouts or 1.12 g fresh florets (considering an average moisture content of 90% for both edible parts) will meet the daily requirement of Se, 55 µg day^{-1} for adults (*3*). This amount of sprouts provides approximately 50.18 µg SeMSCys and 17.12 mg glucoraphanin (molecular weight = 437), and this amount of florets offers approximately 12.16 µg SeMSCys and 0.32 mg glucoraphanin. Thus, Se-biofortified broccoli that provides adequate levels of anticancer agents could be an excellent source of chemopreventive compounds.

ABBREVIATIONS USED

Se, selenium; S, sulfur; SeMSCys, *Se*-methylselenocysteine

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ARTIGO 3

Effect of phosphite supply in nutrient solution on yield, phosphorus nutrition and enzymatic behavior in common bean (*Phaseolus vulgaris* L.) plants

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Phosphite supply in nutrient solution on yield, phosphorus nutrition and enzymatic behavior in common bean (*Phaseolus vulgaris* L.) plants

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Abstract

Aim of this study was to (i) understand the phosphite action used as P source on growth and grain yield, (ii) measure P concentration and accumulation in shoot and root, and (iii) evaluate enzymatic behaviour in common bean (*Phaseolus vulgaris* L.) plants grown in nutrient solution under phosphate starvation. Experimental design was completely randomised with 7 levels of phosphite (0, 16, 32, 64, 128, 256 and 512 µM) and 2 levels of phosphate (80 and 800 µM, corresponding to phosphate-starved plants and phosphate-sufficient plants,

respectively) in nutrient solution. Common bean plants were evaluated at 2 different growth stages: flowering and mature grain stages. For plants harvested at the mature grain stage, two more treatments (additional treatments) were added: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. This study revealed that growth and grain yield in plants grown under phosphate starvation presented negative repercussions on these parameters, in which treatments with 64, 128, 256 and 512 µM of phosphite resulted in no-filled grains. Concentration and accumulation of P in shoot and root of phosphate-starved plants was increased with increasing phosphite levels in nutrient solution, but this additional P concentration did not convert into grain yield. The phosphite application in phosphate-starved plants promoted a decrease in acid phosphatase (EC 3.1.3.4.1) activity, while catalase (EC 1.11.1.6) activity was increased up to 32 µM of phosphite and was reduced at higher levels of phosphite.

Keywords: Antioxidant enzyme; biostimulant; dry mass; grain; leguminous; nutrient solution; *Phaseolus* bean; phosphate; pod.

Abbreviations: CAT-catalase; P-phosphorus; Phi-phosphite; Pi-phosphate.

Introduction

Weathered soils such as Oxisols of tropical and subtropical regions of the world often exhibit low phosphorus (P) availability to plants, due mainly the high rates of soil P fixation and formation of insoluble complexes with aluminum and iron ions under acidic conditions (Wissuwa, 2003). Thus most P in these soils is

present in a form that is unavailable to plants, and this fact has limited the yield of agricultural crops in developing countries.

Phosphate (Pi) and phosphite (Phi) are the two main P forms used in agriculture, in which there are several P forms present in the environment. Phosphate anion ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) is undoubtedly the major form of P utilised by plants for their adequate growth and development, while Phi anion ($H_2PO_3^-$ and HPO_3^{2-}) is effective in controlling some important plant diseases, especially those caused by organisms taxonomically classified in the phylum Oomycota, such as *Phytophthora* sp. The action of Phi is based on two mechanisms, being direct through effects on the pathogen, and indirect effects on the plant, because Phi positively stimulates metabolism of plants such as increasing defense responses during situations of abiotic and biotic stresses (Wilkinson et al., 2001; Reuveni et al., 2003; Shearer and Fairman, 2007; Orbović et al., 2008; Cook et al., 2009; Silva et al., 2011; Olivieri et al., 2012).

The stimulant effect of Phi on metabolism of plants was reported by Lovatt and Mikkelsen (2006) who mentioned that Phi may influence the sugar metabolism, cause internal hormonal and chemical change, and stimulate shikimic acid pathway, resulting in increased floral intensity, and fruit yield and quality, such as soluble solid content. Since shikimic acid pathway, which is a pathway of plant secondary metabolism, is responsible for the biosynthesis of several aromatic compounds, there is the presupposition that Phi may induce the reinforcement of cell wall polymers with a deposition of lignin derivatives. In agreement with these previous reports, Olivieri et al. (2012) found that Phi positively stimulated structural and biochemical changes in periderm and cortex of *Solanum tuberosum*. Moreover, there was also reported that Phi may increase the activity of phenylalanine ammonia-lyase (PAL) and the biosynthesis of

phytoalexins (Saindrenan and Guest, 1995), activity of guaiacol peroxidase in maize plants (Ávila et al., 2011), and content of ascorbic acid in strawberry plants (Moor et al., 2009). Hence, in Pi-sufficient plants, these biochemistry and structural alterations induced by Phi support the metabolic advantages of Phi against stress agents such as reducing the incidence of some plant diseases, as already reported here.

Besides being used as fungicides and biostimulants, recently Phi-based products have also been marketed in the world as fertilizers for foliar spray, fertigation and direct soil application (Thao and Yamakawa, 2009). Phosphite salts are recommended as a fertilizer because they contain a cation that may be a plant nutrient, such as K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+} or Zn^{2+}, and often Phi is also marketed as an additional source of P for plant nutrition. Considering that P is one of the most common limiting plant nutrients in the tropics, the divulgation of the Phi-based products as a possible source of P for crop nutrition is particularly interesting for marketing purposes.

Common bean (*Phaseolus vulgaris* L.) crop is one of the most important grain legumes for human consumption, and it is a major source of protein in many parts of the world, especially in developing countries (Graham and Ranalli, 1997; Broughton et al., 2003). However its productivity is low in these regions due to diseases and low soil fertility (Allen et al., 1998; Hillocks et al., 2006). Phosphite-based products have been recommended for common bean crop as fungicides, plant biostimulants, or P fertilizers, and this is probably due to susceptibility of this crop to various diseases, and also due to P deprivation of the tropical soils.

Lovatt (1990a, b) reported that the application of Phi improved fruit set and yield of *Persea americana* (avocado), and restored normal growth of Pi-

starved *Citrus spp*. Similarly, Albrigo (1999), Rickard (2000) and Watanabe (2005) also mentioned positive effects of Phi on plant P nutrition or yield in some crops. On the other hand, others studies have indicated that the Phi anion may not be used by plants as a P nutrient, even though it is well absorbed by leaves and roots (Thao and Yamakawa, 2009). In addition, Phi supply may cause growth depression in Pi-starved plants (Schroetter et al., 2006; Thao et al., 2008; Thao et al., 2009; Zambrosi et al., 2011). In this case, it appears that Phi acts as a repressor of plant responses to P starvation, by decreasing acid phosphatase activity (Ticconi et al., 2001; Varadarajan et al., 2002).

Several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism (Apel and Hirt, 2004). Antioxidant enzymes have an important role in the plant cellular protection against stress-induced cell damage caused by formation of free radicals, mainly in the form of ROS. As a result, it have been related that increase of the activity of antioxidant enzymes may improve the growth and yield of the crops. In agreement with this, Ramos et al. (2010) related that increased SOD and CAT activity induced by supply of low selenium concentrations improved the lettuce (*Lactuca sativa* L.) leaf yield. In Pi-sufficient plants, stimulation of secondary metabolism by Phi may potentially increase the activities of important antioxidant enzymes, such as the catalase enzyme. Nonetheless, there is still insufficient information to test the hypothesis that Phi may stimulate activity of antioxidant enzymes.

The aim of this study was to (i) understand the Phi action used as P source on growth and grain yield, (ii) measure P concentration and accumulation in shoot and root, and (iii) evaluate enzymatic behavior in common bean (*Phaseolus vulgaris* L.) plants grown in nutrient solution under Pi starvation.

Results

Influence of phosphite and phosphate on shoot and root

Shoot and root weights of Pi-sufficient *Phaseolus vulgaris* plants were not affected by Phi levels in nutrient solution (Fig. 1 A, B, C, and D). However, high Phi levels (256 and 512 µM Phi) decreased shoot and root dry weight in Pi-starved plants. At higher level (512 µM Phi), there was a reduction in shoot dry weight of 43 and 55% for Pi-starved plants evaluated in flowering and mature stages, respectively, compared with the control; while for root dry weights were showed decreases of 25 and 44% during flowering and mature stages, respectively. These Pi-starved plants at 512 µM Phi also exhibited Phi-toxicity symptoms such as curved and malformed leaves, and necrosis in older leaves. According to this Phi at higher level was toxic for Pi-starved plants. Pi-sufficient common bean plants exhibited much higher shoot dry weight than Pi-starved common bean plants, and there was no significant variation of shoot dry weight between the two growth stages (Fig. 1 A and B). Root dry weight was increased in Pi-sufficient common bean plants at mature grain stage (Fig. 1 D), but interestingly root dry weight did not vary between Pi-starved and Pi-sufficient plants at flowering stage, with the exception of plants grown under 512 µM Phi. In additional treatments, which were applied only in plants evaluated at mature grain stage, no P supply and P supply using only Phi (800 µM Phi) decreased the shoot and root dry weight by around 93 and 81% (Fig. 1 B and D), respectively, compared with plants grown under 800 µM Pi (Pi-sufficient plants).

