ANA PAULA BRANCO CORGUINHA

BIOFORTIFICAÇÃO DE MANDIOCA:
PERSPECTIVAS PARA O ENRIQUECIMENTO COM FERRO E ZINCO

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 Recursos Ambientais e Uso da Terra, para a obtenção do título de Doutor.

Orientador

Dr. Luiz Roberto Guimarães Guilherme

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Dr. Eduardo Alano Viera

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APROVADA em 27 de julho de 2015.

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RESUMO GERAL

As deficiências de zinco (Zn) e ferro (Fe) são problemas bem conhecidos de saúde humana, especialmente em países em desenvolvimento. A biofortificação é uma estratégia que objetiva aumentar o teor de micronutrientes selecionados, incluindo Fe e Zn, em alimentos de grande consumo. A mandioca (*Manihot esculenta* Crantz) é uma cultura de grande importância em países tropicais e subtropicais em desenvolvimento, alimentando cerca de 600 milhões de pessoas por dia no mundo. Entretanto, a qualidade nutricional das raízes de mandioca não é suficiente para suprir todas as necessidades da dieta e estudos em biofortificação (agronômica e genética) nessa cultura podem levar a melhorias em sua qualidade nutricional. Neste contexto, nesse trabalho buscou informações e alternativas para a biofortificação de mandioca com Fe e Zn, através de programas de seleção de cultivares e por programas de manejo do solo. No primeiro estudo, foram quantificados os teores de Fe e Zn em clones de mandioca doce enriquecidos com carotenoides cultivados em experimentos de campo durante duas estações. No segundo estudo, foram selecionados acessos de mandioca do Banco Ativo de Germoplasma de Mandiocas do Cerrado (BGMC), localizado na Embrapa Cerrados, Planaltina, DF, para avaliar a variação genotípica e teor de micronutrientes nas raízes e folhas, a fim de fornecer informações para seleção de acessos de mandioca com capacidade de absorver minerais e para biofortificação da cultura. Por fim, no terceiro estudo, avaliou-se o teor de Zn em clones de mandioca enriquecidos com carotenoides cultivados sobre diferentes manejos do solo em diferentes áreas do Cerrado brasileiro, a fim de avaliar a influência da variação genotípica e do manejo agronômico no status de Zn na planta. Dessa forma, buscou-se promover conhecimento a cerca da nutrição de mandioca e contribuir para garantir segurança alimentar e nutricional da população brasileira, sobretudo daquela de baixa renda.

Palavras-Chave: biofortificação. *Manihot esculenta*. Micronutrientes. Variação genotípica. Manejo do solo.

GENERAL ABSTRACT

Iron (Fe) and zinc (Zn) deficiencies are well-known health problems in humans, especially in populations from developing countries. Biofortification is a strategy that aims to increase the content of selected micronutrients, including Fe and Zn, in staple foods. Cassava (*Manihot esculenta* Crantz) is a staple crop of tropical and subtropical developing countries, feeding more than 600 million people a day worldwide and can be a good source of nutrient and vitamins enrichment for the population. However, the nutritional quality of the cassava roots is not sufficient to meet all dietary needs and more studies on biofortification (genetic and agronomic) of this crop can lead to an improvement of its nutritional quality. In the present study, we sought to information and alternatives for cassava biofortification with Fe and Zn through cultivar selection and soil management programs. In the first study, we assessed the Fe and Zn contents in sweet cassava clones enriched with carotenoids grown in field trials during two seasons. In the second study, we selected accessions from the Cerrado´s Cassava Germplasm Bank (BGMC), located on Embrapa Cerrados, Planaltina, DF, Brazil, to evaluate the genotypic variation and micronutrients contents in roots and leaves in order to provide the information for selection of cassava accessions with substantial capacity to acquire minerals and for crop biofortification. Finally, in the third study, we investigated the Zn contents in cassava clones enriched with carotenoids cultivated under different soil management in different areas from the Brazilian Cerrado in order to evaluate the influence of genotypic variation and agronomic intervention on Zn status in the plant. Thus, this work aims to promote knowledge about cassava nutrition and contributes to guarantee food and nutritional security of Brazilian population, especially of the poor people.

Keywords: biofortification. *Manihot esculenta*. Micronutrients. Genotypic variation. Soil management.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A alimentação é um fator primordial na rotina diária da humanidade e um dos seus maiores desafios é produzir alimentos em quantidades suficientes para o suprimento de toda a população mundial. Estima-se que para atender a demanda de alimentos para uma população estimada em 9,1 bilhões de pessoas em 2050, a produção mundial de alimentos terá que crescer cerca de 70% (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAO, 2009).

Embora a produção vegetal esteja acompanhando o crescimento populacional, aproximadamente metade da população mundial vem sofrendo com deficiências nutricionais, principalmente mulheres grávidas, adolescentes e crianças em situações de extrema pobreza (WELCH; GRAHAM, 2004; MORAES, 2008). Ainda segundo Moraes (2008), as causas de maior preocupação em relação à saúde humana são as deficiências de ferro (Fe), iodo (I), selênio (Se), vitamina A e zinco (Zn), principalmente em países em desenvolvimento. Em parte, essa deficiência ocorre devido ao fato das pesquisas agrícolas serem voltadas para aumento de produção e não para a qualidade nutricional (MILLER; WELCH, 2013). Segundo estudos de Garvin, Welch e Finley (2006), ao longo dos anos, o ganho em produtividade obtido através do melhoramento vegetal apresenta relação inversa ao teor de micronutrientes em grãos de trigo. Além disso, grande parte da população, especialmente de países em desenvolvimento, alimenta-se de produtos de origem vegetal, os quais apresentam baixos teores de nutrientes, quando comparados aos de origem animal. A biodisponibilidade desses nutrientes nos alimentos vegetais

é geralmente baixa, o que agrava mais o problema da desnutrição (WELCH et al., 2005).

Deve-se então, buscar a produção de alimentos em quantidade e com qualidade, através de práticas de manejo da fertilidade do solo e de melhoramento de plantas que visem não somente as exigências das culturas ou o aumento da produtividade, mas também o fornecimento adequado de nutrientes aos animais e aos seres humanos (MORAES, 2008).

Com esta preocupação, vem crescendo o número de estudos relacionados à biofortificação de produtos de alto consumo mundial, sobretudo daqueles que compõem a dieta básica das populações de baixa renda. A mandioca (*Manihot esculenta*), por exemplo, é uma fonte de alimento potencialmente valiosa em países em desenvolvimento, cultivada em áreas tropicais e subtropicais, além de ser tolerante à seca (MONTAGNAC; DAVIS; TANUMIHARDJO, 2009). Cerca de 600 milhões de pessoas têm como principal fonte de nutrientes a mandioca, sendo mais de 200 milhões dessas, habitantes da África Subsaariana (SAUTTER et al., 2006).

Focar em pesquisas que permitam melhorar a absorção de nutrientes (e.g., Fe e Zn) em produtos agrícolas de interesse alimentar e econômico é de suma importância para oferecer alimentos de alto valor nutricional a toda a população mundial. Sendo assim, o presente estudo visa buscar informações e alternativas para a biofortificação de mandioca com Fe e Zn, tanto através de programas de seleção de cultivares, quanto por programas de manejo do solo. Espera-se, com isso contribuir para garantir segurança alimentar e nutricional da população brasileira, sobretudo daquela de baixa renda.

2 REFERENCIAL TEÓRICO

2.1 Deficiências de Micronutrientes e Vitaminas

Os humanos necessitam de nutrientes e vitaminas (tabela 1) que permitem o metabolismo adequado do organismo (WELCH; GRAHAM, 2004). Entretanto, diversas dietas falham no fornecimento da quantidade adequada desses nutrientes para a população, principalmente em países em desenvolvimento (WELCH, 2001).

A variação no teor de micronutrientes nos alimentos pode ser atribuída a características da planta, como idade, estágio de maturação, variedade, além de características do ambiente (e.g., solos e clima) e fatores do processamento, como temperatura, método de preservação e preparo do alimento (WELCH et al., 2005).

O acesso restrito das populações aos alimentos ricos em micronutrientes e a presença de inibidores e antinutrientes nas dietas, além da baixa biodisponibilidade dos minerais, são causas atribuídas às deficiências desses elementos na população humana. O consumo inadequado de um dos elementos necessários à saúde causa distúrbios no metabolismo, provocando perda de peso, letargia, apatia, falta de energia para as atividades normais, susceptibilidade a doenças e problemas de saúde (FAO, 2000). Tais problemas levam a um aumento da taxa de mortalidade, doenças crônicas, perda de habilidades cognitivas, entre outros. Além disso, a deficiência nutricional provoca problemas econômicos ao país, causados pelo menor rendimento dos trabalhadores e aumento dos custos (WELCH, 2001).

Tabela 1 – Lista de nutrientes e vitaminas essenciais e benéficos à vida humana[*]

Macronutrientes	**Micronutrientes**	**Vitaminas**
Nitrogênio (N)	Ferro (Fe)	A
Fósforo (P)	Zinco (Zn)	D
Potássio (K)	Cobre (Cu)	E
Cálcio (Ca)	Manganês (Mn)	K
Magnésio (Mg)	Iodo (I)	C
Enxofre (S)	Flúor (F)	B_1
	Boro (B)	B_2
	Selênio (Se)	B_3
	Molibdênio (Mo)	Niacina
	Níquel (Ni)	B_6
	Cromo (Cr)	Folato
	Silício (Si)	Biotina
	Estanho (Sn)	B_{12}
	Cobalto (Co)	

[*]Welch; Graham, 2004

Mais de três bilhões de pessoas no mundo sofrem de desnutrição e estimativas indicam que 805 milhões de pessoas apresentam desnutrição crônica, sendo que, dentre elas, cerca de 98% vivem em países em desenvolvimento (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS – FAO/ THE INTERNATIONAL FUND FOR AGRICULTURAL DEVELOPMENT – IFAD/ WORLD FOOD PROGRAMME – WFP, 2014). Segundo a FAO (2000), a deficiência de nutrientes e vitaminas é mais comum em mulher em idades reprodutivas, crianças e pessoas com doenças que comprometam o sistema imunológico, como a AIDS. Dentre as mortes anuais por deficiência, mais de 5 milhões dos mortos são crianças (BOUIS; WELCH, 2010).

As doses de recomendação diária para Fe, I, Se, vitamina A e Zn são listadas na tabela 2, baseada em dados da FAO e World Health Organization – FAO/WHO (2000). Vitamina A, Fe e Zn são considerados prioridades no combate à desnutrição humana (GRAHAM; WELCH; BOUIS, 2001).

Tabela 2 – Ingestão diária recomendada para homens e mulheres com idade entre 25 a 50 anos*.

Nutrientes	Homens	Mulheres
Vitamina A (μg retinol equivalente)	1000	800
Ferro (mg)	10	15
Zinco (mg)	15	12
Se (μg)	70	55
I (μg)	150	150

* FAO/WHO (2000).

2.1.1 Deficiência de Ferro (Fe)

O Fe é um metal de transição capaz de formar ligações com átomos doadores de elétrons, podendo formar grupos heme, os quais fazem parte de diferentes proteínas, além de existir em diferentes estados de oxidação, o que permite que ele seja um eficiente doador e receptor de elétrons (BARKER; PILBRAM, 2007). Este elemento apresenta um importante envolvimento no metabolismo, por ser um componente da hemoglobina, sendo cerca de 67% do seu teor total presente nestas proteínas (ANDERSON, 2005). O Fe atua no transporte de oxigênio e dióxido de carbono para todas as células do corpo, estando assim, diretamente, envolvido no processo de respiração celular (HEMALATHA; PLATEL; SRINIVASA, 2007), além de estar envolvido na síntese de purina, colágeno e neurotransmissores (ANDERSON, 2005). Ainda segundo Anderson (2005), há também o Fe enzimático, o qual corresponde a 0,2% do Fe total e atua no transporte de elétrons da cadeia respiratória.

Devido à sua atuação na hemoglobina, a deficiência de Fe é a principal causa de anemia no mundo (WHO, 2007). Estima-se que cerca de 50% da população mundial sofra de deficiência de Fe e que, por ano, 800 mil mortes são decorrentes da deficiência deste elemento (MAYER; PFEIFFER; BEYER,

2008). A anemia provoca retardamento das funções motoras e mentais em crianças, além de afetar a memória e outras funções mentais de adolescentes e provocar a fadiga em adultos, prejudicando a habilidade para realização de atividades físicas. Além disso, a deficiência de Fe durante a gravidez pode aumentar o risco de nascimento de bebês prematuros, sendo estes mais propensos a problemas de saúde ou a morte no primeiro ano de vida (CLARK, 2008).

A deficiência deste importante nutriente é, na maioria das vezes, associada às baixas concentrações deste elemento em alimentos vegetais (KABATA-PENDIAS; MUKHERJEE, 2007). O Fe em produtos de origem vegetal encontra-se na forma não-heme, forma esta de menor absorção pelo organismo humano (absorvido 5%, enquanto o Fe heme é absorvido 25%) (ANDERSON, 2005). Entretanto, a absorção desta forma de Fe pode ser aumentada pela presença de ácido ascórbico e outros ácidos orgânicos e também pela presença de vitamina A (TEUCHER; OLIVARES; CORI, 2004). Por outro lado, a absorção pode ser reduzida pela presença de certas substâncias, como o fitato, um componente presente em cereais e legumes capaz de formar complexos com o Fe e impedir sua absorção adequada (ZHAO; SHEWRY, 2011).

Assim, países em desenvolvimento, onde a dieta da população geralmente baseia-se em produtos vegetais, apresentam altos índices de desnutrição pela ausência de Fe. Segundo dados da WHO (2001), o índice médio de crianças anêmicas em países em desenvolvimento foi de 39%, enquanto que nos países industrializados este índice caiu para 20,1%. No Brasil, anemia decorrente da deficiência de Fe, em 2009, atingiu cerca de 20% das crianças e 30% das mulheres (BRASIL, 2009). A elevada incidência e as graves consequências da deficiência deste elemento fizeram com que diversos órgãos

nacionais e internacionais estabelecessem como meta a eliminação desta carência nutricional (FAO/IFAD/WFP, 2014).

2.1.2 Deficiência de Zinco (Zn)

O Zn é um elemento que atua em diferentes processos bioquímicos, sendo um componente essencial para diversas proteínas e enzimas (NRIAGU, 2007), além de estar envolvido na síntese de ácidos nucléicos (DNA e RNA) e no crescimento e diferenciação celular (MAYER; PFEIFFER; BEYER, 2008).

Em países em desenvolvimento, a deficiência de Zn encontra-se na quinta posição de fator de risco à saúde humana, logo após o uso de cigarro. No mundo, esta deficiência encontra-se na 11ª posição de fator de risco à saúde humana (WHO, 2002), afetando, aproximadamente, um terço da população mundial. Tal deficiência pode promover uma série de processos metabólicos (NRIAGU, 2007), sendo capaz de afetar o sistema imunológico, alterando a cicatrização de feridas, além de prejudicar a síntese de DNA e alterar os sentidos do paladar e olfato (FRAGA, 2005). Segundo Cakmak et al. (2010), as maiores consequências da deficiência de Zn ao organismo humano são danos às funções do cérebro, ao sistema imunológico e ao crescimento físico.

Segundo dados da WHO (2007), a deficiência de Zn é responsável por aproximadamente 16% das infecções respiratórias, 18% dos casos de malária e 10% das diarreias no mundo. Em geral, 1,4% do total das mortes são atribuídas à carência de Zn. Contudo, as informações a respeito da deficiência de Zn, tanto em âmbito nacional como mundial, ainda são escassas (Hess et al., 2009).