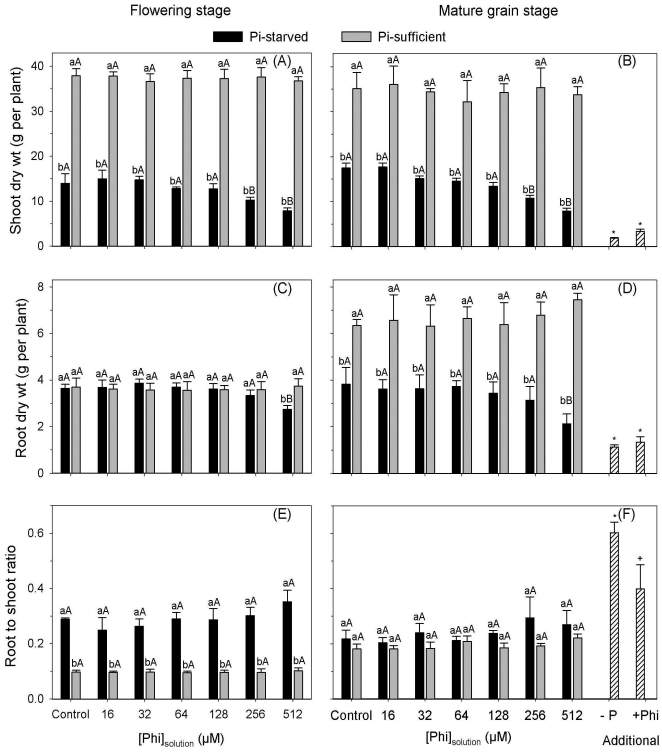

Fig 1. Shoot dry weight (A and B), root dry weight (C and D), and root to shoot ratio (E and F) at 2 different growth stages (flowering and mature grain stages) of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). For plants harvested at mature grain stage, additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi.

Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$). Values marked by asterisks (*) indicate significant differences ($p \leq 0.05$) between the factorial experiment treatments and the two additional treatments (-P and +Phi). In figure 1D, value marked by plus (+) indicate significant difference between the two additional treatments ($p \leq 0.05$).

Root to shoot ratios (Fig. 1 E and F) of common bean plants were not affected significantly by Phi supply in nutrient solution. Root to shoot ratio was 3-fold higher in Pi-starved plants than Pi-sufficient plants at flowering stage, but this ratio did not differ significantly when the plants were evaluated at mature grain stage. Interesting, Pi-starved plants and Pi-sufficient plants exhibited higher and lower values of root to shoot ratio, respectively, at flowering and mature grain stages. No P supply and P supply only as Phi in nutrient solution (additional treatments) increased root to shoot ratio by around 3.3- and 2.2-fold respectively, compared with Pi-sufficient plants grown under control treatment (Fig. 1 F). In general, both additional treatments considerably increased root to shoot ratio of the plants at mature grain stage.

Interference induced by phosphite and phosphate on tissue P concentration and total P accumulation

The values of tissue P concentration and total P accumulation in shoot and root were evaluated only when the plants were at flowering stage, the stage in which common bean exhibits high metabolic activity. Tissue P concentration and total P accumulation in shoot and root of Pi-sufficient plants were not significantly ($p > 0.05$) affected by Phi treatments applied in nutrient solution (Fig. 2 A, B, C and D). Nevertheless, in Pi-starved plants, shoot and root exhibited a progressive increase in tissue P concentration from 32 and 128 µM Phi, respectively. At the highest Phi level (512 µM Phi) there was a substantial increase, corresponding to 7.2-fold in shoot and 11.7-fold in root in tissue P concentration of these Pi-starved plants (Fig. 2 A and C), compared with the control. Total P accumulation in shoot and root (Fig. 2 B and D) of Pi-starved

plants were also increased from 128 and 256 µM Phi, respectively; but the differences were of smaller magnitude than those found for the tissue P concentrations. At the highest Phi level, the values of total P accumulation in shoot and root of these plants were 4.5- and 8.7-fold higher, respectively, compared with the control.

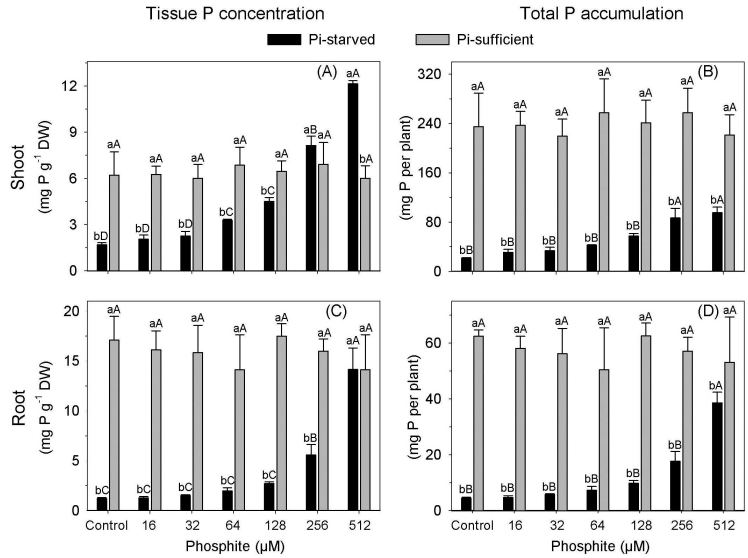

Fig 2. Shoot P concentration (A), shoot P accumulation (B), root P concentration (C), and root P accumulation (D) at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).

Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

At the control treatments (without application of Phi in nutrient solution), Pi-sufficient plants exhibited much higher tissue P concentration and

total P accumulation than Pi-starved plants. However, in the treatment with 512 µM Phi, the tissue P concentration in shoot was higher for Pi-starved plants, and tissue P concentration in root did not differ between Pi-sufficient and Pi starved plants. These effects were mainly due to large increase of tissue P concentration in Pi-starved plants.

Effect of phosphite and phosphate on P nutrition indexes

The indexes of P uptake efficiency, which represent the ability to take up the P from nutrient solution, P utilization efficiency, that is the ability to produce biomass for a given P concentration (Siddiqi and Glass, 1981), and P transport from root to shoot, were measured at flowering stage of the common bean plants (Fig. 3).

The index of P uptake efficiency of the Pi-sufficient plants did not differ significantly between control and all the Phi levels (Fig. 3 A). However in Pi-starved plants, from 128 µM Phi this index increased with increasing the levels of Phi in nutrient solution. Phosphate-starved plants grown under 512 µM Phi exhibited 7-fold higher P uptake efficiency than Pi-starved plants grown under control treatment (without Phi supply in nutrient solution). In general Pi-sufficient common bean showed much higher values of P uptake efficiency in all Phi treatments. In control treatments, for example, P uptake efficiency of Pi-sufficient plants was 11.5-fold higher than Pi-starved plants.

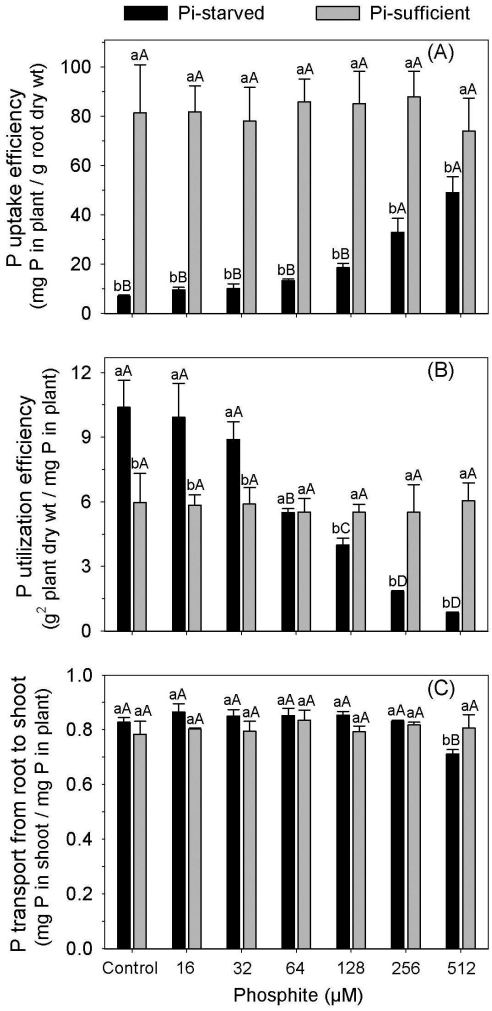

Fig 3. P uptake efficiency (A), P utilisation efficiency (B) and P transport from root to shoot (C) at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).

Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

The P utilisation efficiency index of the Pi-sufficient plants was also not influenced by Phi levels. However for the Pi-starved plants, from 32 µM Phi, this index was considerably decreased with increasing the levels of Phi in nutrient solution (Fig. 3 B). Phosphorus utilisation efficiency of Pi-starved plants grown at the higher Phi level (512 µM Phi) was 12.4-fold lower than that of Pi-starved plants grown under control treatment (without Phi application in nutrient solution). Considering only the control treatments, P utilisation efficiency of Pi-starved plants was around 1.7-fold higher than that of Pi-sufficient plants. But at the higher Phi level the P utilisation efficiency of Pi-starved plants was around 7.2-fold lower than that of Pi-sufficient plants, due to considerable negative effect of the maximum Phi level on this important P nutrition index.

The index of P transport from root to shoot did not vary between the control and all the Phi levels for Pi-sufficient common bean (Fig. 3 C), while in Pi-starved plants this index was affected significantly only in 512 µM Phi. At this Phi level, P transport from root to shoot of Pi-starved plants was slightly reduced (by 14%), compared with the control. In general the values of P transport from root to shoot in the common bean plants were around 0.8 (about 80% of the total P taken up by plants were transported from root to shoot), and there was no significant difference of P transport between Pi-sufficient and Pi-starved plants, with exception of higher Phi level at which the value of P transport of Pi-starved plants was slightly decreased.

Modifications produced by phosphite and phosphate on acid phosphatase activity

The acid phosphatase activity of Pi-sufficient plants was not affected with the Phi treatments (Fig. 4). However for Pi-starved plants the application of Phi levels in the nutrient solution decreased the acid phosphatase activity in concentrations of 16 µM Phi, compared with the control, but only from 32 µM Phi there was significant ($p < 0.05$) decrease in comparison to control (0 µM Phi). In Pi-starved plants, the acid phosphatase activity of the two higher Phi levels (256 and 512 µM Phi) was around 48% lower than that of the control treatment (without Phi supply). Considering only the control treatments, the acid phosphatase activity of Pi-starved plants was 56% higher than that of Pi-sufficient plants. Nevertheless, in concentrations of 256 and 512 µM Phi the activity values of this enzyme did not differ between Pi-starved plants and Pi-sufficient plants.

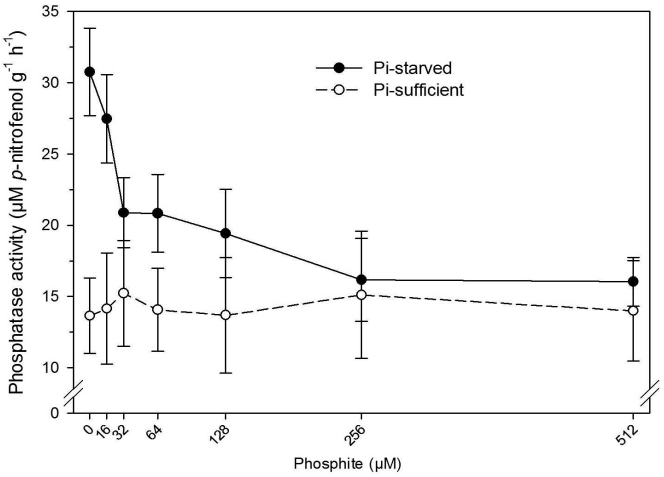

Fig 4. *In vivo* acid phosphatase activity in youngest mature trifoliate leaf at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).
Values represent the mean value of 3 replicates ± SD (Standard deviation).

Impact of phosphite and phosphate on catalase enzyme

The values of catalase (CAT) activity in Pi-sufficient plants was affected significantly only in 512 µM Phi, in which the activity of this enzyme was 71% higher compared with Pi-sufficient plants grown under control treatment (0 µM Phi) (Fig. 5). Nevertheless, CAT activity of Pi-starved plants increased substantially with increasing the Phi levels until 32 µM Phi, and decreased progressively in concentrations from 64 µM Phi. Thus, Pi-starved and Pi-sufficient plants exhibited higher CAT activity in concentrations of 32 and 512 µM Phi, respectively.

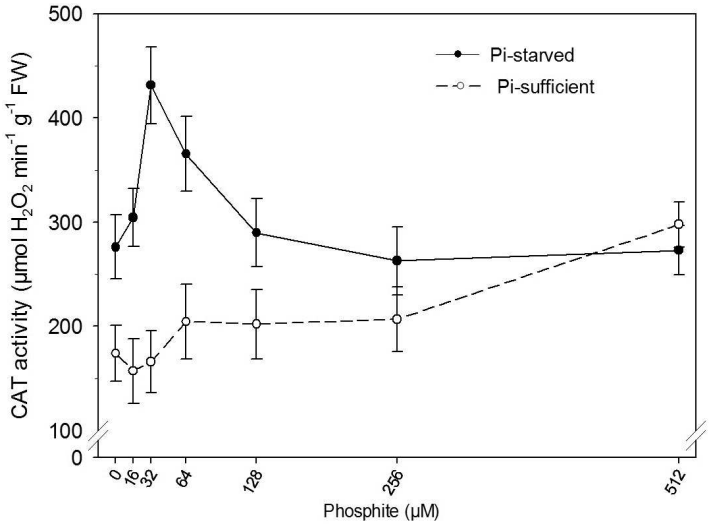

Fig 5. Catalase activity in youngest mature trifoliate leaf at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).
Values represent the mean value of 3 replicates ± SD (Standard deviation).

In general, Pi-starved common bean plants exhibited considerably higher CAT activity in concentrations up to 64 µM Phi, and at the two higher Phi levels (256 and 512 µM Phi) there was no significant difference ($p > 0.05$) of CAT activity values between Pi-starved and Pi-sufficient plants.

Impact produced by phosphite and phosphate on grain yield

Grain dry weight of Pi-sufficient common bean plants did not vary significantly with any of the levels of Phi in nutrient solution (Fig. 6). However Pi-starved common bean plants exhibited decreased grain dry weight when grown under 32 µM Phi, and these plants did not produce grains when grown from 64 µM Phi.

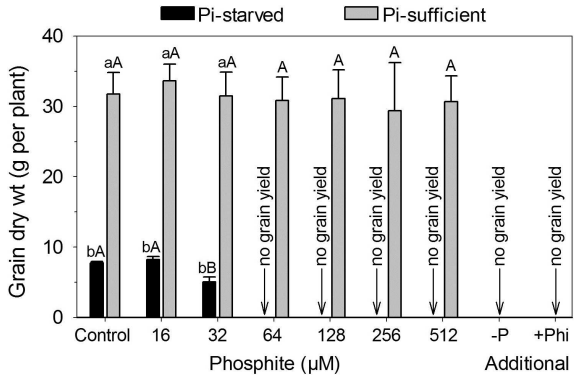

Fig 6. Grain dry weight at mature grain stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). Additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi.

Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

Treatment under 16 µM Phi did not influence the grain yield of the Pi-starved plants. Common bean plants grown under both additional treatments (–P = no P supply, and +Phi = supply of 800 µM of P only as Phi) also did not produce grains. In general, grain yield in Pi-sufficient plants was 4-fold higher than Pi-starved plants grown under control treatment (without supply of Phi in nutrient solution).

Visual appearance of pod induced by phosphite

Toxicity symptoms of Phi on grain yield of Pi-starved common bean were also supported by the visual appearance of these plants (Fig. 7).

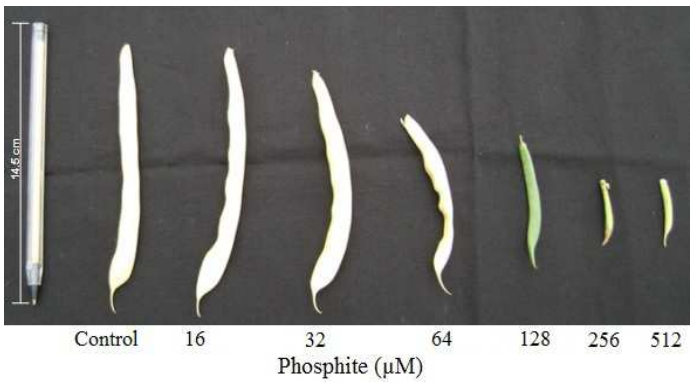

Fig 7. Toxicity symptoms on grain yield at *Phaseolus vulgaris* plants grown in nutrient solution under low phosphate level (Pi-starved plants), as affected by 6 phosphite (Phi) levels + control (without Phi supply).

Development of pods in Pi-sufficient plants was not altered by Phi treatments. On the other hand, Phi-starved common bean exhibited much more pods per plant in treatments with 64, 128, 256 and 512 µM Phi, but these pods

were small and malformed that resulted in no-filled grains (Fig. 7). This harmful effect of Phi on development of pods in Pi-starved plants was increased with increasing the levels of Phi in nutrient solution.

Effects promoted by phosphite and phosphate on P concentration and P accumulation in grain

Fig. 8 shows the values of tissue P concentration and total P accumulation in common bean grains, from plants harvested at mature grain stage.

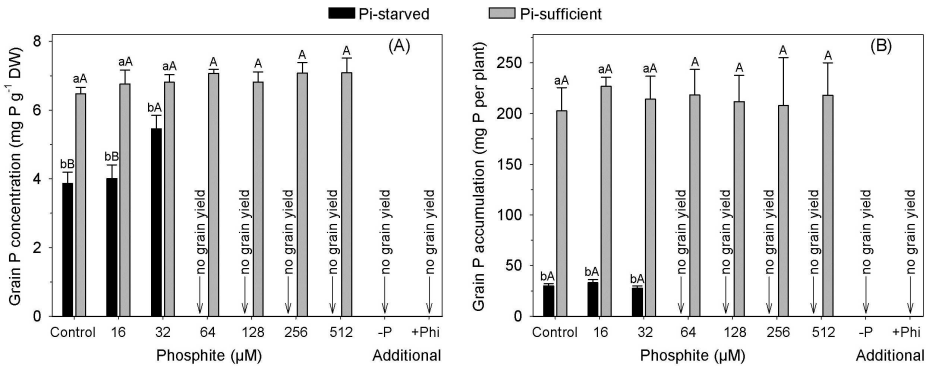

Fig 8. Grain P concentration (A) and grain P accumulation (B) at mature grain stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). Additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi.

Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

Tissue P concentration and total P accumulation in grains of Pi-sufficient plants were not significantly affected by all Phi levels. For Pi-starved plants, tissue P concentration and total P accumulation were studied only in the control treatment (without Phi supply in nutrient solution) and in treatments corresponding to 16 and 32 µM Phi, since there was not grain yield in the other Phi treatments, as well as in both additional treatments (–P = no P supply, and +Phi = supply of 800 µM of P only as Phi). Pi-starved plants exhibited significantly higher tissue P concentration in the grain, when grown under 32 µM Phi, while 16 µM Phi did not alter the tissue P concentration in the grain of these plants compared with the control (Fig. 8 A). Additionally, total P accumulation in grains of Pi-starved plants did not vary with the supply of the two first Phi levels compared with the control (Fig. 8 B).