Como na deficiência de Fe, a deficiência de Zn é maior em países em desenvolvimento. Tal fato ocorre devido à dieta da população desses países ser baseada em produtos vegetais, os quais apresentam baixa biodisponibilidade de

Zn (CAKMAK et al., 2010). Essa biodisponibilidade pode estar relacionada não apenas à baixa ingestão do elemento, mas também pode ser causada pela presença de inibidores na absorção deste (LÖNNERAL, 2000). Assim como para o Fe, o fitato forma complexos fortes com o Zn, o que provoca um efeito negativo na absorção deste elemento pelo organismo (ZHAO; SHEWRY, 2011).

2.2 Mandioca

A mandioca (*Manihot esculenta* Cranz) é uma planta perene e arbustiva, que apresenta raízes tuberosas ricas em amido (SOUSA; AGUIAR; LÔBO, 2011) e folhas ricas em proteínas, vitaminas e nutrientes (MONTAGNAC; DAVIS; TANUMIHARDJO, 2009). Originada na América Tropical, esta cultura é cultivada principalmente em áreas tropicais e subtropicais, sendo uma importante fonte de alimentação da população de países em desenvolvimento, especialmente da África Subsaariana (HARVESTPLUS, 2005; SOUSA; AGUIAR; LÔBO, 2011). Aproximadamente 70 milhões de pessoas obtém da mandioca mais de 500 calorias por dia (HARVESTPLUS, 2005). A mandioca é uma cultura capaz de resistir a doenças e pragas, além de ser flexível quanto à época de colheita (HARVESTPLUS, 2005). Seu desenvolvimento ocorre até mesmo em solos com limitações nutricionais e na presença de alumínio, características comuns em solos tropicais. Em condições de restrição de umidade no solo, a mandioca perde suas folhas, porém quando o ambiente torna-se suficientemente úmido produz folhas novas. Além disso, parte do seu caule colhido pode ser transplantada, produzindo novas raízes e plantas (SAUTTER et al., 2006).

Devido sua importância na alimentação humana, a produção de raízes de mandioca no mundo cresceu em média 2,5% ao ano, entre 1961 a 2007,

alcançando uma produção de, aproximadamente, 215 milhões de toneladas e a décima posição no *ranking* das maiores produções agrícolas no mundo (SOUSA; AGUIAR; LÔBO, 2011). Ainda segundo esses autores, o Brasil ocupa a terceira posição no *ranking* mundial de produção, com um total produzido de 26 milhões de toneladas no ano de 2007. A região Nordeste é responsável por cerca de 37% da produção do país e o consumo per capita chega a 46 kg por ano (GONZALÉZ; JOHNSON; QAIM, 2009).

2.3 Biofortificação

A Revolução Verde promoveu avanços na agricultura, como melhoria nos sistemas de cultivo, que permitiram maior produção e oferta de alimentos, diminuindo a fome em massa. Os cereais produzidos forneciam quantidades suficientes de carboidratos e uma menor quantidade de proteínas, porém quase nenhum nutriente era suprido nesses alimentos. Tal mudança nos sistemas de cultivo contribuiu para redução de micronutrientes, sendo um dos fatores para a desnutrição (WELCH, 2001).

O grande desafio da agricultura moderna é aliar quantidade a qualidade, permitindo alta produção de alimentos que garantam a segurança alimentar. O conceito de Segurança Alimentar implica, de acordo com a *I Conferência Nacional de Segurança Alimentar*, realizada em julho de 1994, no "acesso em quantidade e qualidade de alimentos requeridos para a saudável reprodução do organismo humano e para uma existência digna" (FAO/WHO, 2000).

Existem três estratégias que podem ser utilizadas para melhorar o conteúdo nutricional de culturas e consequentemente da nutrição humana: i) reduzir a concentração de antinutrientes, que inibem a biodisponibilidade de minerais e vitaminas; ii) aumentar as concentrações de nutrientes e de

compostos que promovam a biodisponibilidade e iii) aumentar a concentração de minerais e vitaminas (BOUIS, 2003).

A primeira estratégia requer uma grande mudança na composição química de sementes e grãos, pois, a maior parte dos antinutrientes conhecidos é encontrada em sementes e desempenham papel benéfico no crescimento vegetal. Como exemplo podemos citar as fitinas, que são compostos primários de fósforo que são requeridos durante a germinação e desenvolvimento vegetal, contribuindo para o vigor e a viabilidade das sementes (BOUIS, 2003). Estes, porém, reduzem a biodisponibilidade de Fe e Zn.

Contudo, determinados aminoácidos, assim como cisteína, aumentam a biodisponibilidade desses elementos (HALLBERG, 1981). Estes aminoácidos ocorrem em muitos vegetais, mas encontram-se em menores concentrações quando comparados a alimentos de origem animal. Um pequeno aumento na concentração de aminoácidos nas plantas pode ter efeitos consideráveis sobre a biodisponibilidade de Fe e Zn na nutrição, pois incrementos de aminoácidos permitem compensar os efeitos negativos dos antinutrientes. Além disso, aminoácidos são normalmente constituintes das plantas, de modo que não trariam consequências negativas no crescimento caso haja aumento na concentração dos tecidos vegetais (WELCH; GRAHAM, 2004).

A segunda e terceira estratégias seriam através da biofortificação, processo pelo qual é feito o aumento do teor de nutrientes em produtos agrícolas a serem colhidos e/ou posterior a essa etapa, na industrialização, visando o enriquecimento do produto final (WELCH, 2001; MAYER; PFEIFFER; BEYER, 2008). Compreender os processos fisiológicos e os componentes moleculares que fundamentam a nutrição mineral de plantas e seu posterior entendimento objetivando o aumento no conteúdo mineral de espécies

agronomicamente importantes tem sido o foco de estudo de diversos pesquisadores (GRUSAK, 2002).

Culturas biofortificadas podem conter características agronômicas e nutricionais melhores do que aquelas não biofortificadas (WELCH; GRAHAM, 2004). Além disso, a fortificação industrial tem sido um dos melhores processos para o controle de deficiências de micronutrientes em crianças em todo o mundo (ZANCUL, 2004).

A fortificação de alimentos é considerada um dos exemplos mais bem sucedidos de alimentos funcionais por se tratar de uma importante estratégia para eliminar deficiências nutricionais (WAHLQVIST; WATTANAPENPAIBOON, 2002). A ideia de plantas enriquecidas com nutrientes e de estratégia de armazenamento deste podem ajudar a atingir o objetivo de fornecer a quantidade adequada do mineral à população e também garantir a sustentabilidade ambiental. Cada objetivo exige a compreensão de como plantas acumulam e armazenam minerais. Isso inclui compreensão da biodisponibilidade do elemento na rizosfera e raiz, bem como sua translocação e armazenamento na parte aérea (GUERINOT; SALT, 2001).

O enriquecimento de alimentos com micronutrientes por meio de métodos tradicionais de produção de vegetais ou via técnicas biomoleculares, é uma poderosa ferramenta de intervenção que visa pessoas mais vulneráveis (BOUIS, 2003). Esta ferramenta deve ser plenamente explorada pela saúde pública para redução do problema da desnutrição, com relação aos micronutrientes, nas comunidades (GRAHAM; WELCH; BOUIS, 2001). Além disso, trata-se de uma intervenção sustentável que depende de suplementação, fortificação e de programas governamentais, os quais não têm sido promissores em muitas nações em desenvolvimento.

A literatura relata ainda que, sementes enriquecidas com micronutrientes, ao serem semeadas em solos pobres, apresentam melhor viabilidade, maior vigor das mudas, estandes mais uniformes, menor densidade de plantas, melhor uso da água e são mais resistentes a doenças (WELCH, 2001). Com esses benefícios, há uma maior produtividade e maior retorno ao agricultor.

Programas de biofortificação requerem que pesquisadores ligados à área agrícola se aproximem cada vez mais de profissionais ligados às áreas de saúde e nutrição humana (BOUIS, 2003). Para tal, é necessário que exista cooperação multidisciplinar, vontade dos pesquisadores de diferentes áreas se comunicarem e incentivos financeiros para apoiar e divulgar os resultados obtidos por meio desse processo (NESTEL et al., 2006).

3 CONSIDERAÇÕES GERAIS

Temas como a biofortificação estão se tornando frequentes no meio científico, principalmente na literatura internacional. Trabalhos envolvendo biofortificação vêm abrangendo diversas culturas básicas. Dentre elas, pode-se citar a mandioca, uma cultura que apresenta grande importância em dietas de populações de países em desenvolvimento, cujos índices de desnutrição são elevados.

O aperfeiçoamento do potencial genético da mandioca em conjunto com o uso de práticas agrícolas eficientes pode ser explorado para promover melhorias na qualidade das raízes dessa cultura e beneficiar a saúde humana, uma vez que seu consumo vem crescendo. Assim, garantir uma dieta adequada e balanceada nutricionalmente pode ser o primeiro passo para auxiliar a erradicação da desnutrição mundial. Ainda, a adoção de políticas públicas de

interesse nacional e internacional, dentre as quais pode-se citar a biofortificação, o mapeamento e distribuição das carências nutricionais e a valorização da agricultura regional são essenciais para o sucesso dessas tecnologias.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Assessing Zinc and Iron Contents in Sweet Cassava Roots Enriched with Carotenoids for Biofortification Purposes

(Normas de publicação da revista *Jounal of Food Composition and Analysis*)

ASSESSING ZINC AND IRON CONTENTS IN SWEET CASSAVA ROOTS ENRICHED WITH CAROTENOIDS FOR BIOFORTIFICATION PURPOSES

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Abstract

Iron (Fe) and zinc (Zn) deficiencies are well-known health problems in humans, especially in populations from developing countries. Biofortification is a strategy that aims to increase the content of selected micronutrients, including Fe and Zn, in staple foods. Cassava (*Manihot esculenta* Crantz) is a staple crop of tropical and subtropical developing countries, feeding more than 600 million people a day worldwide and can be a good source of nutrient and vitamins enrichment for the population. This study used a combined approach for biofortification, assessing Fe and Zn contents in sweet cassava clones enriched with β-carotene and lycopene, precursors of vitamin A, grown in field trials in the central Brazilian Savanna during two seasons (2010/2011 – S1; 2011/2012 – S2). In the experiment with cassava roots enriched with β-carotene (yellow-colored pulps), the Zn contents ranged from 3.20 to 7.56 mg kg^{-1} dry weight

(DW), while the Fe content varied from 3.20 to 8.73 mg kg^{-1} DW. Clones 94, 215, and 246 were the most promising to be used for biofortification since they presented highest Zn and Fe contents in both seasons. In the experiment with cassava roots enriched with lycopene (pinkish-colored pulps) the Zn contents varied from 1.85 to 5.22 mg kg^{-1} DW and Fe contents ranged from 2.09 to 7.90 mg kg^{-1} DW. The BGMC 753, which was used as control, presented better results than the clones with pinkish-colored pulps, showing that they are not good for biofortification purposes.

Keywords: micronutrients, β-carotene, lycopene, biofortification, human nutrition.

1. Introduction

The food system is dysfunctional and is not able to delivery enough micronutrients to the nutritional requirement, which is the main cause of micronutrient malnutrition (Welch, 2005). This problem affects more than three billion people worldwide, mainly in developing countries (98%), where ~ 805 million people are chronically undernourished (FAO, IFAD and WFP, 2014). In these countries a great majority of the population is feed with vegetable and cereals, which have low mineral contents and bioavailability (Miller and Welch, 2013; Welch, 2005). Biofortification is a strategy used to alleviate malnutrition, by increasing the contents of nutrients or other compounds (e.g. lycopene, β-carotene) in staple food crops thru genetic or agronomic approaches (Cakmak, 2008; Hess et al., 2005; Miller and Welch, 2013). The targets of biofortification programs are staple foods due to their predominance in the diet of the poor people (FAO, IFAD and WFP, 2014).

Iron (Fe) and zinc (Zn) deficiencies are a major human health concern worldwide (Joint FAO/WHO, 2001; White and Broadley, 2009). Iron plays an important role in the metabolism as a component of hemoglobin (Anderson, 2005), and the most severe consequence of Fe deficiency is anemia, which is estimated to cause 800,000 deaths per year (Mayer et al., 2008). Zinc operates on different biochemical processes, being an essential component of proteins and enzymes (Nriagu, 2007). Besides is also involved in the synthesis of nucleic acids and cell growth and differentiation (Mayer et al., 2008) and its deficiency affects the immunological system, growth, and development of one out of every three people in the world (Cakmak et al., 2010).

Cassava (*Manihot esculenta Crantz*) is an important staple foods and have been the focus of several studies (Mayer et al., 2008; Montagnac et al., 2009; Sautter et al., 2006). Around 70 million people in the world consume more than 500 calories per day from cassava (HarvestPlus, 2005). Owing to that, this crop can be a good candidate for nutrient enrichment with easy access by the population of developing countries. Besides the nutrient supply, cassava can also be used as a source for enrichment of β-carotene (roots with yellow-colored pulps) and lycopene (roots with pinkish-colored pulps). These carotenoids are important in the human diet, since β-carotene has benefits as a vitamin A precursor, in the fight against the night blindness, growth disorders, and learning difficulties (Underwood and Smitasii, 1999), and lycopene as an antioxidant, in the fight against early aging and prostate cancer prevention (Shami and Moreira, 2004).

Biofortification of cassava can lead to an improvement of its nutritional quality, which may well reduce the deficiencies of minerals and vitamins in areas where cassava is a staple food. Therefore, this study used a combined approach for biofortification, assessing the contents of Fe and Zn in Brazilian

cassava clones enriched with β-carotene and lycopene from the cassava-breeding program of Embrapa Cerrados, Brazil. To obtain a better understanding of nutrient contents and improve the database, we investigate for two growing seasons the genetic variation and micronutrient contents in 20 cassava clones. This study provides important information for breeding cassava with the capacity of simultaneous accumulation of micronutrients and vitamins in edible parts.

2. Materials and Methods

2.1 Study area

Soil and cassava samples were obtained from an experimental field at Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cerrados) experimental station, located in Planaltina (DF), Brazil, during the 2011/2012 (S1) and 2012/2013 (S2) growing seasons. Composite soil samples (500 g) were made up of fifteen separate subsamples, which were collected from the 0-20 cm soil layer and then storage in plastic bags until analysis.

Two experiments were performed in a randomized block design with three replicates, one with cassava clones of yellow-colored pulps (enriched with β-carotene) and other with pinkish-colored pulps (enriched with lycopene). Each field plot of 1.20 m × 0.8 m consisted of 4 lines with 10 plants, and the useful area of each plot was made of 16 central plants. The experiment with yellow-colored pulps tested 12 sweet cassava clones (2008 generation): 26, 90, 91, 94, 215, 246, 259, 272, 273, 446, 450, and 497. The experiment with pinkish-colored pulps evaluated 8 sweet cassava clones (2008 generation): 341, 345, 378, 387, 390, 395, 406, and 413. For both experiments, the cassava cultivar IAC 576-70, popularly known as Japonesinha, was used as control. This cultivar has natural cream-colored pulps. It is indicated for commercial cultivation in the

Brazilian Federal District region (Fialho et al., 2009) and is identified as BGMC 753 in the Cassava Germplasm Bank of Embrapa Cerrados. The cultural practices followed the recommendation for cultivating cassava in the Cerrados region (Fialho et al., 2011; Fialho and Vieira, 2011).

Composite samples of cassava tubers (900-1000 g) were harvested by hand at the mature stage from each experimental field plot, washed with distilled water, peeled, and stored in a cold chamber (4ºC) for subsequent analyses.