Discussion

In Pi-sufficient and Pi-starved plants regardless of the applied Phi treatments, we found different root growth responses between flowering and mature grain stages. At the flowering stage, shoot biomass weight was much higher at the Pi-sufficient plants but root biomass weight was little altered between Pi-sufficient and Pi-starved plants, thereby showing that Pi-starved common bean plants exhibited increased root growth rate at the expense of the shoot growth rate. These data were confirmed with data of root to shoot ratio at flowering stage, in which Pi-starved plants exhibited higher root to shoot ratio than Pi-sufficient plants, and plants grown under additional treatments, mainly under the first additional treatment (no P supply in nutrient solution), exhibited higher values of root to shoot ratio than those of all others treatments. These increased values

of root to shoot ratio in Pi-starved plants at flowering stage is a mechanism for overcoming P deprivation from growth medium (Ticconi et al., 2001; Devaiah et al., 2007).

For plant biomass weight at mature grain stage, we observed that there was no significant variation of the shoot biomass yield from full flowering stage. But, interestingly, there was considerable increase in root biomass yield from full flowering stage to mature grain stage at the Pi-sufficient plants, while at the Pi-starved plants this increase was not significant. Thus, at the mature grain stage both shoot and root growth were increased at the Pi-sufficient plants, while data of root to shoot ratio did not differ significantly between Pi-sufficient and Pi-starved plants. Hence, in this study the evaluation of the plant biomass at two different periods of time (flowering and mature grain stages) was crucial to show the variations of shoot and root growth between Pi-starved and Pi-sufficient common bean.

Supply of Phi in nutrient solution in general did not affect the tissue P concentration and total P accumulation in shoot, root and grain of Pi-sufficient common bean plants. These data disagree with those of Thao et al. (2009), which noticed that tissue P concentration in Pi-sufficient hydroponic lettuce was increased with increasing the Phi levels in nutrient solution. However we found that Pi-starved common bean plants exhibited much higher tissue P concentration in shoot and root when grown under high Phi levels. Although Phi-starved plants did not produce grains at the high Phi levels, increased tissue P concentration in grains of Pi-starved plants was observed at the second Phi level. This higher tissue P concentration of Pi-starved plants grown under high Phi levels was not due only to “concentration effect” (caused by inhibitory effect of Phi on growth and yield of the Phi-starved plants) but also due to

increased uptake of P from nutrient solution, since total P accumulation (i.e. estimation of the amount of P taken up) of the Pi-starved plants did not decrease with the Phi levels applied (actually the contrary was observed, in which total P accumulation in shoot and root of Pi-starved plants was increased at the high Phi levels). In this case, our data of tissue P concentration for Pi-starved common bean plants are in agreement with those of Thao et al. (2009) who also observed that tissue P concentration in Pi-starved hydroponic lettuce was increased with increasing the Phi levels in nutrient solution.

We also showed that application of Phi in the growth medium (nutrient solution) did not influenced the P nutrition indexes of the Pi-sufficient common bean plants, but increased the P uptake efficiency and, at the same time, decreased the P utilization efficiency of the Pi-starved common bean plants. In this study the P uptake efficiency refers to total P taken up by plant per root weight unit, and the P utilization efficiency refers to the plant's ability to produce biomass for a given P concentration according to Siddiqi and Glass (1981). Thus our data showed that the high Phi levels from nutrient solution enhanced the uptake of P per unit of root mass but did not improve the P nutrition of the Pi-starved plants. When only plants from control treatments (without Phi supply) were considered, we observed that Pi-starved plants exhibited much lower P uptake efficiency (due to low availability of P from nutrient solution) but at the same time these plants exhibited higher P utilization efficiency, compared with the Pi-sufficient plants. This increase of the P utilization efficiency by Pi-starved common bean plants was a response to Pi deprivation from growth medium. Rouached et al. (2010) mentioned several molecular mechanisms that regulate gene expression in plants are modified during Pi starvation.

When Phi anion was not added in nutrient solution, *in vivo* acid phosphatase activity of Pi-starved plants was higher than that of Pi-sufficient plants. This is also an adaptive mechanism of plants in order to grow better in P-deficient environment (Tadano et al., 1993), in which acid phosphatase may hydrolyse organic P compounds within the plant and rhizosphere (acid phosphatase secreted by the roots) and liberate inorganic P (Haussling and Marschner, 1989; Tadano et al., 1993; Yun and Kaeppler, 2001; Louw-Gaume et al., 2010). However, we found that *in vivo* acid phosphatase activity of Pi-starved common bean plants was considerably decreased with increasing Phi levels in nutrient solution. Thus these data of *in vivo* acid phosphatase activity corroborated the presupposition that Phi anion was taken up by Pi-starved plants reducing their acid phosphatase activity, but at the same time these plants did not utilize Phi anion as a P source for its growth and development. These data agree with those previously reported by Ticconi et al. (2001) and Varadarajan et al. (2002) who found that the addition of Phi in the growth medium inhibited mechanisms of overcoming P starvation in Pi-starved *Arabidopsis* and tomato, such as gene expression and activities of acid phosphatase enzymes.

This study showed that, in Pi-starved plants, catalase (CAT) activity was considerably higher when Phi was applied in low levels, while medium and high Phi levels reduced substantially the activity of this enzyme. Antioxidant enzymes, such as CAT, have an important role in the plant cellular protection against stress-induced cell damage caused by formation of free radicals, mainly in the form of ROS. Catalase is an important antioxidant enzyme involved in ROS detoxification. However, in Pi-sufficient plants, CAT activity did not vary in the low and medium Phi levels, while the supply of high Phi level increased its activity by 71%. This data indicate a possible beneficial effect of Phi on Pi-

sufficient common bean plants, although growth and grain yield of these Pi sufficient plants were neither increased nor decreased at all the Phi levels. Recent studies have shown that the Phi anion may induce molecular alterations that increase resistance to stress agents, such as stimulation of guaiacol peroxidase activity and lignin biosynthesis in maize (Ávila et al., 2011), and structural and biochemical changes in potato tuber periderm and cortex (Olivieri et al., 2012). On the other hand, studies that relate Phi effects on antioxidant enzymes are still rare. In agreement with our data, Moor et al. (2009) found that soaking strawberry plants in Phi solution resulted in increased content of ascorbic acid (compounds known to have antioxidant properties) in the fruits.

In this investigation, we showed that growth and grain yield of the common bean plants grown under adequate Pi supply (Pi-sufficient plant) were not affected by Phi levels applied in the growth medium (nutrient solution). Although Lovatt and Mikkelsen (2006) reported that Phi anion may stimulate growth of some crop when grown under adequate Pi conditions, our data indicated that Phi supply did not provide stimulation on growth and grain yield of the Pi-sufficient common bean plants grown under greenhouse environment conditions and in the absence of pathogens. In agreement with these results, Thao et al. (2009) did not observe a stimulant effect of Phi anion on hydroponic lettuce growth.

Nonetheless, grain yield of the common bean plants grown under low Pi supply (Pi-starved plant) was strongly inhibited from the low Phi levels, as shown in our results, although shoot and root growth of these plants at both growth stages (flowering and mature grain stages) decreased significantly only at the higher Phi level. Thus, besides not being a P source for common bean plants, Phi anion was strongly harmful to grain yield of the Pi-starved plants.

This conclusion was also supported by the visual aspect of the pods of these plants (Fig. 7). Hence, in this work the evaluation of the grain (edible part) yield was crucial to show the harmful effects of Phi anion on yield of the Pi-starved common bean plants. Although there is little information in the literature comparing the effects of Phi supply on grain yield in leguminous crops, there are some previous studies with *Arabidopsis,* vegetables and some others crops that are in agreement with our data (Ticconi et al., 2001; Varadarajan et al., 2002; Lee et al., 2005; Schroetter et al., 2006; Devaiah et al., 2007; Thao et al., 2008; Thao et al., 2009). The causes of this harmful effect of Phi anion on Pi-starved plants are not well understood yet. The most plausible hypothesis to date is that, although Pi and Phi appear to be indistinguishable by Pi uptake transporter sites, plants are unable to metabolise Phi anion, which, after uptake, this anion remains stable in the cell compartments. Furthermore, Phi anion suppresses some plant responses to Pi deprivation, such as syntheses of acid phosphatases, phosphodiesterases, nucleases, and high-affinity P transporters (Ticconi et al., 2001; Abel et al., 2002; Varadarajan et al., 2002; Lee et al., 2005; Ávila et al., 2011). In agreement with this explanation, based on the kinetic studies of the Pi transport system of tobacco BY-2 cells, Danova-Alt et al. (2008) demonstrated that Phi inhibited Pi uptake in a competitive manner. Within the plant, the same authors also showed by *in vivo* ^{31}P nuclear magnetic resonance spectroscopy that there is a intracellular accumulation of Phi in Pi-starved cells, but the Pi resupply results in a rapid efflux of Phi from apoplast and cytoplasm. Thereby the authors related that tobacco BY-2 cells predominantly accumulate Phi in the cytoplasm in Pi-starved cells, but, in contrast, Phi accumulates almost exclusively into vacuoles in Pi-sufficient cells. Thus the results of Danova-Alt et al. (2008) may help to explain, in part, the

harmful effects of Phi in Pi-starved plants, while in Pi-sufficient plants these harmful effects have not been reported. Moreover, in Pi-sufficient plants, Phi may be benefic such as reducing the incidence of some plant diseases and inducing some molecular mechanisms that increase resistance to stress agents, as has already been showed.