2.2 Sample preparation, chemical and statistics analysis

The samples were prepared and analyzed following a rigid quality assurance/quality control (QA/QC) program to ensure accurate and reliable analytical data. Soil samples were air-dried, ground and sieved to <2 mm, while cassava samples were rinsed with distilled water, oven dried at 65°C, and ground to <0.38 mm using a stainless steel mill before analysis.

The chemical properties of the soil samples were determined following the methodology proposed by Silva (2009). Briefly, soil pH was determined in a 1:2.5 soil:water suspension. Soil organic matter content was determined using the potassium dichromate ($K_2Cr_2O_7$) method, which consists in oxidizing an aliquot of soil (0.5 g) with a solution of $K_2Cr_2O_7 + H_2SO_4$ at 160ºC. The excess of dichromate is titrated with 0.25 mol L^{-1} $FeSO_4$. Calcium and Mg were extracted mixing a volumetric soil aliquot (10 cm^3) with 100 mL of KCl (1 mol L^{-1}) at room temperature overnight. Available soil P, K, Fe, Zn, Mn, and Cu were extracted using 100 mL of the Mehlich^{-1} solution (0.05 mol L^{-1} HCl + 0.0125 mol L^{-1} H_2SO_4) reacted with 10 cm^3 of soil sample. The CEC was calculated as the sum of Ca, Mg, K, and Al (extracted by 1 mol L^{-1} KCl) (Silva, 2009). Calcium, Mg, Fe, Mn, Zn, and Cu concentrations in the extracted solutions were determined by flame atomic absorption spectrometer (F-AAS,

Perkin-Elmer® AAnalyst™800 – Waltham, MA, USA), using either certified or Sigma Aldrich® single elements AAS standards for QA/QC. Available K was measured by flame photometry and available P was determined by colorimetry.

The soil and cassava samples were microwave-digested according to the USEPA Method 3051A (USEPA, 1998) using a CEM® Mars-5 microwave system. An aliquot of a 0.5-g sample was combined with 10 mL of HNO_3 in Teflon® PTFE vessels and digested for 10 minutes in the microwave. All reagents used (Sigma-Aldrich® or Merck®) were of high purity, and the HNO_3 was distilled prior to its use in digestions. The total mineral contents (Cu, Fe, Mn, and Zn) in acid-digested solutions were determined using flame atomic absorption spectrometer (F-AAS). Standard reference materials from the Institute for Reference Materials and Measurements (IRMM-804 Rice flour and BCR® 142R Sandy Soil) and from the National Institute of Standards & Technology (SRM 1573a Tomato Leaves and SRM 2710 Montana Soil) were used to substantiate the accuracy of the analytical results obtained. Blank and certified reference samples were analyzed along with every batch of digestion.

The GENES software (Cruz, 2001) was used for the statistical analysis. The differences were determined by ANOVA (individual and joint analysis of variance) and Scott-Knott´s test to investigate statistically significant differences at $p<0.05$. Principal components analysis (PCA) was performed to identify the clones that presented the highest values for Zn and Fe in both seasons. The average of each clone and $n \times p$ matrix was obtained, where n are the clones ($n=13$ for cassava of yellow-colored pulps and $n=9$ for pinkish-colored pulps) and p are the parameters measured ($p=4$). Then, the dataset was standardized for mean 0 and variance 1 to minimize in the first component the effects of variance of individual parameters and the scale of each parameter. From the standardized dataset of the covariance matrix (which equals the correlation in this case), the

eigenvalues and eingenvectors were calculated and the biplot was made and improved from an Excel macro proposed by Lipkovich and Smith (2002). The number of components that explains the dataset was obtained considering eigenvalues higher then 1 (Kaiser, 1958).

3. Results

The mean Fe, Mn, Cu, and Zn contents obtained from the repeated analysis (n=8) of the standard reference materials are presented in Table 1. The micronutrients content recoveries in the certified samples exhibited reliable analytical data accuracy for Fe, Mn, Cu, and Zn analysis.

3. 1 Cassava with yellow-colored pulps experiment

Table 2 presents the soil chemical properties, with the available Fe, Zn, Mn, and Cu contents and table 3 displays the total contents of Fe, Zn, Mn, and Cu in the 2010/2011 (S1) and 2011/2012 (S2) seasons. The total micronutrient contents in the soil showed a slight variation between the seasons, except for Cu, which was 3-fold higher in the 2011/2012 season (S2) than in the 2010/2011 season (S1) (Table 3). However, the available content of this element did not vary between the seasons (Table 2). Total soil Zn did not vary between the seasons, while the available content of this element was higher in season S1. There were positive correlations between Zn vs Mn and Zn vs Cu contents in the soil in season S1 and Zn vs Cu and Zn vs Fe contents in the soil in season S2. However, the correlation coefficients were low (S1: Zn vs Mn, $r = 0.32$; Zn vs Cu, $r = 0.38$; S2: Zn vs Cu, $r = 0.42$; Zn vs Fe, $r = 0.39$).

The Zn contents in the roots ranged from 3.20 to 7.56 mg kg^{-1} dry weight (DW) (Figure 1). The average of Zn content in season S1 was 5.22 mg kg^{-1} DW, which was higher than that found in season S2 (4.60 mg kg^{-1} DW).

Two clones showed highest values (clones 91 and 246) in season S1, while four clones (clones 94, 246, 259, and 450) were found in season S2. The clone 246 showed the highest values in both seasons (7.56 and 6.20 mg kg^{-1} DW, S1 and S2, respectively). Some β–carotene enriched cassava clones had Zn values higher than that found in the BGMC 753, which was used as reference (Figure 1). These clones showed that have potential for use in plant breeding and can offer micronutrients, besides vitamins.

The Fe contents found in this study ranged from 3.20 to 8.73 mg kg^{-1} DW (Figure 2). As well as found for the Zn contents, the average values between the seasons also showed significant difference for Fe contents (S1: 6.6 mg kg^{-1} DW and S2: 4.0 mg kg^{-1} DW). In season S1, a significant difference among the clones was observed and five clones (clones 91, 94, 215, 246, and 272) showed highest amounts of Fe (Figure 2). However, the Fe contents in season S2 did not vary significantly. Comparing the values in the clones with the found value for the reference (BGMC 753) in the season S1, we detected that the clones with highest amounts of Fe showed values 2-fold higher than the reference.

In the present study, there were significant correlations between Zn vs Fe contents in both seasons but the correlations coefficients were low (S1: $p = 0.02$; $r = 0.38$; S2: $p = 0.01$; $r = 0.41$). There were no significant correlations for other micronutrients, except the correlations between Mn vs Zn and Mn vs Fe in season S1. However the correlations coefficients were also low (Table 4).
According to the PCA analysis, the clones 94, 215 and 246 showed the highest values for Zn and Fe contents in both seasons (Figure 3).

3.2 Cassava with pinkish-colored pulps experiment

In the soil analysis, the soil chemical properties with available micronutrient contents (Table 5) and their total contents (Table 6) in this experiment showed the same tendency as the experiment with clones with yellow-colored pulps. The Mn and Cu contents were higher in season S2, but the available value did not vary for Cu and was lower in season S2 for Mn. There were positive correlations between Zn vs Mn, Zn vs Cu, Fe vs Mn, and Fe vs Cu contents in the soil in season S1 and Zn vs Cu and Mn vs Fe contents in the soil in season S2 (S1: Zn x Mn, r = 0.54; Zn x Cu, r = 0.64; Fe x Mn, r = 0.51, and Fe x Cu, r = 0.56; S2: Zn x Cu, r = 0.56; Fe x Mn, r = -0.66).

The contents of Zn in the roots varied from 1.85 to 5.22 mg kg^{-1} DW (Figure 4). Similarly to the yellow-pulps clones experiment, the Zn average content in season S1 (4.07 mg kg^{-1} DW) was higher than in season S2 (2.88 mg kg^{-1} DW), but both values were lower than that found in the first experiment.

Season S1 showed no significant differences in Zn contents among clones, while clones 345, 387, 390, and 413 presented the lowest Zn amounts in season S2. However, the clones did not show higher amounts of Zn when compared with the reference (BGMC 753).

The Fe contents observed in this experiment varied from 2.09 to 7.9 mg kg^{-1} DW (Figure 5). The average values followed the same trend seen in the yellow-pulps experiment, with season S1 average content (6.7 mg kg^{-1} DW) higher than season S2 (3.42 mg kg^{-1} DW). Clone 390 showed the lowest Fe content in season S1 and clones 413 and BGMC 753 showed the highest in season S2 (Figure 5).

In relation to nutrient interactions, there was correlation between Zn vs Fe in season S2 ($p = 0.04$, $r = 0.40$), but not in season S1 ($p = 0.16$). Also, the interaction between Zn vs Mn in both seasons, Fe vs Mn, and Fe vs Cu in season

S2 was not significant, whereas Zn vs Cu (S1 and S2), Fe vs Mn (S1) and Fe vs Cu (S1) showed positive correlation (Table 7).

The PCA analysis showed that the BGMC 753 has the highest values for Zn and Fe, followed by the clone 315 (Figure 6), which indicates that the clones enriched with lycopene are not good ones for biofortification purposes.

4. Discussion

Nutrients statuses in the soil are important to provide good nutrition for the plants. Cassava should not be cultivated in the same area 2 years consecutively, i.e., cassava production requires crop rotation, which is a management practice that helps preventing the depletion of specific elements (e.g., micronutrients) caused by single crops (Fialho et al., 2011). Thus, the experiments were conducted in different areas to avoid soil exhaustion. The results of soil chemical analysis in the cassava yellow-colored pulps experiment showed a 3-fold difference in total soil Cu content in season/soil S2 when compared with season/soil S1, but the available content did not show significant differences between the seasons/soils. We also observed a variation in the available content of Zn between the seasons, while total soil Zn contents did not vary.

The soil chemical analysis of the cassava with pinkish-colored pulps experiment showed similar results as the ones found in the soil cultivated with the cassava with yellow-colored pulps, showing total Cu varying between the seasons/soils (S2 > S1), but no variation for the available contents. The available contents of Mn and Zn were lower in season/soil S2. The availability of micronutrients can be affected by different aspects, as pH, i.e., a lower availability of most cationic micronutrients is expected as soil pH increases (Abreu et al., 2007). In fact, soil pH in season S2 was higher than that found in

season S1, which can explain the difference found for Zn availability. However, it could not provide a rationale for the difference found between the available contents of Cu, which were already very low in both seasons/soils. As one of the most important soil chemical attributes affecting trace metals solubility, a higher soil pH is also a major factor increasing adsorption of these elements to soil constituents (Cakmak, 2008). This is especially true for Cu when compared with Zn, as the first element tends to be specifically adsorbed to most soil colloids, including the organic ones (Sparks, 2003). In reality, even though total soil Cu in season/soil 2 was higher, the higher content of soil organic matter as well higher cationic exchange capacity of this soil, compared with season/soil 1 may hinder the availability of Cu in season 2.

Cassava is a good energy source, however contains little zinc, iron and β-carotene (Maziya-Dixon, 2000). Combining vitamins with micronutrient enrichment can be a good alternative to fortify cassava roots/tubers. Several factors can influence the composition of cassava, including environmental conditions, geographic location, plant age, as well as variety (Montagnac et al., 2009). Such combination of events leads to significant differences in the values found for Zn contents in many studies worldwide. Chávez et al. (2005) evaluated the mineral contents in roots of 600 cassava genotypes and detected Zn values ranging from 2.63 to 37.5 mg kg^{-1} DW, while a study evaluating 20 genotypes from the CIAT core collection showed Zn contents varying from 4.4 to 8.6 mg kg^{-1} DW (Chávez et al., 2000). In the study of Ssemakula et al. (2008) the Zn content in the roots of 27 cassava clones ranged from 7.6 to 13.9 mg kg^{-1} DW.

Besides β-carotene, cassava also contains other carotenoids, as lycopene, which has antioxidant effects (Stahl and Sies, 1996). In this study, we analyzed 8 cassava clones enriched with lycopene, a carotenoid that yields pinkish-colored pulps. Yet, we could not find any studies analyzing

micronutrient contents (e.g., Fe and Zn) in this kind of cassava. The Zn contents were lower for pinkish-colored when compared with yellow-colored pulps, as well as lower than values reported for a core collection from CIAT, Colombia (Chávez et al., 2000; Chávez et al,. 2005) and the reference (BGMC 753) used in our study. So, we conclude that pinkish-colored clones are not good candidates to be used for Zn biofortification.

The difference of the mean Zn contents in the cassava roots we found between the seasons, in both experiments, may be related to the lower availability of this micronutrient in the season-S2 soil. These results are below those found by (Montagnac et al., 2009), who reported a Zn content of 41 mg kg^{-1} DW, i.e., up to 8-fold higher than what we found in the present study.

Besides the differences between the seasons, we also found significant differences in Zn contents between the studied clones, which agrees with data from a study conducted in Nigeria (with a reported range of concentrations varying from 4.3 to 18 mg kg^{-1} DW) (Maziya-Dixon et al., 2000) and also by Adeniji et al. (2007), who found values varying from 5.3 to 8.5 mg kg^{-1} in six cassava cultivars.

The results of Fe contents found in our study were lower than those reported for cassava cultivars cultivated in Ibadan, Nigeria, which presented Fe contents varying from 4 to 49 mg kg^{-1} DW (Maziya-Dixon et al., 2000). In the study of Adeniji et al. (2007), the highest amount of Fe (184 mg kg^{-1} DW) was 21-fold and 23-fold higher than the highest value found in the yellow-colored pulps (8.73 mg kg^{-1} DW) and pinkish-colored pulps studies (7.9 mg kg^{-1} DW), respectively. The average values for Fe in the present study were slightly higher than those reported by Mitchikpe et al. (2008) (3.00 mg kg^{-1} DW) and lower than those found by Maziya-Dixon et al. (2000) (18.8 mg kg^{-1} DW) and Ssemakula et al. (2008) (10 mg kg^{-1} DW). Adeniji et al. (2007) also reported

significant differences for Fe contents among cassava varieties. In our study, we observed significant difference in Fe contents in both seasons for clones with pinkish-colored pulps and only in season S1 for clones with yellow-colored pulps. As observed for Zn, the cultivar BGMC 753 showed better results for Fe contents than the clones with pinkish-colored pulps. Such results may indicate that clones enriched with lycopene are also not good for Fe biofortification purposes.

Fortification strategies need to consider the potential risk of negative interactions among micronutrients (Sandström, 2001). Iron status has antagonistic influence in Zn absorption and vice-versa. However, we could not see negative correlation between contents of these elements in any season. According to Sandström (2001), micronutrients present in foods may not be vulnerable to negative interactions if they have specific absorption mechanisms.

Finally, clones with highest amounts of Zn and Fe in both seasons, i.e., with greatest potential for biofortification could be identified via PCA analysis. With that, we conclude that yellow-colored cassava clones 94, 215, and 246 are the best ones to be used for biofortification purposes. However, we could not select any cassava clones with pinkish-colored pulps, as they presented values for Zn and Fe smaller than those found for the reference material BGMC 753, confirming the hypothesis that cassava clones with pinkish-colored pulps are not good candidates for biofortification.

5. Conclusions

This investigation suggests that Zn and Fe contents in cassava clones are strongly influenced by genetic variability. Cassava accessions enriched with β-carotene have a great potential to be explored to improve their nutrients and vitamins content, since they presented higher values for both nutrients than the

accession with cream-colored pulps (used as reference), especially the clones 94, 215 and 246, which presented the greatest values for both Zn and Fe. Thus, there is a potential to develop cassava clones enriched with β-carotene, Zn and Fe. However, the clones with pinkish-colored pulps are not good candidates for Fe and Zn biofortification purposes, as they did not present greater values than the reference.