In this investigation, additional treatments were conducted to confirm that the Phi anion did not replace Pi anion in common bean P nutrition. When data from the second additional treatment (supply of 800 µM of P only as Phi) were compared with those of Pi-sufficient plants (supply of 800 µM of P only as Pi), we verified that growth parameters and grain yield of the plants grown under second additional treatment were strongly inhibited. Moreover, the growth of the plants grown under the second additional treatment was similar with that of plants grown under the first additional treatment (no P supply in nutrient solution), and all plants grown in both additional treatments did not produce grains. These data corroborate those of Lee et al. (2005) for *Ulva lactuca*, Schröetter et al. (2006) for *Zea mays*, Thao et al. (2008) for *Brassica rapa*, Ávila et al. (2011) with *Zea mays*, Zambrosi et al. (2011) for *Citrus spp.* rootstocks, and Hirosse et al. (2012) for *Ipomoea batatas* tissue cultures. These authors found that the Phi anion did not replace Pi anion in plant P nutrition. Furthermore they also reported that the use of Phi as sole P source generally caused a significant reduction in plant growth compared with the treatments with either null or insufficient Pi fertilization.

Materials and methods

Experiment localisation, plant material, and seedling obtaining

Study was conducted in Departamento de Ciência do Solo of the Universidade Federal de Lavras, Brazil (21°14' S; 45°00' W; 915 m asl). The plants were grown in a greenhouse environment. Photoperiod was 12 h of light. Seeds of the common bean (*Phaseolus vulgaris* L.) cv. Radiante were germinated in expanded polystyrene trays containing 128 compartments filled with vermiculite and irrigated with distilled water. Five days after emergence, seedlings were transferred to plastic box containing 36 L of one-fourth-strength modified Hoagland's solution (Jones Junior, 1983).

Experimental design and treatments

This study was conducted in a completely randomised experimental design with 3 replicates, being 7 phosphite (Phi) levels (0, 16, 32, 64, 128, 256 and 512 µM) and 2 phosphate (Pi) levels (80 and 800 µM, these levels considered Pi-starved plants and Pi-sufficient plants, respectively) in nutrient solution. Common bean plants were evaluated at 2 different growth stages: flowering and mature grain stages. For plants harvested at the mature grain stage, two more treatments (additional treatments) were added: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. Each experimental unit consisted of one common bean plant per pot.

Plant culture and treatment applications

The 10-day-old young seedlings were selected for regular leaf size and area and transplanted to plastic pots containing 3 L of half-strength modified Hoagland’s solution. Five days after transplanting, these plants were grown in full-strength

modified Hoagland's solution with the Phi and Pi treatments. Phosphite used in the experiment was obtained by the reaction of phosphorous acid with potassium hydroxide, resulting in potassium Phi. The nutrient solution was changed twice each week. The volume of the nutrient solution in each plastic pot was supplemented daily with deionised water and pH was adjusted to 5.5 (± 0.3) by adding 0.5 M NaOH or HCl. Throughout the experimental period the nutrient solution was constantly aerated.

Harvest, biomass yield and phosphorus nutrition

Three replicates of each treatment were harvested when plants were at full flowering stage. Shoot and root dry weight of these plants were measured, after drying in a forced air oven at 60 °C until there was no change in weight, and their tissue P concentrations determined by colorimetry (Murphy and Riley, 1962) after nitric-perchloric digestion of the plant material (Johnson and Ulrich, 1959). Data from shoot and root dry weight, and tissue P concentration were used to calculate total P accumulation in shoot and root, as well as P uptake efficiency (total P accumulation in plant / root dry wt) (Swiader et al., 1994), P utilisation efficiency [(plant dry wt)^2 / (total P accumulation in plant] (Siddiqi and Glass, 1981) , and index of P transport from root to shoot (total P accumulation in shoot / total P accumulation in plant). The other 3 replicates of each treatment were harvested when plants were at mature grain stage. Grain dry weight, and grain P concentration and accumulation in these plants were also determined.

In vivo acid phosphatase and catalase activities

All enzymatic analyses were only done in plants evaluated at flowering stage. Prior to harvest of the plants, one youngest mature trifoliate leaf was collected in 3 replicates of each treatment to evaluate the *in vivo* acid phosphatase (EC 3.1.3.4.1) activity, according to Besford (1980) with minor modifications (Silva and Basso, 1993).

Catalase (CAT) (EC 1.11.1.6) activity was performed according to Ramos et al. (2010) with minor modifications. During plant harvest, one youngest mature trifoliate leaf was collected in 3 replicates of each treatment and was immediately wrapped in aluminum foil, submerged in liquid nitrogen and stored in a freezer, at –80 °C. Posteriorly, frozen tissues were homogenised in a cooled 0.1 mol/L Tris–HCl buffer at pH 7.8 containing 1 mmol/L EDTA, 1 mmol/L dithiothreitol and 5 ml of 4% polyvinyl pyrrolidone per gram of fresh weight. The homogenate was filtered through a nylon mesh and centrifuged at 14000 rpm for 30 min at 4 °C. The supernatant was used to measure enzymatic activity of the CAT by observing H_2O_2 consumption at 240 nm for 5 min (Rao et al., 1997). The reaction mixture (3 ml total volume) contained 25 mM Tris-acetate buffer (pH 7.0), 0.8 mM EDTA-Na, 20 mM H_2O_2, and enzymatic assay was carried out at 25 °C.

Data analysis

Data were submitted to variance analysis (ANOVA, $p \leq 0.05$) using SAS software (SAS Institute, 1996), and when significant differences occurred the data were applied to Scott Knott's test ($p \leq 0.05$) (Scott and Knott, 1974). For plants harvested at the mature grain stage, statistical comparisons between the additional treatments, as well as between additional treatment and factorial

experiment, were evaluated according to Healy (1956). Standard deviation (± SD) was calculated for all means (three replicates).

Conclusion

This study revealed that growth and grain yield in *Phaseolus vulgaris* plants grown under Pi starvation presented negative repercussion on these parameters, in which treatments at 64, 128, 256 and 512 µM Phi resulted in no-filled grains. Concentration and accumulation of P in shoot and root of Pi-starved plants was increased with increasing the Phi levels in nutrient solution, but this additional P concentration did not convert into increased growth or grain yield. Application of Phi in Pi-starved plants promoted a decrease in acid phosphatase activity, while catalase activity had increased up to 32 µM Phi and was reduced at higher levels of Phi.

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ARTIGO 4

Phosphite as phosphorus source to grain yield of common bean plants grown in soils under low or adequate phosphate availability

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PHOSPHITE AS PHOSPHORUS SOURCE TO GRAIN YIELD OF COMMON BEAN PLANTS GROWN IN SOILS UNDER LOW OR ADEQUATE PHOSPHATE AVAILABILITY

Fosfito como fonte de fósforo para produção de grãos em feijoeiro cultivado em solos sob baixa ou adequada disponibilidade de fosfato

Fabricio William Ávila[1], Valdemar Faquin[2], Douglas Ramos Guelfi Silva[2], Carla Elisa Alves Bastos[3], Nilma Portela Oliveira[2], Danilo Araújo Soares[2]

ABSTRACT

The effects of foliar and soil applied phosphite on grain yield in common bean (*Phaseolus vulgaris* L.) grown in a weathered soil under low and adequate phosphate availability were evaluated. In the first experiment, treatments were composed of a 2 x 7 + 2 factorial scheme, with 2 soil P levels supplied as phosphate (40 e 200 mg P dm^{-3} soil), 7 soil P levels supplied as phosphite (0-100 mg P dm^{-3} soil), and 2 additional treatments (without P supply in soil, and all P supplied as phosphite). In the second experiment, treatments were composed of a 2 x 3 x 2 factorial scheme, with 2 soil phosphate levels (40 e 200 mg P dm^{-3} soil), combined with 3 nutrient sources applied via foliar

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sprays (potassium phosphite, potassium phosphate, and potassium chloride as a control), and 2 foliar application numbers (single and two application). Additional treatments showed that phosphite is not P source for common bean nutrition. Phosphite supply in soil increased the P content in shoot (at full physiological maturity stage) and grains, but at the same time considerably decreased grain yield, regardless of the soil phosphate availability. Foliar sprays of phosphite decreased grain yield in plants grown under low soil phosphate availability, but no effect was observed in plants grown under adequate soil phosphate availability. In general, foliar sprays of phosphate did not satisfactorily improve grain yield of the common bean plants grown under low soil phosphate availability.

Index terms: *Phaseolus vulgaris*, tropical soil, foliar fertilizer, plant nutrition.