The genetic variation can be explored to improve the nutritional quality of cassava roots. Therefore, there is a great potential to explore the Brazilian cassava clones in order to optimize the nutritive genetic potential for biofortification purposes, since cassava is a staple food not only in Brazil, but also in many developing countries.

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Tables

Table 1. Certified value. determined concentration and Fe. Zn. Mn and Cu recovery on certified materials.

		Certified value (mg kg^{-1})	Determined concentration* (mg kg^{-1})	Mean recovery (%)
IRMM-804	Fe	---	---	---
	Zn	23.1±1.9	20.6±0.6	89
	Mn	34.2±2.3	29±0.5	85
	Cu	2.74±0.24	2.1±0.9	75
BCR® - 142R	Fe	---	---	---
	Zn	93.3±2.7	82.4±8	88
	Mn	800±50	732±97	91
	Cu	69.8±1	50±1.7	71
SRM 1573a	Fe	368±7	268±7	73
	Zn	30.9±0.7	30.5±1.5	99
	Mn	246±8	253±10	103
	Cu	4.7±0.14	3.42±0.09	73
NIST 2710	Fe	43200±800	35070±993	81
	Zn	4180±20	3392±76	81
	Mn	2140±60	1765±158	82
	Cu	3420±50	2780±68	82

*mean of 8 measurements of standard reference material samples. Mean Recovery (%) = (Mean $_{\text{Determined concentration}}$/Mean $_{\text{Certified value}}$) x 100%; ---: value not certified.

Table 2. Soil chemical characteristics of sweet cassava yellow-colored pulps areas, during the 2010/2011 (S1) and 2011/2012 (S2) seasons.

Soil	pH	P	K	Ca	Mg	CEC	OM	Fe	Cu	Mn	Zn
		---------- mg dm^{-3} ----------			cmol dm^{-3}	$cmol_c$ dm^{-3}	g kg^{-1}	---------- mg dm^{-3} ----------			
S1	5.1±0.3	11±6	63±20	1.9±0.5	0.7±0.2	3.3±0.6	32±1.6	46±8	0.4±0.1	11±2	3.3±1.3
S2	5.6±0.1	5.3±1.5	54±11	3±0.3	1.2±0.2	4.6±0.4	29±0.2	35±5	0.6±0.1	4.1±0.9	0.7±0.2

Values are the means ± standard deviation (SD) (n=3). Data for Fe, Cu, Mn, and Zn express plant-available contents.

Table 3. Total zinc, iron, manganese, and copper contents in soils cultivated with sweet cassava with yellow-colored pulps, during the 2010/2011 (S1) and 2011/2012 (S2) seasons.

Seasons	Micronutrient			
	Zn	Mn	Cu	Fe
	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(g kg^{-1})
S1	12.4±0.3	23.5±3.5	4.8±0.1	4.8±0.1
S2	11.4±0.2	18.7±2.0	16.7±0.4	4.4±0.2
Average	11.9±0.2	21.1±2.6	10.7±0.7	4.6±0.1

Values are the means ± standard deviation (SD) (n=3).

Table 4. Simple descriptive statistics (minimum, maximum, mean and standard error) for Mn and Cu contents (dry weight basis) in roots of sweet cassava clones with yellow-colored pulps cultivated during 2010/2011 (S1) and 2011/2012 (S2) seasons, and their correlations with Zn and Fe contents.

						Correlations with*			
					Standard	**S1**		**S2**	
	Micronutrient	**Min**	**Max**	**Mean**	**Error**	**Zn**	**Fe**	**Zn**	**Fe**
S1	**Mn**	1.18	2.23	1.70	0.09	0.009 (0.41)*	0.0008 (0.51)	---	---
	Cu	0.88	0.34	0.56	0.03	0.32 (0.16)	0.41 (0.14)	---	---
S2	**Mn**	0.81	2.18	1.66	0.09	---	---	0.71 (0.06)	0.96(-0.007)
	Cu	0.55	2.21	1.31	0.07	---	---	0.43 (0.13)	0.06 (-0.30)

* *p* value (correlation coefficient)

Table 5. Soil chemical characteristics of sweet cassava pinkish-colored pulps areas, during the 2010/2011 (S1) and 2011/2012 (S2) seasons.

Soil	**pH**	**P**	**K**	**Ca**	**Mg**	**CEC**	**OM**	**Fe**	**Cu**	**Mn**	**Zn**
		---------- mg dm^{-3} ----------			cmol dm^{-3}	$cmol_c$ dm^{-3}	g kg^{-1}	---------- mg dm^{-3} ----------			
S1	5.4±0.1	18±6	68±14	2.2±0.3	0.6±0.1	3.3±0.4	23±0.2	45±7	0.4±0.2	10±3	3.7±1
S2	5.9±0.1	4.9±1.3	67±18	3.4±0.4	1.6±0.1	5.3±0.1	28±0.2	34±6	0.4±0.1	4.6±0.9	0.7±0.3

Values are the means ± standard deviation (SD) (n=3). Data for Fe, Cu, Mn, and Zn express plant-available contents.

Table 6. Total zinc, iron, manganese, and copper contents in soils cultivated with cassava with sweet pinkish-colored pulps, during 2010/2011 (S1) and 2011/2012 (S2) seasons.

Seasons	Micronutrient			
	Zn	Mn	Cu	Fe
	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(g kg^{-1})
S1	11.9±0.3	20.7±0.5	3.4±0.1	4.7±0.1
S2	9.0±0.2	25.4±0.8	13.9±0.6	4.9±0.2
Average	10.5±0.4	23.1±0.8	8.7±1.1	4.8±0.2

Values are the means ± standard deviation (SD) (n=3).

Table 7. Simple descriptive statistics (minimum, maximum, mean and standard error) for Mn and Cu contents (dry weight basis) in roots of sweet cassava clones with pinkish-colored pulps cultivated during 2010/2011 (S1) and 2011/2012 (S2) seasons, and their correlations with Zn and Fe contents.

						Correlations with*			
						S1		**S2**	
	Micronutrient	**Minimum**	**Maximum**	**Mean**	**Standard Error**	**Zn**	**Fe**	**Zn**	**Fe**
S1	**Mn**	0.81	1.50	1.17	0.06	0.76 (-0.06)	0.04 (0.40)	---	---
	Cu	0.21	0.61	0.40	0.03	0.009 (0.49)	0.008 (0.50)	---	---
S2	**Mn**	0.59	1.70	0.95	0.08	---	---	0.48 (-0.14)	0.18 (-0.26)
	Cu	0.67	1.38	1.03	0.07	---	---	0.04 (0.40)	0.19 (0.26)

* *p* value (correlation coefficient)

Figures

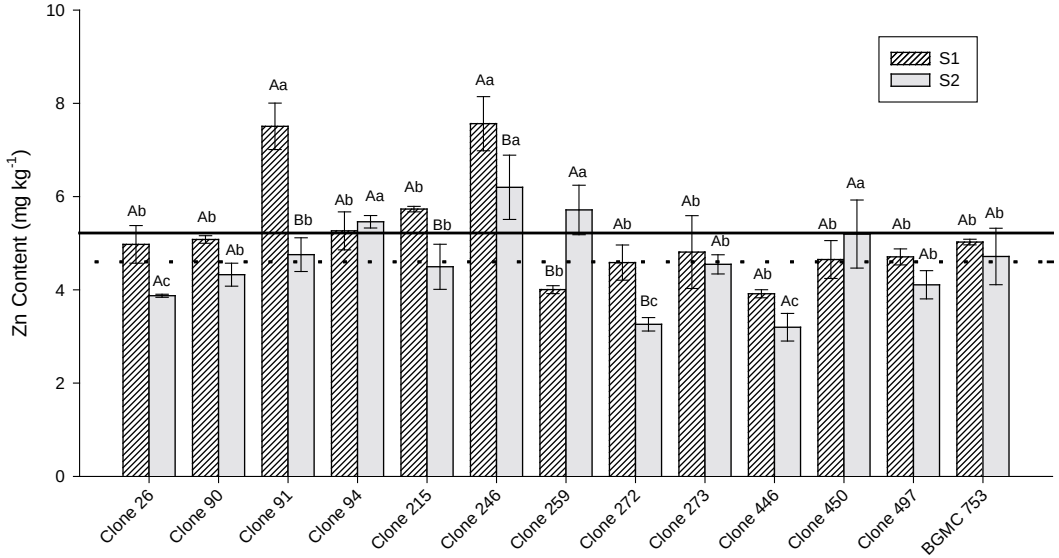

Figure 1. Genotypic variation of Zn contents of roots of sweet cassava clones with yellow-colored pulps grown in Planaltina, DF, Brazil, during the 2010/2011 (S1) and 2011/2012 (S2) seasons. Values shown are mean ± SE (n=3; p≤0.05). Solid line represents the average in season S1 and dash line is the average in season S2. Values followed by the same capital letters between the seasons in the each clone and lower case letters between the clones in each season do not significantly differ by the Scott-Knott test (p<0.05);

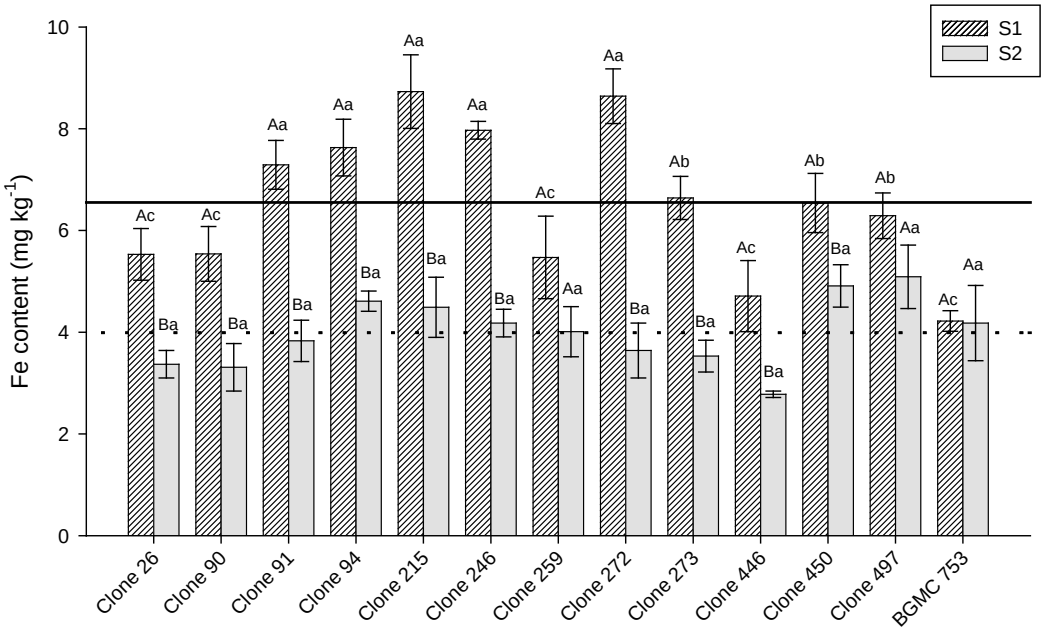

Figure 2. Genotypic variation of Fe contents of roots of sweet cassava clones with yellow-colored pulps grown in Planaltina, DF, Brazil, during the 2010/2011 (S1) and 2011/2012 (S2) seasons. Values shown are mean ± SE (n=3; p≤0.05). Solid line represents the average in season S1 and dash line is the average in season S2. Values followed by the same capital letters between the seasons in the each clone and lower case letters between the clones in each season do not significantly differ by the Scott-Knott test (p<0.05);

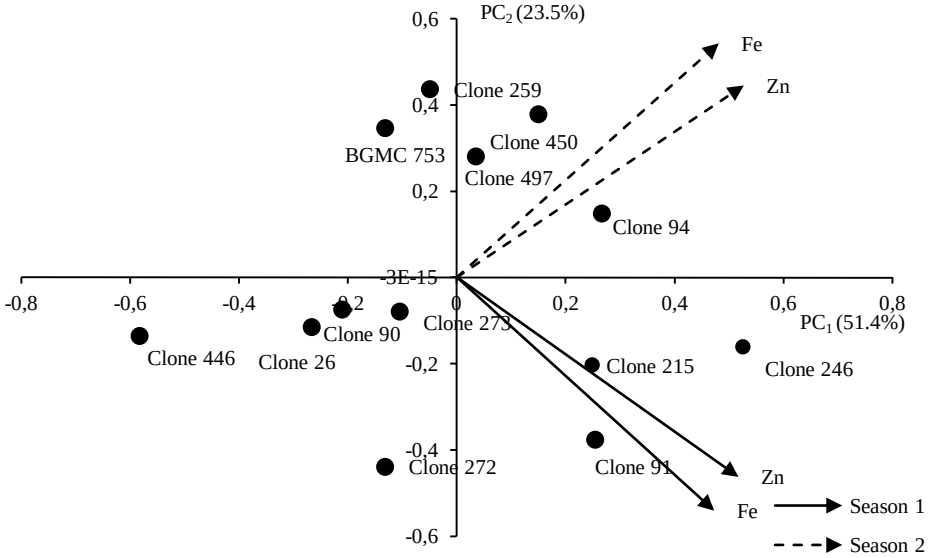

Figure 3. Graphic representation of the principal component analysis (PCA) of sweet cassava yellow-pulps clones according to the Zn and Fe contents in two seasons.

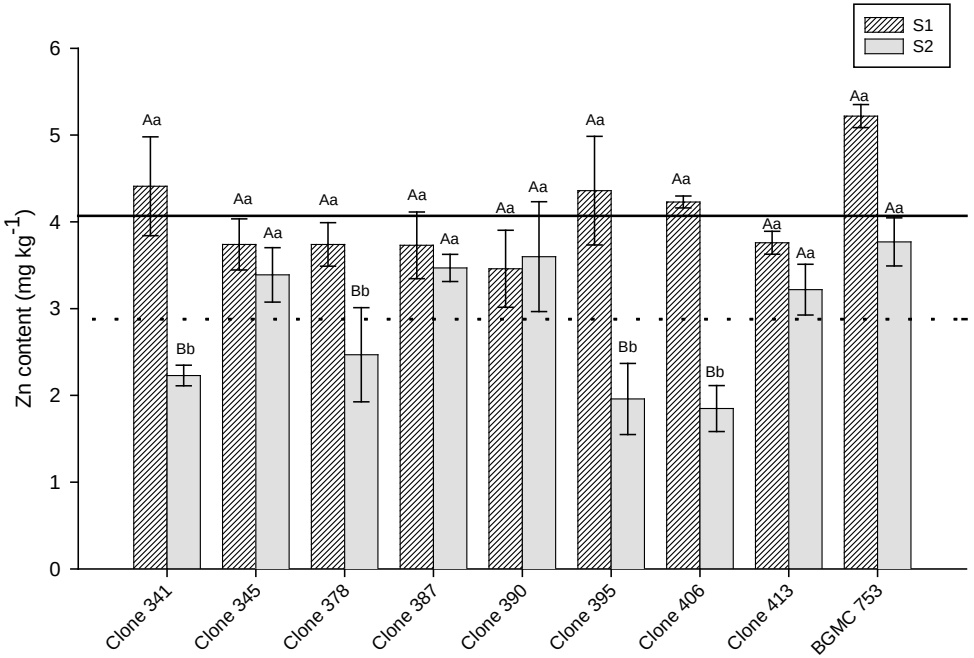

Figure 4. Genotypic variation of Zn contents of roots of cassava clones with pinkish-colored pulps grown in Planaltina, DF, Brazil, during the 2010/2011 (S1) and 2011/2012 (S2) seasons. Values shown are mean ± SE (n=3; p≤0.05). Solid line represents the average in season S1 and dash line is the average in season S2. Values followed by the same capital letters the seasons in the each clone and lower case letters between the clones in each season do not significantly differ by the Scott-Knott test (p<0.05);