RESUMO

Os efeitos de fosfito aplicado via solo ou foliar sobre produção de grãos em feijoeiro (*Phaseolus vulgaris* L.), cultivado em um solo intemperizado sob baixa ou adequada disponibilidade de fosfato foram avaliados. No primeiro experimento, o delineamento consistiu de um esquema fatorial 2 x 7 + 2, sendo 2 doses de P fornecidas na forma de fosfato (40 e 200 mg P dm^{-3} de solo) x 7 doses de P no solo fornecidas na forma de fosfito (0-100 mg P dm^{-3} de solo), mais 2 tratamentos adicionais (sem fornecimento de P no solo, e todo o P fornecido na forma de fosfito). No segundo experimento, o delineamento consistiu de um esquema fatorial 2 x 3 x 2, com 2 doses de P no solo na forma de fosfato (40 e 200 mg P dm^{-3} de solo), combinados com 3 fontes de nutrientes aplicados via pulverização foliar (fosfito de potássio, fosfato de potássio, e cloreto de potássio como um controle), e 2 números de aplicações foliares (uma

e duas aplicações). Os tratamentos adicionais evidenciaram que o fosfito não é uma fonte de P para a nutrição do feijoeiro. O fornecimento de fosfito no solo aumentou o teor de P na parte aérea (no estágio de maturidade fisiológica) e nos grãos, mas, ao mesmo tempo, consideravelmente reduziu a produção de grãos, independentemente da disponibilidade de fosfato no solo. As pulverizações foliares de fosfito diminuiram a produção de grãos em plantas cultivadas com baixa disponibilidade de fosfato no solo, mas esse efeito não foi observado em plantas cultivadas com adequada disponibilidade de fosfato no solo. Em geral, as pulverizações foliares de fosfato não supriram adequadamente as necessidades de P pelo feijoeiro.

Termos para indexação: *Phaseolus vulgaris*, solo tropical, fertilizante foliar, nutrição vegetal.

INTRODUCTION

Subtropical and tropical regions of the word often exhibit highly weathered soils (e.g. Typic Haplustox) that are characterized by low natural fertility, especially by phosphorus (P) deprivation to plant nutrition. Phosphate anion ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) is major P form metabolized by plants for their adequate growth and development (Novais and Smyth, 1999; Ticconi et al., 2001), while phosphite anion ($H_2PO_3^-$ and HPO_3^{2-}) is used as fungicide to control some important plant diseases, such as *Phytophthora* sp (Deliopoulos et al., 2010).

Nevertheless, besides fungicide, recently phosphite-based products also have been marketed as fertilizers for foliar spray, fertigation and direct soil application (Thao and Yamakawa, 2009). Phosphite salts are marketed as

fertilizer because they contain a cation that may be plant nutrient, such as K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+} or Zn^{2+}, and often phosphite also is recommended as additional source of P for plant nutrition (Ávila et al., 2012a). But, phosphite effects on P nutrition of the crops still are inconclusive. It was reported that supply of phosphite improved avocado yield (Lovatt, 1990a), and restored normal growth in phosphate-deficient citrus (Lovatt, 1990b). Others authors also mentioned beneficial effects of phosphite on yield and P nutrition in some crops (Albrigo, 1999; Rickard, 2000). In contrast, the literature also shows that phosphite anion does not replace phosphate anion in P nutrition of the plants (Thao and Yamakawa, 2009), and still some works indicated that phosphite supply may cause growth depression in phosphate-deficient plants (Schröetter et al., 2006; Thao et al., 2008; Thao et al., 2009; Ávila et al., 2011; Zambrosi et al., 2011). However, most of the studies that evaluated the effects of phosphite anion on plant P nutrition were related to Arabidopsis, vegetables, seedlings, and some citrus and cereals; but there is still little knowledge about effects of phosphite on leguminous grain yield.

Common bean (*Phaseolus vulgaris* L.) is the most important leguminous crop in many subtropical and tropical regions of the world (Graham and Ranalli, 1997; Broughton et al., 2003). Previously, it was shown the effects of phosphite on growth and nutrition of common bean plants at flowering stage (Ávila et al., 2012a, b). In this study, the aim was to evaluate the effects of foliar-and soil-applied phosphite on grain yield in common bean plants grown in a weathered soil under low and adequate phosphate availability.

MATERIAL AND METHODS

The study was carried out at the Soil Science Department of the Federal University of Lavras (Lavras city, Minas Gerais State, Brazil) using samples of a low-fertility soil classified as Typic Haplustox (Soil Survey Staff, 1999) or sandy loam dystrophic Red-Yellow Latosol (Embrapa, 2006). Surface soil (0–20 cm depth) was collected from a non-cultivated field with natural Brazilian Cerrado vegetation. Later, after sieving through 4-mm mesh sieve, soil subsamples were characterized chemically, physically and mineralogically (Table 1), using the same methodology described in Souza et al. (2011).

Table 1. Chemical, physical and mineralogical attributes of the soil (Typic Haplustox) samples, prior to treatments.

Chemical [1]															
pH	P	K	Zn	Cu	Mn	Fe	EP	Ca	Mg	Al	H+Al	T	m	V	MPAC
	----------mg dm^{-3} of soil----------						mg L^{-1}	----------$emol_c$ dm^{-3} of soil------					------%-----		mg kg^{-1}
5.4	0.9	22	0.5	0.7	0.4	27.4	20.5	0.1	0.1	0.1	1.7	2	28	13.3	396
Physical [2]															
Sand				Silt				Clay					OM		
----------%----------															
60				17				23					0.8		
Mineralogical [3]															
SiO_2	Al_2O_3	Fe_2O_3	TiO_2	P_2O_5	Fe_d	Fe_o	Ct	Gb	Ki	Kr					
----------g kg^{-1} of clay----------															
95.1	97.4	36.2	6.2	0.0	10.8	0.1	752.0	63.0	0.98	0.71					

[1] pH in water, EP = P in the equilibrium solution; OM = level of organic matter; T = cation exchange capacity at pH 7.0; m = aluminum saturation index; V = vase saturation index; MPAC = maximum P adsorption capacity according to Olsen and Watanabe (1957); Ct = kaolinite; and Gb = gibbsite. Ki = SiO_2 / Al_2O_3; and Kr = SiO_2 / (Al_2O_3 + Fe_2O_3).

Posteriorly, soil samples were transferred into plastic pots (6 dm^3 of soil per pot), and then mixed with $CaCO_3$ and MgO (stoichiometric ratio of Ca:Mg = 4:1) to raise soil base saturation to 60% of cation exchange capacity at pH 7.0. After 30 days of incubation of the soil, two independent greenhouse experiments with common bean (*Phaseolus vulgaris* L.) cv. Radiante BRS were

simultaneously carried out in a completely randomized design with three replications. Each experimental unit consisted of one pot containing two common bean plants, and all measured variables were expressed as mean of two plants.

In first experiment, whose aim was to evaluate the effects of phosphite application in low-fertility soil on common bean plants, treatments were composed of a 2 x 7 + 2 factorial scheme, with 2 P levels supplied as phosphate anion (low phosphate availability = 40 mg P dm^{-3} soil, and adequate phosphate availability = 200 mg P dm^{-3} soil), 7 P levels supplied as phosphite anion (0; 3.125; 6.25; 12.5; 25; 50 and 100 mg P dm^{-3} soil), and 2 additional treatments: without supply of P in the soil, and all P (200 mg P dm^{-3} soil) from soil supplied as phosphite. Treatments were applied as basal dressing and then soil was homogenized, prior to sowing of the seeds. The phosphate levels (40 and 200 mg P dm^{-3} soil) were selected based on the growth response of common bean in a preliminary experiment to ensure an inadequate and adequate supply of P for maximum plant growth. Phosphate anion was supplied as KH_2PO_4 and $NH_4H_2PO_4$, and phosphite anion was supplied as KH_2PO_3 (monobasic potassium phosphite). KH_2PO_3 was obtained by the reaction of H_3PO_3 (phosphorous acid) with the KOH. All reagents were of pa grade.

In second experiment, whose aim was to evaluate the effects of foliar application of phosphite on common bean plants, the treatments were composed of a 2 x 3 x 2 factorial scheme, with 2 soil phosphate levels (low phosphate availability = 40 mg P dm^{-3} soil, and adequate phosphate availability = 200 mg P dm^{-3} soil), combined with 3 nutrient sources supplied via foliar application (KH_2PO_3, KH_2PO_4, and KCl used as control of K), and 2 foliar application numbers (single and two application). Single application was implemented

when plants presented fourth trifoliate leaf stage, and two applications was carried out in stage of fourth trifoliate leaf and another application in the beginning flowering stage. Solutions of KH_2PO_3, KH_2PO_4 and KCl, all of pa grade, were sprayed at concentration of 40 µM, using a manual backpack sprayer. Concentration of P equals the used dose of approximately 3 L of commercial potassium phosphite to 400 L of water, which is usually recommended for growing beans.

In both experiments, pots received application of fertilizers as basal dressing, made up of (in mg dm^{-3} of soil): 126 N, 126 K, 40 S, 6 Zn, 6 Mn, 2.5 Cu, 1.25 B and 0.25 Mo, all added as nutrient solutions that were prepared with pa grade reagents of the following: KH_2PO_3 (K added together with phosphite treatments from soil), KH_2PO_4 (K added together with phosphate treatments from soil), $NH_4H_2PO_4$ (N added together with phosphate treatments from soil), NH_4NO_3, $(NH_4)_2SO_4$, KNO_3, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $H_2MoO_4.2H_2O$, H_3BO_3 and $ZnSO_4.7H_2O$. Some of these sources were combined differently for each phosphate and phosphite treatment from soil. During the experimental period, fertilization as top dressing also was supplied individually to each pot at following rates (in mg dm^{-3} of soil): 210 N, 180 K and 42 S, using solutions of KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$. This fertilization was split among into three applications throughout the experiment. Soil water content was maintained at around 60% of the total pore volume by periodic weighing of the pots and adding deionized water to compensate for weight loss. Common bean plants were harvested at full physiological maturity, and grains were separated from shoot. Plant shoot and grains were dried for 72 hours at 60-65°C in a forced draught oven, weighed (for obtaining the dry mass weight) and triturated in a

Wiley-type mill. Samples of shoot and grains were analyzed for P content (Murphy and Riley, 1962) after nitric-perchloric digestion of the plant material.