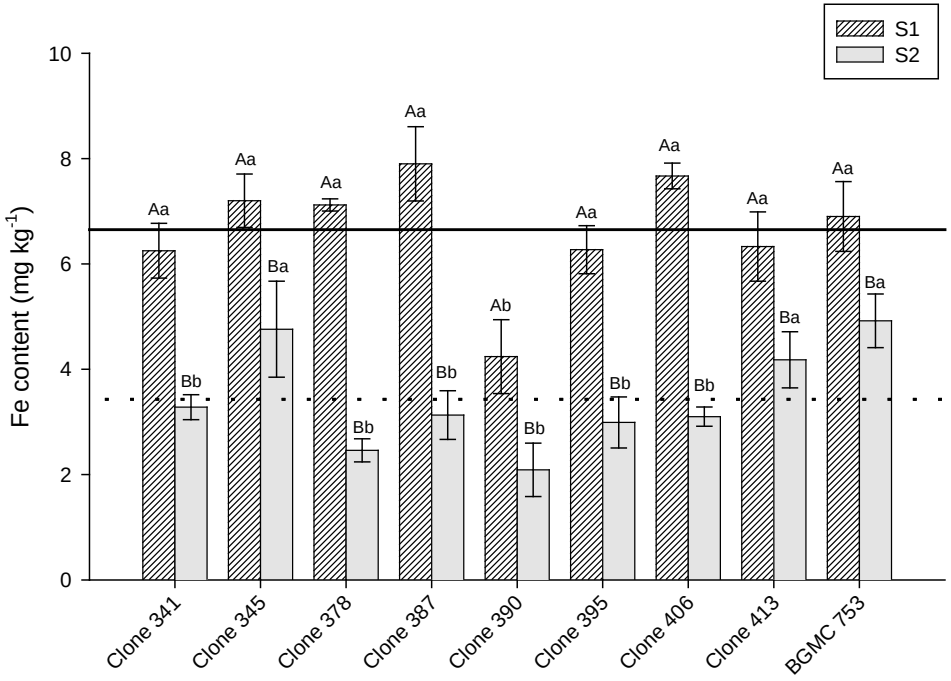

Figure 5. Genotypic variation of Fe contents of roots of cassava clones with pinkish-colored pulps grown in Planaltina, DF, Brazil, during the 2010/2011 (S1) and 2011/2012 (S2) seasons. Values shown are mean ± SE (n=3; p≤0.05). Solid line represents the average in season S1 and dash line is the average in season S2. Values followed by the same capital letters the seasons in the each clone and lower case letters between the clones in each season in each clone do not significantly differ by the Scott-Knott test (p<0.05);

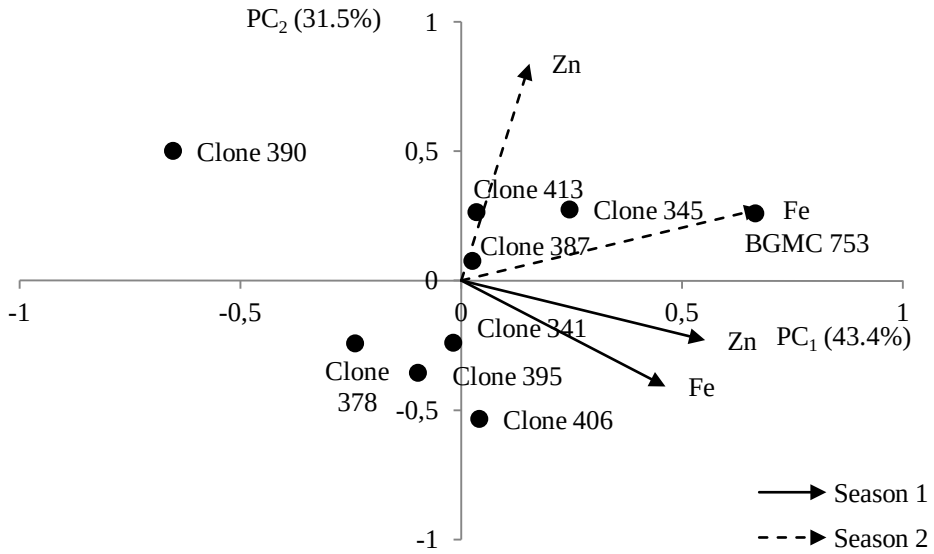

Figure 6. Graphic representation of the principal component analysis (PCA) of sweet cassava yellow-pulps clones according to the Zn and Fe contents in two seasons.

ARTIGO 2

Cassava Roots and Leaves as Alternative to Complement the Nutritional Quality of Diets

(Normas de publicação da revista *Plant Foods for Human Nutrition*)

CASSAVA ROOTS AND LEAVES AS ALTERNATIVE TO COMPLEMENT THE NUTRITIONAL QUALITY OF DIETS

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Abstract

Cassava (*Manihot esculenta* Crantz) is a staple crop of tropical and subtropical developing countries, feeding some 600 million people a day worldwide. However, the nutritional quality of the cassava roots is not sufficient to meet all dietary needs and biofortification can improve the nutritional quality of this crop. This work evaluated genotypic variation and nutrient content in cassava roots and leaves of selected accessions from the Cerrado´s Cassava Germplasm Bank, located on Embrapa Cerrados, Planaltina, DF, Brazil. The zinc (Zn) content in cassava leaves (n=464) varied from 16 to 105 mg kg^{-1} dry weight (DW), while in the roots (n=53), the values ranged from 5 to 16 mg kg^{-1} DW. These results show that cassava leaves are an alternative to improve the diet of undernourished people. Iron (Fe), manganese (Mn), and copper (Cu) contents in the roots also varied among the accessions, ranging from 3.2 to 28, 0.5 to 4.4, and 0.3 to 2.4 mg kg^{-1} DW, respectively. The hierarchical cluster

analysis (HCA) and principal component analysis (PCA) showed that cluster 13 is the one with the greater potential for enrichment with Fe and Mn, while cluster 10 is the best for providing Zn and Cu. Thus, there is a high potential to explore the BGMC in order to increase micronutrient contents for biofortification purposes using genetic strategies.

Key-words: micronutrients; biofortification; genetic variability; *Manihot esculenta*

1. Introduction

Cassava (*Manihot esculenta Crantz*) is an important staple food cultivated in tropical and subtropical areas, mainly in developing countries. It is estimate that 600 million people have their starch-rich storage roots as the main source of energy [1]. Cassava roots production is estimated as 215 million tones per year and it has the 10º position in the ranking of the biggest agricultural productions in the world [2]. The success of this crop has been attributed to its drought tolerance and its ability to grow in soils with low fertility, a characteristic of tropical soils [3].

Regardless of its importance, cassava is one of the poorest known staple foods in terms of nutritional quality, since its roots, which are the main part of consumption, have low protein and nutrients contents. However, consumption of cassava leaves can be an alternative to increase the nutrient intake, since they are richer in proteins, minerals, vitamins, and fibers [1,4]. To increase nutrients and vitamins in diets, the concept of supplementation with “multi-mixture flour" emerged, consisting in the introduction of a flour made with by-products of staple crops, such as leaves, peels, and seeds in human diets [5]. According to

these authors, this multi-mixture flour can be an important tool to reduce the malnutrition in people with limited access to food.

On the other hand, an alternative to increase the micronutrient content in staple foods is biofortification, which can be achieved through agronomic or genetic approaches [6-9]. Agronomic biofortification consists in agricultural practices that can lead to micronutrients improvement in food, through some techniques as fertilization and crop diversification [10], while genetic biofortification consists in plant breeding strategies, which increase the nutritional quality of crops [7].

Still, the first step to improve the density and balance of micronutrients through genetic biofortification is to exploit the amounts of micronutrients and the degree of genetic variability [11]. Therefore, we developed this work with selected accessions from the Cerrado´s Cassava Germplasm Bank (BGMC), located on Embrapa Cerrados, Planaltina, DF, Brazil, in order to evaluate genotypic variation and micronutrient contents in roots and leaves aiming to provide basic information for selection of cassava accessions with substantial capacity to acquire minerals and for crop biofortification.

2. Materials and Methods

2.1. Study area

Leaves and cassava roots samples were obtained from the Cerrado´s Cassava Germplasm Bank (BGMC) of Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cerrados), Planaltina (DF), Brazil. Crop management practices were applied following the recommendation for cultivating cassava in Central Brazil [12]. The cassava genotypes were cultivated in field plots of 1.20

m x 0.80 m (with 10 plants in each line), from which we have harvested 3 replicates.

Composite cassava leaves samples (200-300 g) were harvested at 5 months after planting (young stage) from 464 cassava accessions by hand, while cassava tubers samples (900-1000 g) were harvested at mature stage from 53 accessions by hand (Table 1). The samples were washed with distilled water, oven-dried at 65°C, and ground to <0.38 mm using a stainless steel mill before analysis.

2.2 Sample preparation, chemical and statistics analysis

The samples were prepared and analyzed following a rigid quality assurance/quality control (QA/QC) program to ensure accurate and reliable analytical data.

The leaves and cassava samples were microwave-digested according to the USEPA Method 3051A (USEPA, 1998) using a CEM® Mars-5 microwave system. An aliquot of a 0.5 g sample was combined with 10 mL of HNO_3 in Teflon® PTFE vessels and digested for 10 minutes in the microwave. All the reagents used (Sigma-Aldrich® or Merck®) were of high purity, and the HNO_3 was distilled prior to its use in the digestions. The Fe and Zn concentrations in the digested solutions were determined by flame atomic absorption spectrometer (F-AAS).

Standard reference materials from the Institute for Reference Materials and Measurements (BCR® - 482 Lichen) and from the National Institute of Standards & Technology (SRM 1573a - Tomato Leaves) were used to substantiate the accuracy of the analytical results obtained. Blank and certified reference samples were analyzed along with every batch of digestion.

The GENES software [13] was used for the statistical analysis. The differences were determined by ANOVA and Scott-Knott test to investigate statistically significant differences at $p<0.05$. The multivariate analysis was performed through hierarchical cluster and principal component analysis. The dataset was obtained from averages of Zn, Fe, Cu, and Mn contents and then standardized for mean 0 and variance 1 to minimize the individual effects of variance and the scale of each parameter. In hierarchical cluster analysis, the distance matrix was created using the Euclidean distance between the accessions and the linkage underweighted pair group method using arithmetic averages (UPGMA). Based on the results from cluster analysis, groups were created using a linkage distance, which explains better the similarity of the accessions. Principal components analysis (PCA) was performed to characterize the groups that presented the highest values for micronutrients. The biplot was made from the covariance matrix and the eigen values and eigen vectors were calculated as proposed by Lipkovich and Smith [14]. The number of components that explains the dataset was obtained considering eigen values higher than 1 [15].

3. Results and Discussion

The mean iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn) contents obtained from the repeated analysis ($n = 8$) of the standard reference materials are presented in Table 2. The micronutrients content recoveries in the certified samples exhibited reliable analytical data accuracy for Fe, Mn, Cu, and Zn analysis.

Cassava leaves are rich in Fe, Zn, and Mn and have been used as a complement in “multi-mixture” supplement [5]. The Zn contents in the cassava leaves of 464 accessions from the BGMC varied from 16 to 105 mg kg^{-1} dry weight (DW) (Figure 1) and presented up to 6.5-fold variation. A previous study

showed that Zn contents in cassava leaves ranged from 43 to 52 mg kg^{-1} DW [4]. These authors found values 3-fold higher than the lowest value found in our study. Montagnac et al. [1] reported Zn value in the leaves 42% higher (250 mg kg^{-1}) than the highest value found in the present study. The leaves provide high levels of minerals per calorie, even with high water content and low mineral density [11].

After analyzing Zn in the leaves, accessions with highest and lowest Zn contents were selected for harvesting the tuberous roots in order to assess their micronutrient contents. With that, a total of 53 accessions of tuberous roots were analyzed (dots inside the circles – Figure 1 and Table 1). Their Zn, Fe, Mn, and Cu contents are presented in the Figure 2 (2A, 2B, 2C, and 2D). Significant differences were found among the accessions for all of the studied micronutrients, which allowed a separation of groups according to the amount of nutrients in the roots.

The Zn contents in roots were divided in four groups, according the statistical differences, (<6.7; 6.7-8.9; 9.0-11.4; and >11.4 mg kg^{-1} of Zn) and varied significantly (a 3.2-fold, i.e., from 5 to 16 mg kg^{-1} DW) among the accessions (Figure 2A). Accessions BGMC 593 and BGMC 1451 showed the highest values. These results are in agreement with those reported by Ssemakula et al. [16], which analyzed Zn contents in roots from 27 cassava genotypes and found significant differences between the genotypes, with values ranging from 7.6 to 14 mg kg^{-1} DW. The Zn contents in 12 cassava clones from a Cassava Breeding Program in Campinas, Brazil, varied from 7 to 18 mg kg^{-1} DW [17], which were also similar to the observed results in the present study. Different genotypes exhibit different abilities to uptake and accumulate nutrients, since genetic factors along with physiological factors play an important role in the crop nutritional quality [9].

In the present study, the highest Zn content in the leaves (105 mg kg^{-1} DW) was 7-fold higher than the highest value observed in the tuberous root (16 mg kg^{-1} DW) and no correlation was detected between the Zn content in the leaves and roots. A 12-fold variation was found in cassava grown in Zambia (leaves: 133 mg kg^{-1} DW; tuberous roots: 11 mg kg^{-1} DW) [18]. Cassava leaves present high amount of fibers, proteins, lipid, minerals, and vitamins, while cassava tuberous roots contain more carbohydrate [1,4]. These results suggest that cassava leaves can be an alternative to improve the diet of undernourished people.

A bigger variability could be observed for Fe, which allowed us to separate the accessions into seven groups with values ranging from 3.2 to 28 mg kg^{-1} DW (up to 9-fold variation) (Figure 2B). The accession BGMC 585 presented the highest Fe content, followed by accession BGMC 1263. A study of 20 genotypes from CIAT collection showed Fe contents ranging from 7.7 to 12.6 mg kg^{-1} DW [11], while the study performed by Mezzete et al. [17] presented Fe contents varying from 10.5 to 19.2 mg kg^{-1} DW.

The Mn and Cu contents were lower than those found for Zn and Fe, varying from 0.5 to 4.4 mg kg^{-1} DW for Mn (Figure 2C) and from 0.3 to 2.4 mg kg^{-1} DW for Cu (Figure 2D). For manganese, these results are in agreement with those found by Cháves et al. [19], who reported Mn contents in cassava varying from 0.5 to 5 mg kg^{-1} DW. On the other hand, the Cu contents ranged from 0.8 to 40 mg kg^{-1} DW, showing up to 50-fold variation. In the present study, no significant correlations were observed for Zn vs. Fe and Fe vs. Cu (data not shown, since all p values were greater than 0.20) and weak relationship were observed for Zn vs. Cu ($p < 0.0001$; $r = 0.47$), Zn vs. Mn ($p = 0.002$; $r = 0.24$), Fe vs. Mn ($p = 0.006$; $r = 0.21$), and Cu vs. Mn ($p = 0.006$; $r = 0.21$).

Hierarchical cluster analysis (HCA) is an analytical tool that uses a set

of selected variables to group a set of data according to their similarity [20]. In the present study, the HCA was applied to a data set of four variables (Zn, Fe, Mn, and Cu contents) and 53 cassava accessions, and, at the linkage distance of 1.8, grouped the accessions into 13 clusters (Table 3), which revels a wide genetic diversity among the analyzed material. Cluster 1 showed the highest amount of similar accessions (n=23), corresponding to 43% of the studied samples, while 7 of the 13 clusters presented just one accession, showing a high heterogeneity.

Finally, accessions with highest amounts of micronutrients could be identified via PCA analysis. Cluster 13, composed by the accession BGMC 585, presented the greater values of Fe content and is a good candidate for Mn indicating that could be used in biofortification programs. Cluster 10, composed by BGMC 593, showed greater values for Zn and Cu (Figure 4), which agrees with values found for micronutrient contents (Figure 2). Clusters 7 (BGMC 893 and BGMC 1263), 8 (BGMC 550 and BGMC 1213), and 9 (BGMC 1234) are possible candidates when considering all the studied micronutrients, although they were not the ones that accumulated the highest amounts.

4. Conclusions

The results suggest that genetic variability strongly influences micronutrient contents in roots and leaves, since we observed a great variance in micronutrient contents among the studied accessions. The Zn contents in the cassava leaves were bigger than the contents in the roots, showing that leaves are an interesting alternative to complement Zn supplementation in diets.

The similarity among the accessions, based on roots micronutrient contents, allowed the separation of 13 clusters. Among these clusters, clusters 13

and 10 were highlighted, since they presented the best results for Fe and Mn and Zn and Cu, respectively.

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Tables

Table 1 List of 53 cassava accessions from the Active Cassava Germplasm Bank of Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cerrados).

Nº	**Accessions**	**Nº**	**Accessions**	**Nº**	**Accessions**	**Nº**	**Accessions**
1	BGMC 8	**15**	BGMC 1049	**29**	BGMC 1263	**43**	BGMC 1448
2	BGMC 350	**16**	BGMC 1056	**30**	BGMC 1270	**44**	BGMC 1449
3	BGMC 550	**17**	BGMC 1100	**31**	BGMC 1277	**45**	BGMC 1450
4	BGMC 585	**18**	BGMC 1102	**32**	BGMC 1283	**46**	BGMC 1451
5	BGMC 593	**19**	BGMC 1120	**33**	BGMC 1288	**47**	BGMC 1452
6	BGMC 753	**20**	BGMC 1140	**34**	BGMC 1320	**48**	BGMC 1453
7	BGMC 758	**21**	BGMC 1179	**35**	BGMC 1341	**49**	BGMC 1454
8	BGMC 771	**22**	BGMC 1196	**36**	BGMC 1344	**50**	BGMC 1456
9	BGMC 834	**23**	BGMC 1203	**37**	BGMC 1377	**51**	BGMC 1460

Continue ...

10	BGMC 882	**24**	BGMC 1223	**38**	BGMC 1379	**52**	BGMC 1461
11	BGMC 890	**25**	BGMC 1227	**39**	BGMC 1386	**53**	BGMC 1464
12	BGMC 893	**26**	BGMC 1232	**40**	BGMC 1434		
13	BGMC 895	**27**	BGMC 1234	**41**	BGMC 1437		
14	BGMC 983	**28**	BGMC 1262	**42**	BGMC 1447		

Table 2 Certified value, determined concentration, and Zn recovery on certified materials.

		Certified value (mg kg^{-1})	Determined concentration (mg kg^{-1})	Mean recovery (%)
BCR® - 482	Fe	---	---	---
	Zn	100.6±2.2	94.6±1.3	94
SRM 1573a	Fe	368±7	282±3	77
	Zn	30.9±0.7	27±1	87
	Mn	246±8	224±24	91
	Cu	4.7±0.14	4.1±0.12	87

*mean of 8 measurements of standard reference material samples. Mean Recovery (%) = (Mean $_{\text{Determined concentration}}$/Mean $_{\text{Certified value}}$) x 100%; ---: value not certified.

Table 3 - Clustering pattern of accessions based on Dendrogram (Linkage Distance <1.8).

Cluster	**Nº of Accessions**	**Accessions**
1	23	BGMC 8; BGMC 753; BGMC 771; BGMC 983; BGMC 1100; BGMC 1179; BGMC 1450; BGMC 1460; BGMC 1341; BGMC 350; BGMC BGMC 1461; BGMC 1447; BGMC 882; BGMC 1448; BGMC 1453; BGMC 1437; BGMC 1454; BGMC 1452; BGMC 1056; BGMC 1320; BGMC 1120; BGMC 1140; BGMC 1464
2	6	BGMC 834; BGMC 1227; BGMC 1454; BGMC 1288; BGMC 1449; BGMC 1456
3	9	BGMC 890; BGMC 1102; BGMC 1270; BGMC 1386; BGMC 1049; BGMC 1196; BGMC 1232; BGMC 1344; BGMC 1379
4	4	BGMC 895; BGMC 1277; BGMC 1283; BGMC 1377
5	1	BGMC 1262
6	1	BGMC 1203
7	2	BGMC 893; BGMC 1263
8	2	BGMC 550; BGMC 1213
9	1	BGMC 1234
10	1	BGMC 593
11	1	BGMC 758
12	1	BGMC 1451
13	1	BGMC 585

Figures

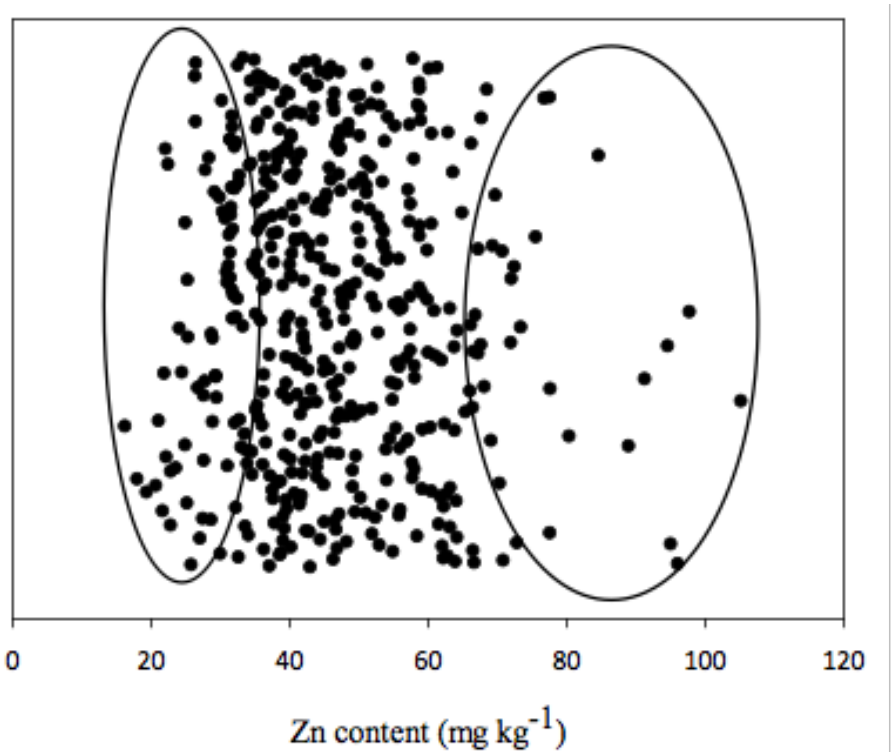

Figure 1 – Zn content in cassava leaves (n=464). Dots inside the circles were selected for further Zn analysis in roots.

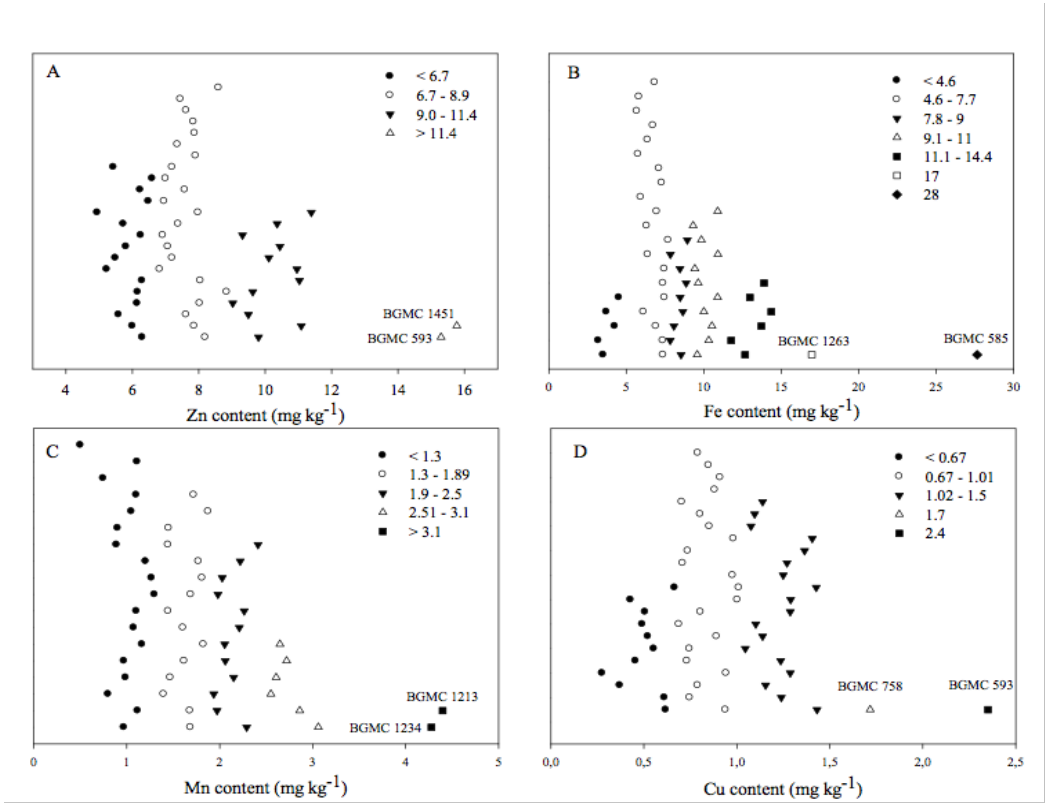

Figure 2 – Micronutrient contents in the cassava tuberous roots of 53 accessions A) Zinc; B) Iron; C) Manganese; D) Copper.

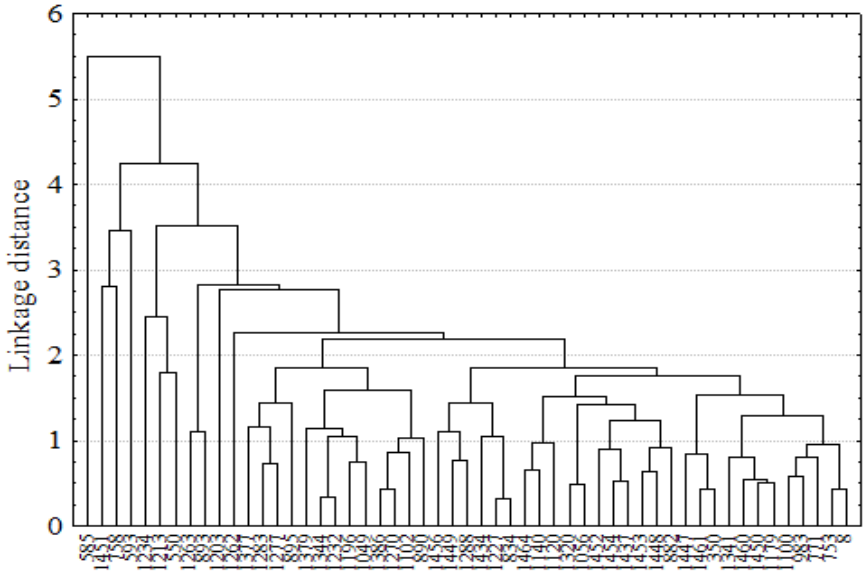

Figure 3 – Dendrogram depicting genetic relationships among 53 cassava accessions.

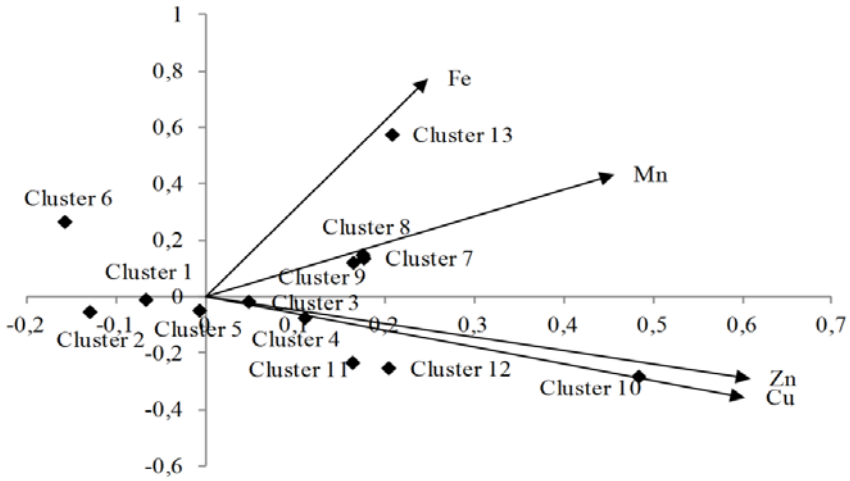

Figure 4 - Graphic representation of the principal component analysis (PCA) of 13 clusters of cassava accessions according to Zn, Fe, Mn, and Cu contents.

ARTIGO 3

Genotypic Variation and Agronomic Intervention as Zinc Biofortification Approaches in Cassava Clones Enriched with Carotenoids

(Normas de publicação da revista *Field Crops Research*)

GENOTYPIC VARIATION AND AGRONOMIC INTERVENTION AS ZINC BIOFORTIFICATION APPROACHES IN CASSAVA CLONES ENRICHED WITH CAROTENOIDS

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Abstract

Zinc (Zn) deficiency is a major human health concern worldwide and biofortification (genetic and agronomic) is a complementary solution that can increase the contents of nutrients, e.g. Zn, or other compounds in staple crops. Cassava has been used for Zn biofortification purposes, since it is a staple crop in most of countries that are affected by malnutrition and Zn deficiency. Thus, studies on biofortification of this crop can lead to an improvement of its nutritional quality. This study investigated Zn contents in cassava clones enriched with β-carotene or lycopene cultivated under different areas and soil managements in order to evaluate the influence of genotypic variation and agronomic intervention on Zn status in the plant. The Zn content in cassava tubers ranged from 3.6 to 24 mg kg^{-1}. The clones 26 and 273 (β-carotene enriched) presented the highest Zn

content in 7 of the 16 studied areas and clone 341 (lycopene enriched) presented highest amounts in 3 of the 7 studied areas. Highest values were found in cassava accessions cultivated in an area receiving application of animal manure, which showed high soil Zn content (76 mg kg^{-1}). These results show that combining genetic and agronomic managements is a good strategy to biofortification purposes.

Key-words: Agronomic management; genetic variability; *Manihot esculenta*; micronutrients.

1. Introduction

Zinc (Zn) is a micronutrient that is component of proteins and enzymes and operates on different biochemical processes (Nriagu, 2007). Synthesis of nucleic acids and cell growth and differentiation are processes that also have Zn involvement (Mayer et al., 2008). Because of its essentiality, Zn deficiency is a major human health concern worldwide (Joint FAO/WHO, 2001; White and Broadley, 2009). In fact, one of every three people in the world presents its deficiency, which affects the immunological system, growth, and development (Cakmak et al., 2010).

Increasing nutrients, e.g.. Zn, or other compounds contents in staple crops is an alternative to alleviate micronutrient deficiency, a complementary solution called biofortification (White and Broadley, 2005; Miller and Welch, 2013). The staple foods are the main target of biofortification programs, in view of the fact of their predominance in the diet of poor people (FAO, IFAD and WFP, 2014).