Data obtained were submitted to variance analysis by F test ($p \leq 0.05$) using the SISVAR software (Ferreira, 2011). In first experiment (application of phosphite in the soil), statistical comparisons between the additional treatments, as well as between additional treatment and factorial experiment, were evaluated according to Healy (1956).

RESULTS AND DISCUSSION

In first experiment, shoot growth (at full physiological maturity stage) and grain yield of the common bean plants were considerably affected by application of medium and high phosphite levels in the soil (Typic Haplustox) (Figure 1).

Supply of low phosphite levels in soil had little effect ($p > 0.05$) on the shoot and grain dry weight of the common bean plants grown under low and adequate phosphate supply. However, in general from medium phosphite levels (25 mg P dm^{-3} soil), it was observed lower shoot and grain dry weight with increasing soil phosphite levels, which was exhibiting phosphite-toxicity symptoms such as both curved and malformed leaves. Values of shoot dry weight at highest phosphite level (100 mg P dm^{-3} soil), in comparison to zero phosphite level (without phosphite supply), were significantly ($p \leq 0.001$) decreased by 65 and 53% for plants grown under low and adequate phosphate availability, respectively (Figure 1a).

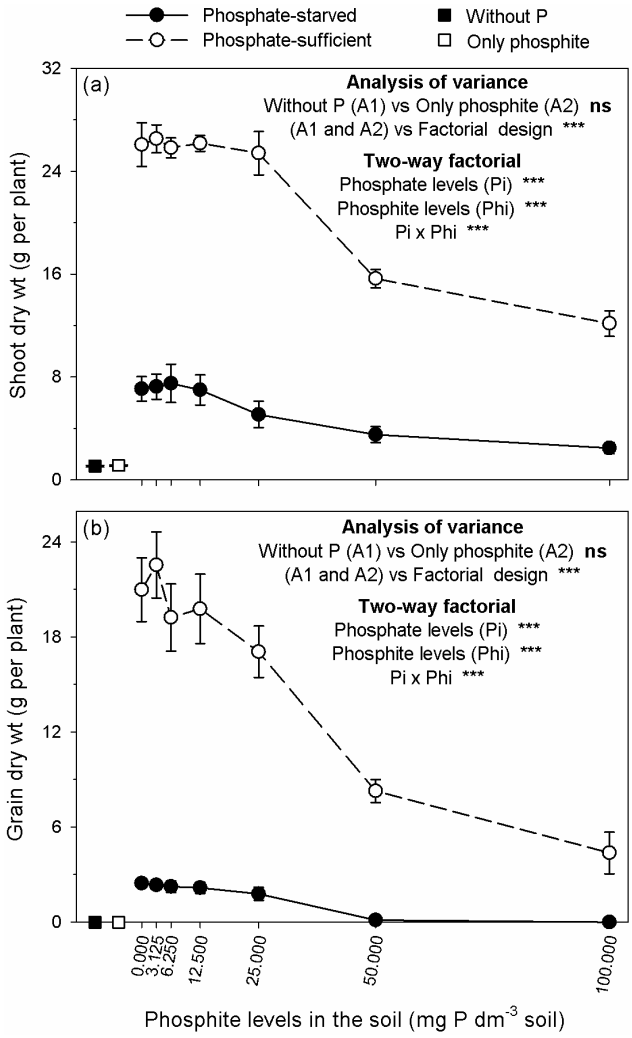

Figure 1. Shoot dry weight (a) and grain dry weight (b) in common bean grown under 2 phosphate levels (phosphate-starved = 40 mg P dm^{-3} soil, and phosphate-sufficient = 200 mg P dm^{-3} soil), and 7 phosphite levels in the soil. Without P = without supply of P in the soil. Only phosphite = supply of phosphite (200 mg P dm^{-3} soil) as source de P. The bars represent the standard error of the mean (n = 3). *** and ns (non-significant) corresponding to $p \leq 0.001$ and $p > 0.05$, respectively.

In addition, values of grain dry weight of the plants grown under adequate phosphate availability were significantly ($p \leq 0.001$) 35% lower at highest phosphite level than those at zero phosphite level, and plants grown under low phosphate availability did not produce grain at two higher phosphite levels (50 and 100 mg P dm^{-3} soil), thus showing 100% of decrease of the grain yield in comparison to zero phosphite level (Figure 1b).

The literature shows that some previous studies also found harmful effects of phosphite anion on plants grown under low phosphate availability, but no harmful effects have been reported when this anion was applied in plants grown under adequate phosphate availability (Ticconi et al., 2001; Varadarajan et al., 2002; Lee et al., 2005; Schröetter et al., 2006; Devaiah et al., 2007; Thao et al., 2008; Moor et al., 2009; Thao et al., 2009; Ávila et al., 2011). However, most of these studies were based in Arabidopsis, vegetables, seedlings, citrus, and some cereals, but there is still little information on the effects of phosphite on leguminous plants, especially on grain yield of leguminous. Moreover, these works studied effects of phosphite on plants grown in soils of low phosphate sorption capacity (soils of temperate regions of the world) or in nutrient solution, and also applied phosphite via foliar spray, but studies that relate effects of phosphite on plants grown in weathered soils of tropical and subtropical regions of the world still are rare (Ávila et al., 2012a).

Additional treatments also affected considerably the growth (at full physiological maturity stage) and yield grain of common bean. No P supply and P supply only as phosphite (200 mg P dm^{-3} soil), decreased by 96% and 100% the weight of shoot and grains (there was not produce grains in additional treatments), in comparison to those of plants grown under adequate phosphate availability (200 mg P dm^{-3} soil) and without phosphite supply (Figure 1). It was

not observed significant ($p > 0.05$) difference of shoot and grain yield between the two additional treatments. Thus, data show that phosphite anion did not replace phosphate anion in P nutrition for grain yield of common bean. These results are in agreement with those reported for other crops, such as *Ulva lactuca* (Lee et al., 2005), maize (Schröetter et al., 2006; Ávila et al., 2011), *Brassica rapa* (Thao et al., 2008), citrus rootstocks (Zambrosi et al., 2011), and sweet potato tissue cultures (Hirosse et al., 2012).

In second experiment, it found that foliar spraying of phosphite affected only the shoot growth (at full physiological maturity stage) and grain yield of the common bean plants grown under low phosphate availability, while no effect was verified for common bean plants grown under adequate phosphate availability (Figure 2). In comparison to control and potassium phosphate spray, regardless of foliar application numbers (single application timing and two application timings), shoot and grain dry weight was not significantly ($p > 0.05$) influenced by potassium phosphite spray when common bean plants grew under adequate phosphate availability in the soil. However, for plants grown under low phosphate availability, shoot and grain dry weight were significantly ($p < 0.05$) limited by foliar-applied phosphite.

The toxic effect of phosphite anion on plants deficient in phosphate anion also was found by others authors, as already was commented. The causes of this effect are not well understood. There is the hypothesis that plants did not metabolize phosphite anion, and this anion may suppress some plant responses to phosphate deficiency, such as synthesis of phosphatases and P transporters (Ticconi et al., 2001; Varadarajan et al., 2002; Ávila et al., 2011).

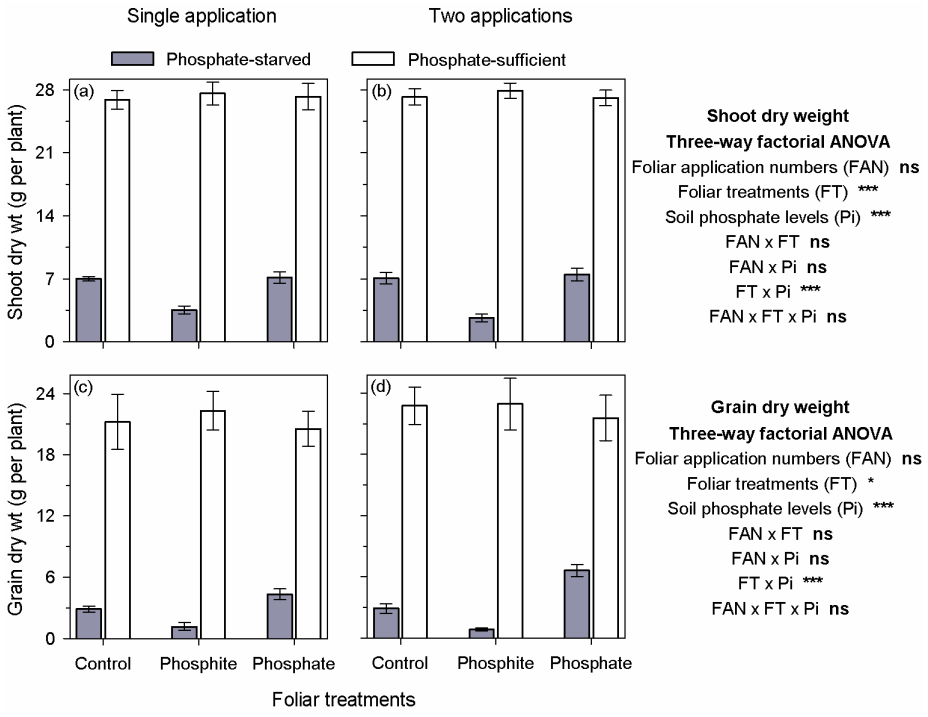

Figure 2. Shoot dry weight (a and b) and grain dry weight (c and d) in common bean grown under 2 phosphate levels (phosphate-starved plants = 40 mg P dm^{-3} soil, and phosphate-sufficient plants = 200 mg P dm^{-3} soil), 3 nutrient sources supplied via foliar application (potassium chloride as a control, potassium phosphite, and potassium phosphate), and 2 foliar application numbers (single and two applications). The bars represent the standard error of the mean (n = 3). ***, * and ns (non-significant) corresponding to $p \leq 0.001$, $p \leq 0.05$ and $p > 0.05$, respectively.