Among the strategies used for biofortification purposes, genetic selection and agronomic intervention are the main ones (Cakmak, 2008; White and Broadley, 2005; Miller and Welch, 2013). Plants present considerable genetic variation in essential nutrients contents (Hirschi, 2009) and this ample natural genetic variability can be used to increase these

nutrient contents, including Zn, in the edible parts (White and Broadley, 2005). However, it is necessary to keep sufficient amounts of available nutrients in the soil in order to improve the nutrient status in the plant, especially in the edible parts. Zinc availability in soil influences the success of Zn biofortification programs (Cakmak, 2008). In view of that, agronomic biofortification is an important tool to improve the Zn status in the plants, using agricultural practices, such as fertilization and crop diversification (Zhao and Shewry, 2011).

One of the most important crops that have been used for Zn biofortification purposes is cassava. This crop is a staple crop in most of the tropical countries, especially the developing ones, which are the most affected by malnutrition and Zn deficiency (Montagnac et al., 2009). As the main source of nutrient supply, cassava can also be used as source for carotenoids enrichment, e.g. β-carotene (roots with yellow-colored pulps) and lycopene (roots with pinkish-colored pulps). They are important in the human diet, since β-carotene, as a vitamin A precursor, can help on difficulty of learning, growth disorders, and in the fight against night blindness (Underwood and Smitasii, 1999). Lycopene, on the other hand, is an antioxidant that helps in prostate cancer prevention and fight against early aging (Shami and Moreira, 2004).

Focusing on cassava biofortification with zinc, this study investigated Zn contents in cassava clones enriched with β-carotene or lycopene cultivated in different areas and under different soil managements in order to evaluate the influence of genotypic variation and agronomic intervention on Zn status in the plant. With that, we plan to understand how a combined genetic and agronomic approach could help us develop a better strategy for biofortification of cassava with Zn.

2. Material and Methods

2.1 Study area

2.1.1 Experiment 1: Cassava enriched with β-carotene

Soil and roots of sweet cassava clones of yellow-colored pulps (enriched with β-carotene) samples were obtained from sixteen areas in the central Brazilian Savanna, Brazilian Cerrado, with different soil management (Table 1).

For each studied area, cassava clones were cultivated in field plots of 1.20 m x 0.80 m with 5 plants in each line (5 lines per plot). The experiment with yellow-colored pulps tested 7 sweet cassava clones (2008 generation): 26, 215, 272, 273, 446, 450, and 497. The cassava cultivar IAC 576-70, popularly known as Japonesinha, was used as control. This cultivar has natural cream-colored pulps. It is indicated for commercial cultivation in the Brazilian Federal District region (Fialho et al., 2009) and is identified as BGMC 753 in the Cassava Germplasm Bank (Banco Regional de Germoplasma de Mandioca do Cerrado - BGMC).

2.1.2 Experiment 2: Cassava enriched with lycopene

Soil and roots of sweet cassava clones of pinkish-colored pulps (enriched with lycopene) samples were obtained from seven areas in the Brazilian Cerrado. No fertilization was done in Area 1, while the other areas (from 2 to 7) were previously fertilized with 300 kg ha^{-1} of the commercial fertilizer formula NPK 04-30-16 + Zn.

The experimental design followed the same one used for experiment 1. The experiment with pinkish-colored pulps tested 8 sweet cassava clones (2008 generation): 341, 345, 378, 387, 390, 395, 406, and 413. The cultivar IAC 576-70 (BGMC 753) was also used as control.

2.1.3 Soil and cassava samples collection

Composite soil samples (500 g) were made up of fifteen separate subsamples, which were collected from the 0-20 cm soil layer and then storage in plastic bags until analysis. Composite samples of cassava tubers (900-1000 g) were harvested by hand at the mature stage from each

experimental field plot, washed with distilled water, peeled, and stored in a cold chamber (4ºC) for subsequent analysis.

2.2 Sample preparation, chemical and statistics analysis

The samples were prepared and analyzed following a rigid quality assurance/quality control (QA/QC) program to ensure accurate and reliable analytical data. Soil samples were air-dried, ground, and sieved to <2 mm, while cassava samples were rinsed with distilled water, oven-dried at 65°C, and ground to <0.38 mm using a stainless steel mill before analysis.

The chemical properties of the soil samples were determined following the methodology proposed by Silva (2009). Briefly, soil pH was determined in a 1:2.5 soil:water suspension. Soil organic matter content was determined using the potassium dichromate ($K_2Cr_2O_7$) method, which consists in oxidizing an aliquot of soil (0.5 g) with a solution of $K_2Cr_2O_7$ + H_2SO_4 at 160ºC. The excess of dichromate is titrated with 0.25 mol L^{-1} $FeSO_4$. Calcium and Mg were extracted mixing a volumetric soil aliquot (10 cm^3) with 100 mL of KCl (1 mol L^{-1}) at room temperature overnight. Available soil P, K, Fe, Zn, Mn, and Cu were extracted using 100 mL of the Mehlich^{-1} solution (0.05 mol L^{-1} HCl + 0.0125 mol L^{-1} H_2SO_4) reacted with 10 cm^3 of soil sample (Silva, 2009). Calcium, Mg, Fe, Mn, Zn, and Cu concentrations in the extracted solutions were determined by flame atomic absorption spectrometer (F-AAS, Perkin-Elmer® AAnalyst™800 – Waltham, MA, USA), using either certified or Sigma Aldrich® single elements AAS standards for QA/QC. Available K was measured by flame photometry and available P was determined by colorimetry.

The soil and cassava samples were microwave-digested according to the USEPA Method 3051A (USEPA, 1998) using a CEM® Mars-5 microwave system. An aliquot of a 0.5-g sample was combined with 10 mL of HNO_3 in Teflon® PTFE vessels and digested for 10 minutes in the microwave. All reagents used (Sigma-Aldrich® or Merck®) were of high purity, and the HNO_3 was distilled prior to its use in digestions. The total Zn

contents in acid-digested solutions were determined using flame atomic absorption spectrometer (F-AAS). Standard reference materials from the National Institute of Standards & Technology (NIST - SRM 1573a Tomato Leaves and SRM 2710 Montana Soil) were used to substantiate the accuracy of the analytical results obtained. Blank and certified reference samples were analyzed along with every batch of digestion.

The GENES software (Cruz, 2001) was used for the statistical analysis. The differences were determined by ANOVA and Scott-Knott´s test to investigate statistically significant differences at $p<0.05$.

3. Results

The Zn certified value for the standard material NIST 2710 Montana Soil is 4,180 mg kg^{-1}, while the certified value for the NIST 1573a Tomato Leaves is 30.9 mg kg^{-1}. The mean Zn contents obtained from the repeated analysis (n=12) of these certified samples were 3,936 mg kg^{-1} for the NIST2710 and 29.3 mg kg^{-1} for the NIST SRM1573a, which means a mean recovery of 94 and 95%, respectively.

3.1 Soils

The soil chemical properties, including the available and total Zn contents, of the sixteen studied areas in the experiment with cassava clones enriched with β-carotene are presented in the Table 2. The soil characteristics showed variation among the sixteen areas. The total Zn values varied from 6.8 to 78 mg kg^{-1} and presented up to 11.5-fold variation. The highest Zn value was found in Area 5, which also presented the highest amount of organic matter (OM) (57 g kg^{-1}) and available Fe (74 mg dm^{-3}), high amounts of P (202 mg dm^{-3}) and an adequate pH (5.6). The available Zn content ranged from 0.9 to 36 mg dm^3 (up to 36-fold variation) with the highest value also found in Area 5. The highest total Zn content was 2-fold higher than the highest available value, while the lowest value of total Zn was 7-fold higher than the available Zn. Besides that, the correlation

between the total and available Zn contents was significant and positive (p<0.005; r=0.92).

In the experiment with cassava enriched with lycopene, there was also variability of the soil chemical properties of the seven studied areas (Table 3). The total Zn contents in the soil ranged from 9.4 to 23 mg kg^{-1}. The lowest value was found in the Area 1, which also presented the lowest value of P (6.3 mg dm^{-3}), the highest Fe content (63 mg dm^{-3}), and a high pH (6.4). Area 5 showed the highest Zn content, an adequate pH (5.9), and a high P content (171 mg dm^{-3}). The available Zn contents varied from 0.8 to 9.0 mg dm^{-3} and followed the same trend of total Zn, with the lowest value in Area 1 and the highest in Area 5. As the experiment 1, the total Zn contents were higher than the available values in the experiment 2, with an up to 12-fold variation. There was also a significant correlation between the total and available Zn contents in the seven studied areas of this experiment (p<0.005; r=0.90).

3.2 Cassava

The Zn contents in the tuberous roots of sweet cassava clones enriched with β-carotene (experiment 1) presented a variation of up to 9-fold, varying from 3.6 to 24 mg kg^{-1} dry weight (DW) (average: 9.2 mg kg^{-1} DW; median: 8.3 mg kg^{-1} DW). About 50% of the studied clones in the 16 areas presented Zn contents between 6.6 to 10.2 mg kg^{-1} DW (Table 4). The highest value was found in clone 273 cultivated in Area 5, while the lowest was found in clone 497 cultivated in Area 16 (Table 5).

There were significant differences among Zn contents in the clones for each area as well as in the areas for each clone. Clones 26 and 273 presented the highest Zn contents in seven of the sixteen studied areas and an average value of 10 mg kg^{-1} DW, approximately, could be observed for both clones. Clones 446, 450, and 497 presented the lowest contents of Zn in Areas 7, 9, and 10, respectively, and averages lower than 8.5 mg kg^{-1} DW. Regarding the areas, clones cultivated in Area 5 presented higher Zn

contents than the other areas and the highest average (17.8 mg kg^{-1} DW), being 3.5-fold higher than the lowest average, which was found in Area 16 (5.0 mg kg^{-1} DW). We also observed a great variability of Zn contents in the clones among the areas with a variation of up to 4.8-fold, with the greatest variation being observed for clones 215 and 450.

In experiment 2, the Zn content in the tuberous roots of the cassava clones enriched with lycopene varied from 3.5 to 16 mg kg^{-1} DW (up to 5-fold variation), with an average of 9.0 mg kg^{-1} DW and a median of 8.7 mg kg^{-1} DW (Table 6). These contents were similar to those observed in experiment 1 with clones enriched with β-carotene. Clone 395 presented the highest content when cultivated in Area 6 and the lowest when cultivated in Area 1 (Table 7). A total of 75% of the studied samples presented values lower than 11.0 mg kg^{-1} DW.

The clones showed great variability on Zn contents, since most of them showed the highest values for Zn content in some areas, yet the lowest in others (Table 7). The clone that presented the lowest values in 4 areas as well as the lowest average (7.5 mg kg-1 DW) was clone 387, while the highest average (10.0 mg kg-1 DW) was found for clone 341, which presented the highest amounts of Zn in 3 areas. Concerning the areas, the lowest average was found in Area 3 (6.0 mg kg-1 DW), being 2-fold lower than the highest average found in Area 7 (12.4 mg kg^{-1} DW). The Zn contents in the clones among the studied areas presented a variation of up to 4.5-fold, which was found for clone 395.

4. Discussion

The Zn content recovery in the certified samples exhibited a mean recovery of almost 100%, which means reliable analytical data accuracy for Zn analysis.

Tropical soils presents low natural fertility, with low pH values, high aluminum (Al) levels, and high capacity of phosphorus (P) fixation. Nutrient deficiencies are widespread in Brazilian soils, especially in Cerrado's soils

(Lopes et al., 2012). Zinc content is one of the major yield constrains in these soils, affecting 90-95% of the native Cerrado soils. Worldwide, it has been reported that 50% of grains in agricultural areas are Zn deficient, resulting in low productivity and low Zn status in plant tissues (Alloway, 2008; Cakmak, 2008). Besides the native Zn deficiency in Brazilian soils, its availability is very dependent on a variety of soil properties, which explains the lower levels found of available Zn compared to total Zn contents in our study. Therefore, an adequate management is required to ensure high yields and a sufficient Zn status in the soil for plant growth.

The recommendations described for the Cerrado Region classify the available Zn values (Mehlich-I extractable) into three levels, which are: low (Zn content below 1.0 mg dm^{-3}), medium (Zn content from 1.0 to 1.6 mg dm^{-3}), and high (Zn content above 1.6 mg dm^{-3}) (Sousa and Lobato, 2004). According to this classification, most of the studied areas presented high Zn values, except for Area 2 from experiment 1 and Area 1 from experiment 2, which showed values below 1.0 mg dm^{-3}. These areas had a record of soil management with no specific fertilization for cassava, although corn had been previously cultivated with the use of chemical fertilizers, i.e., cassava plants were grown with the remaining nutrients in the soil. These areas presented high pH (>6.0), which plays an important role in Zn solubility, since higher pH can increase the adsorption of Zn to the soil constituents, thus decreasing its availability to plants (Cakmak, 2008).

Organic manure enhances organic matter content, while improving the nutrient status in the soil, including its micronutrient levels (John et al., 2005). Additionally, organic compounds can serve as chelate for Zn, forming soluble organic Zn complexes and increasing its solubility and availability to plants (Alloway, 2008). Animal manure was used in Area 5 of experiment 1 and it may have contributed to the higher amounts of available Zn

Cassava is an important source of energy and it is poor in nutritional quality (Maziya-Dixon, 2000). However, it is known that root crops present

higher Fe and Zn contents than cereals grains (White and Broadley, 2009). Therefore, studies focusing on nutritional enrichment of cassava roots, combining vitamins and micronutrients, can be an alternative to cassava biofortification. The composition of cassava can be influenced by several factors, as environmental conditions, geographic location, plant age, and variety, and the combination of these events leads to differences in the Zn status in the plant (Montagnac et al., 2009).

Genetic and physiological factors play an important role in nutritional quality of crops, since the ability to uptake and accumulate nutrients vary according to the plant genotype (White and Broadley, 2009). Significant differences between cassava genotypes for Zn contents are commonly found (Chávez et al., 2000; Chávez et al., 2005; Ssemakula et al., 2008), which agree with the results found for both experiments in the present study.

At least 75% of the studied samples (quartile 25%: 6.6 and 6.9 mg kg^{-1}, experiments 1 and 2, respectively) presented Zn contents higher than the average value found by Chávez et al. (2000) (6.4 mg kg^{-1} DW). Besides that, the variation in the root Zn content among cassava clones found in the present study (9- and 5-fold variation in the roots from experiment 1 and 2, respectively) was higher than the variation among cassava accessions studied by Maziya-Dixon et al. (2000) (4-fold variation). This genetic variation shows a great potential to breed for improved Zn contents in cassava roots, since natural variability is the first requirement for developing genotypes with high Zn contents in the edible parts in genetic biofortification approaches (Mayer et al., 2008).

Nevertheless, breeding strategies require long-term studies to have applicability and genetic variability alone does not guarantee the success of biofortification programs, since nutrient status in the plant is dependent on other conditions, as environmental factors, especially soil-available minerals and nutrients (Ortiz-Monasterio et al., 2007; Cakmak, 2008). The highest Zn contents in cassava clones roots were found in the area with highest Zn

content (total and available) in the soil (Area 5). These results suggests that biofortification through agronomic management can lead to improved zinc contents in cassava.

The combination of genetic and agronomic biofortification can increase the yield of crops and improve the nutritional quality in edible portions of staple crops (White and Broadley, 2009). In the present study, the cassava clones showed variable responses to the environmental conditions, since the Zn contents in the clones among the areas were different. Ssemakula et al. (2008) also observed variability on Zn contents in cassava roots among the locations and a previous study conducted in Embrapa Cerrados experimental areas showed Zn content in cassava clones roots varying from 1.8 to 7.6 mg kg^{-1} DW (Corguinha et al., unpublished data), values up to 3-fold lower than the highest found in the current study. These results show that the environment influenced Zn status in the clones, showing that the use of agronomic strategies is important to assure adequate nutritional balance in the plants.