In relation to results from potassium phosphate spray, it was found that foliar-applied phosphate did not alter shoot growth and grain yield of the plants grown under adequate phosphate availability in the soil. However, although shoot growth was not influenced, the grain yield of the plants grown under low phosphate availability was a little higher ($p < 0.05$) with two foliar sprays of

phosphate, but this increase was not sufficient to compensate the low productivity caused by phosphate deficiency in the soil. Therefore, several foliar sprays of phosphate may be necessary to adequately correct a plant P deficiency (Faquin, 2005); this has been economically impractical (Ávila et al., 2012b).

The values of P content in shoot (at full physiological maturity stage) and grains of the common bean plants were considerably increased from medium phosphite levels in the soil (in general from 25 mg P dm^{-3} soil), regardless of the soil phosphate availability (Figure 3).

Shoot P content of the common bean plants grown under low and adequate phosphate availability was, respectively, 7- and 2-fold higher at maximum phosphite level than that at the zero phosphite level (Figure 3a). Besides the “concentration effect”, caused by growth reduction of the plants (Faquin, 2005), this relevant increase in shoot P content of the plants at the maximum phosphite level also was due to uptake of phosphite from soil by plants. Probably the common bean plants took up phosphite anion from soil, since medium and high soil phosphite levels were considerably toxic for plant growth. This supposition is confirmed by results of shoot P content in additional treatments. While shoot P content in the first additional treatment (without supply of P in the soil) was much lower than that in the second additional treatment (all P from soil was supplied as phosphite), the shoot dry weight did not vary between both additional treatments (in this case there was not influence of “concentration effect” in shoot of plants grown in the second additional treatment), showing that common bean takes phosphite anion from soil.

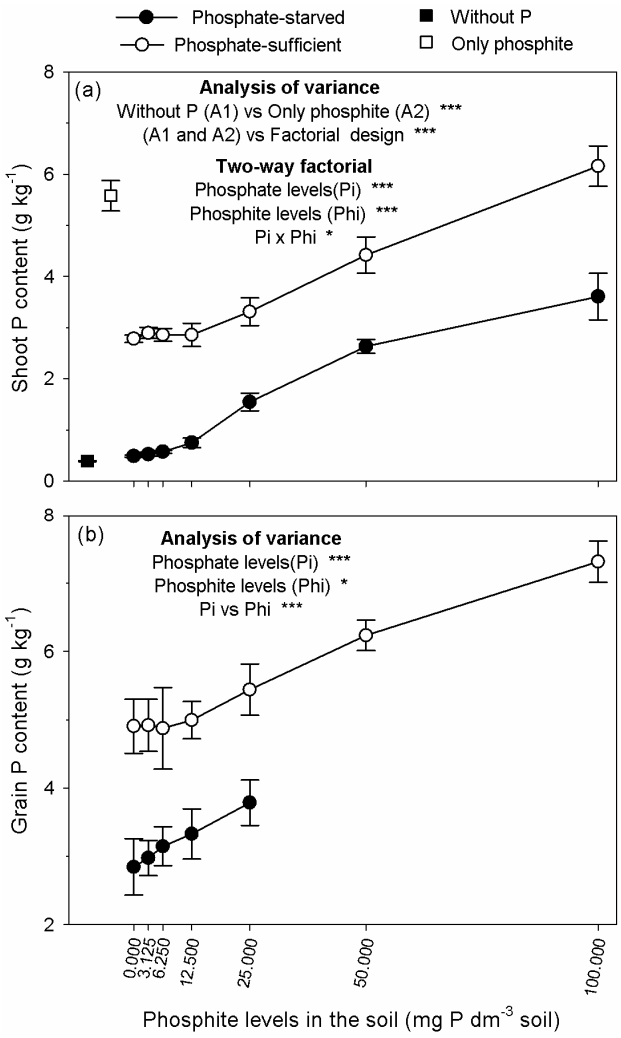

Figure 3. Shoot P content (a) and grain P content (b) in common bean grown under 2 phosphate levels (phosphate-starved plants = 40 mg P dm^{-3} soil, and phosphate-sufficient plants = 200 mg P dm^{-3} soil), and 7 phosphite levels in the soil. Without P = without supply of P in the soil. Only phosphite = supply of phosphite (200 mg P dm^{-3} soil) as source de P. The bars represent the standard error of the mean (n = 3). *** and * corresponding to $p \leq 0.001$ and $p \leq 0.05$, respectively.

In agreement with this study, Thao et al. (2009) also found increase in P content of hydroponic lettuce with supply of phosphite in nutrient solution. Moreover, in this work, grain P content of the plants grown under adequate phosphate availability was 1.5-fold higher at maximum phosphite level than that at zero phosphite level (Figure 3b). For plants grown under low soil phosphate availability, grain P content at level of 25 mg P dm^{-3} soil (maximum phosphite level in which there was grain yield of the deficient plants in phosphate) was 1.3-fold higher than that at the zero phosphite level.

Unlike the supply of phosphite in weathered soil, one or two foliar sprays of potassium phosphite did not influence significantly ($p > 0.05$) P content in shoot (at full physiological maturity stage) and grains of the common bean plants grown under low and adequate phosphate availability in the soil, when compared with the foliar sprays of potassium chloride or phosphate (Figure 4). However, in this study, foliar sprays of phosphite reduced shoot growth and grain yield, as already was commented, showing that common bean plants taken up the foliar-applied phosphite, but there was not variation in shoot and grain P content.

Foliar sprays of potassium phosphate also did not alter significantly ($p > 0.05$) P content in shoot (at full physiological maturity stage) and grains of the common bean plants, regardless of the soil phosphate availability (Figure 4). These results are in agreement with those obtained by Conte e Castro and Boaretto (2001) who found that three foliar application timing of phosphate did not affect P content in grains of common bean grown under field conditions. Finally, in both experiments, in general plants exposed to phosphate starvation exhibited lower P content in shoot (at full physiological maturity stage) and grains, showing that low phosphate availability from soil decreased the P uptake

by the common bean plants and, consequently, reduced their shoot growth and grain yield.

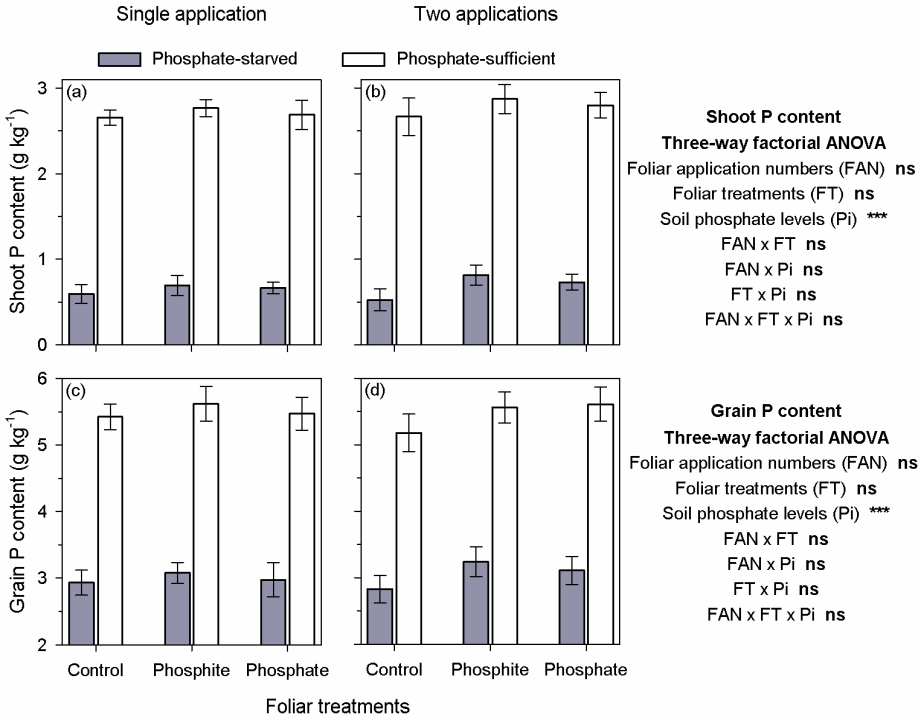

Figure 4. Shoot P content (a and b) and grain P content (c and d) in common bean grown under 2 phosphate levels (phosphate-starved plants = 40 mg P dm^{-3} soil, and phosphate-sufficient plants = 200 mg P dm^{-3} soil), 3 nutrient sources supplied via foliar application (potassium chloride as a control, potassium phosphite, and potassium phosphate), and 2 foliar application numbers (single and two applications). The bars represent the standard error of the mean (n = 3). *** and ns (non-significant) corresponding to $p \leq 0.001$ and $p \geq 0.05$, respectively.

CONCLUSIONS

Phosphite is not adequate P source to common bean crop.

Supply of medium and high phosphite levels in weathered soil decreased grain yield of common bean, regardless of soil phosphate availability.

Foliar sprays of phosphite decreased grain yield of the plants grown under low soil phosphate availability, but no effect was observed in plants grown under adequate soil phosphate availability. Thus, foliar sprays of phosphite in common bean crop to other purposes (e.g. fungicide) require adequate plant phosphate nutrition.

Either one or two foliar sprays of phosphate did not satisfactorily improve grain yield of the common bean plants grown under low soil phosphate availability.

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