5. Conclusions

The results showed that Zn content in cassava clones roots is influenced by genetic variation as well as by agronomic intervention. The genetic variation is a good tool to be used for biofortification purposes, being important to increase nutrient contents in edible parts of staple crops. However, breeding approaches do not produce the best results alone, and it is necessary to consider other practices, as agronomic management.

By optimizing the genetic potential of cassava clones with agronomic management for biofortification purposes, it is possible to improve the nutritional quality of cassava roots and provide better products for consumption by people who have this crop as the main source of energy, reducing the deficiency of Zn.

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Tables

Table 1 – Soil managements in the studied areas

Areas	**Management**
Area 1	green manure (sunnhemp + Brachiaria + millet)
Area 2	no fertilization; previous crop: corn
Area 3	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 4	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 5	5 t ha^{-1} animal manure + 3 t ha^{-1} liming
Area 6	120 L manure
Area 7	16 kg ha^{-1} NPK 2:8:4 + 4 kg ha^{-1} humus + 30 kg Borax
Area 8	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 9	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 10	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 11	400 kg ha^{-1} thermophosphate fertilizer with 175 g kg^{-1} P_2O_5 + 280 g kg^{-1} CaO + 145 g kg^{-1} MgO + 5.5 g kg^{-1} Zn
Area 12	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 13	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 14	300 kg ha^{-1} NPK 4:30:16 + Zn
Area 15	400 kg ha^{-1} thermophosphate fertilizer with 175 g kg^{-1} P_2O_5 + 280 g kg^{-1} CaO + 145 g kg^{-1} MgO + 5.5 g kg^{-1} Zn
Area 16	300 kg ha^{-1} NPK 4:30:16 + Zn

Table 2 – Soil chemical properties from cassava enriched with β-carotene areas.

Area	pH	P	K	Ca	Mg	OM	Cu	Mn	Fe	Zn (available)	Zn (total)
		-----mg dm^{-3}-----		------cmol dm^{-3}-----		g kg^{-1}	----------mg dm^{-3}----------				mg kg^{-1}
Area 1	6.5±0.03	32±2	153±5	3.7±0.2	2.0±0.1	35±2	5.1±0.7	14±0.5	34±1.3	10±0.4	19±1.5
Area 2	6.2±0.07	7.3±0.3	183±9	7.0±0.4	1.5±0.1	32±3	2.1±0.5	45±6	67±1.7	1.0±0.2	11±1.4
Area 3	6.9±0.1	28±2	132±2	7.7±0.6	1.3±0.03	25±2	1.4±0.1	15±0.5	42±2.3	3.7±0.5	15±1.0
Area 4	6.7±0.1	11±0.3	157±1	3.6±0.1	1.1±0.01	30±0.4	1.8±0.07	21±0.9	25±0.5	2.3±0.2	11±1.0
Area 5	5.6±0.2	202±6	154±6	5.0±0.5	1.4±0.2	57±3	3.6±0.2	9.8±1.1	74±1.8	36±0.8	78±5.4
Area 6	5.7±0.03	6.2±0.6	41±2	2.5±0.1	0.8±0.03	45±1	0.8±0.07	9.4±0.4	43±2.6	3.5±0.3	12±2.1
Area 7	6.0±0.2	67±4	187±3	4.7±0.2	1.5±0.1	29±0.1	4.4±0.2	25±2.8	33±1.0	12±0.4	41±1.2
Area 8	5.4±0.1	55±12	119±73	3.6±0.7	1.3±0.2	36±2	11.3±5.6	16±5	30±3.4	26±13	32±3.6
Area 9	6.0±0.03	156±12	93±22	9.8±0.7	1.7±0.1	43±3	0.8±0.06	34±7	33±7.0	3.1±0.7	11±1.7
Area 10	6.2±0.03	145±11	71±20	6.6±1.3	1.0±0.1	31±1	2.0±0.2	15±1.4	20±2.3	6.0±1.0	17±1.6
Area 11	6.0±0.1	148±12	3.5±0.3	5.2±0.1	2.4±0.1	39±1	0.8±0.04	54±10	19±1.7	1.8±0.1	12±0.6
Area 12	5.6±0.1	156±14	105±6	5.3±0.1	1.5±0.01	33±2	4.8±0.04	25±0.8	29±0.2	8.4±0.5	22±1.2
Area 13	5.7±0.1	130±14	58±7	4.7±0.2	0.7±0.03	41±1	2.1±0.2	16±2	22±1.6	5.5±0.3	18±0.7
Area 14	5.3±0.1	49±6	14±5	1.7±0.1	0.5±0.03	48±6	1.7±0.1	8.1±1	44±2.2	4.4±0.6	11±0.7
Area 15	5.5±0.3	31±3	5.1±0.6	2.8±0.5	0.9±0.1	35±1	1.4±0.4	13±2	38±1.3	3.4±0.7	9.2±1.3
Area 16	5.3±0.03	65±14	11±5	3.1±0.2	0.6±0.1	42±3	0.5±0.08	5.8±0.5	27±3.7	3.5±2.1	6.8±1.5

Table 3 – Soil chemical properties from cassava enriched with lycopene areas.

Area	pH	P	K	Ca	Mg	OM	Cu	Mn	Fe	Zn (available)	Zn (total)
		------mg dm^{-3}-----		------cmol dm^{-3}-----		g kg^{-1}	----------mg dm^{-3}----------				mg kg^{-1}
Area 1	6.4±0.03	6.3±0.7	228±10	6.0±0.5	1.5±0.1	32±0.5	1.2±0.1	38±2.3	63±2.0	0.8±0.1	9.4±0.6
Area 2	7.1±0.03	28±2.0	121±6	6.6±0.5	1.3±0.1	23±1.1	1.8±0.3	17±1.2	49±3.0	4.3±0.5	18±0.3
Area 3	5.9±0.03	228±4	23±11	10±1.1	1.6±0.2	52±4.4	0.8±0.1	64±5.6	13±1.4	3.1±0.4	13±0.5
Area 4	6.0±0.06	137±11	31±1	4.0±0.2	1.1±0.1	30±1.4	1.4±0.1	8.7±0.1	25±3.4	3.7±0.4	13±0.6
Area 5	5.9±0.07	171±21	101±6	5.5±0.1	1.8±0.1	33±3.2	4.7±0.3	28±1.7	29±2.1	9.0±0.3	23±1.4
Area 6	5.5±0.03	205±14	41±5	4.6±0.1	0.8±0.1	56±7.0	2.0±0.1	19±1.1	22±1.5	5.0±0.1	18±0.3
Area 7	5.2±0.03	43±5	14±3	1.8±0.2	0.6±0.2	35±1.0	1.7±0.1	8.5±0.3	39±1.4	5.9±1.1	14±0.6

Table 4 – Descriptive statistical analysis of Zn contents in tuberous roots of sweet cassava clones enriched with β-carotene (mg kg^{-1} DW).

Statistical Parameter	Zn
Average	9.2
Median	8.3
Standard deviation	3.7
Minimum	3.6
Maximum	24.0
Quartile 25	6.6
Quartile 75	10.2

Table 5 – Zn contents in tuberous roots of sweet cassava clones enriched with β-carotene

Area	Clones								
	Clone 26	**Clone 215**	**Clone 272**	**Clone 273**	**Clone 446**	**Clone 450**	**Clone 497**	**BGMC753**	**Average**
Area 1	11.8±0.4 *Ca*	6.5±0.8 *Ec*	8.3±0.5 *Db*	9.0±0.2 *Db*	6.2±0.8 *Dc*	6.0±0.2 *Ec*	6.8±0.6 *Dc*	9.7±0.3 *Db*	8.1±0.7
Area 2	6.8±0.3 *Ec*	6.1±0.2 *Fc*	10.1±0.1 *Ca*	8.5±1.2 *Db*	8.8±0.2 *Cb*	6.0±0.3 *Ec*	8.3±0.3 *Cb*	8.3±0.1 *Eb*	7.9±0.5
Area 3	13.2±1.0 *Bb*	8.7±0.1 *Dc*	7.2±0.1 *Ec*	6.9±0.8 *Ed*	9.6±1.1 *Cc*	8.5±0.1 *Dc*	10.0±0.01 *Bc*	18.6±0.9 *Aa*	10.3±1.4
Area 4	6.0±0.8 *Fb*	5.4±0.2 *Fb*	6.6±0.1 *Eb*	9.5±0.3 *Da*	5.9±0.2 *Db*	6.0±0.5 *Eb*	4.4±0.2 *Fb*	6.0±0.6 *Fb*	6.2±0.5
Area 5	18.2±0.3 *Ac*	21.0±0.3 *Ab*	13.5±0.7 *Ad*	24.0±1.0 *Aa*	17.0±0.3 *Ac*	17.7±0.4 *Ac*	13.3±0.1 *Ad*	17.6±0.6 *Bc*	17.8±1.2
Area 6	10.1±0.2 *Da*	7.8±0.2 *Eb*	9.0±0.2 *Db*	7.2±0.6 *Ec*	5.3±0.2 *Dd*	5.6±0.8 *Ed*	5.7±0.5 *Ed*	8.4±0.4 *Eb*	7.4±0.6
Area 7	7.0±0.4 *Eb*	9.0±0.03 *Da*	7.7±0.1 *Ea*	6.9±0.3 *Eb*	5.2±0.1 *Dc*	7.5±0.2 *Da*	8.3±0.4 *Ca*	8.5±0.3 *Ea*	7.6±0.5
Area 8	16.7±0.7 *Aa*	12.2±0.4 *Bb*	12.5±0.3 *Bb*	11.1±0.3 *Cc*	6.2±0.1 *De*	13.5±0.2 *Cb*	8.1±0.1 *Cd*	9.1±0.3 *Dd*	11.2±1.2
Area 9	8.1±0.2 *Ea*	4.3±0.1 *Fc*	4.6±0.2 *Fc*	6.5±0.3 *Eb*	6.9±0.3 *Db*	6.7±0.2 *Eb*	6.3±0.2 *Db*	7.8±0.8 *Ea*	6.4±0.5
Area 10	9.8±0.1 *Da*	7.4±0.4 *Eb*	7.5±0.7 *Eb*	11.3±1.2 *Ca*	6.1±0.6 *Dc*	6.4±0.7 *Ec*	6.6±0.8 *Dc*	5.6±0.6 *Fc*	7.6±0.7
Area 11	9.6±0.02 *Dc*	10.1±0.4 *Dc*	14.1±0.4 *Ab*	9.5±0.6 *Dc*	17.1±0.7 *Aa*	9.2±0.1 *Dc*	10.0±0.03 *Bc*	8.8±0.1 *Dc*	11.0±1.0

Continue...

Area 12	9.0±0.1 *Dc*	10.5±0.2 *Cb*	9.9±0.5 *Cb*	11.9±0.6 *Ca*	7.9±1.0 *Cc*	7.7±0.3 *Dc*	7.8±0.3 *Cc*	7.8±1.0 *Ec*	9.1±0.6
Area 13	17.7±0.4 *Aa*	10.7±0.3 *Cb*	11.9±0.3 *Bb*	9.0±0.3 *Dc*	5.9±0.2 *Dd*	6.9±0.4 *Ed*	6.8±0.4 *Dd*	7.5±0.1 *Ed*	9.6±1.4
Area 14	11.7±2.0 *Cb*	9.2±0.2 *Dc*	12.1±0.2 *Bb*	15.6±0.1 *Ba*	8.5±0.2 *Cc*	15.3±0.2 *Ba*	13.3±0.03 *Ab*	12.3±0.4 *Cb*	12.3±0.9
Area 15	9.4±0.7 *Db*	9.2±0.4 *Db*	8.5±0.2 *Dc*	12.2±0.5 *Ca*	11.6±0.8 *Ba*	8.1±0.5 *Dc*	7.4±0.4 *Cc*	9.8±0.7 *Db*	9.5±0.6
Area 16	5.0±0.3 *Fa*	5.6±0.4 *Fa*	5.3±0.2 *Fa*	5.7±0.3 *Ea*	4.9±0.5 *Da*	4.0±0.2 *Fb*	3.5±0.3 *Fb*	5.7±0.2 *Fa*	5.0±0.3
Average	10.6±1.0	9.0±1.0	9.3±0.7	10.4±1.1	8.3±1.0	8.4±1.0	8.0±0.6	9.5±1.0	

Table 6 – Descriptive statistical analysis of Zn contents in tuberous roots of sweet cassava clones enriched with lycopene (mg kg^{-1} DW).

Statistical Parameter	Zn
Average	9.0
Median	8.7
Standard deviation	3.0
Minimum	3.5
Maximum	15.7
Quartile 25	6.9
Quartile 75	11.0

Table 7 – Zn contents in tuberous roots of sweet cassava clones enriched with lycopene

Area	**Clones**									
	Clone 341	**Clone 345**	**Clone 378**	**Clone 387**	**Clone 390**	**Clone 395**	**Clone 406**	**Clone 413**	**BGMC 753**	**Average**
Area 1	9.3±0.1 *Ca*	8.4±0.4 *Cb*	6.7±0.6 *Cc*	5.2±0.1 *Dd*	7.1±0.1 *Cc*	3.5±0.2 *Ee*	10.1±0.6 *Ca*	8.0±0.1 *Cc*	8.6±0.1 *Db*	7.4±0.7
Area 2	7.0±0.4 *Dc*	8.0±0.7 *Cc*	9.8±0.8 *Bb*	11.3±0.3 *Aa*	10.5±0.9 *Ab*	11.5±0.4 *Ba*	12.1±0.8 *Ba*	8.5±0.2 *Cc*	12.4±0.2 *Ba*	10.1±0.6
Area 3	7.4±0.3 *Ca*	6.2±0.3 *Db*	4.9±0.2 *Dc*	5.3±0.6 *Db*	8.7±0.2 *Ba*	4.0±0.2 *Ec*	5.8±0.2 *Eb*	5.4±0.1 *Db*	6.3±0.6 *Fb*	6.0±0.5
Area 4	9.3±0.2 *Cb*	6.8±0.04 *Dc*	7.1±0.4 *Cc*	5.0±0.2 *Dd*	10.7±0.2 *Aa*	5.6±0.6 *Dd*	5.9±0.2 *Ed*	5.1±0.3 *Dd*	7.6±0.5 *Ec*	7.0±0.7
Area 5	15.0±0.8 *Aa*	9.1±0.3 *Bb*	7.4±0.3 *Cc*	7.5±0.5 *Cc*	8.7±0.4 *Bb*	9.2±0.1 *Cb*	6.7±0.4 *Dc*	7.9±0.3 *Cc*	7.7±0.7 *Ec*	8.8±0.8
Area 6	10.0±0.3 *Cd*	13.9±0.7 *Ab*	9.6±0.2 *Bd*	8.8±0.2 *Bd*	11.6±0.5 *Ac*	15.7±1.7 *Aa*	7.3±0.2 *De*	11.8±0.5 *Bc*	9.8±0.1 *Cd*	11.0±0.9
Area 7	11.3±0.3 *Bb*	9.5±0.2 *Bc*	14.5±0.1 *Aa*	9.5±0.2 *Bc*	11.3±0.1 *Ab*	11.7±0.2 *Bb*	15.5±0.7 *Aa*	14.4±0.2 *Aa*	13.7±0.1 *Aa*	12.4±0.7
Average	10.0±0.7	9.0±0.6	8.6±0.8	7.5±0.6	9.8±0.4	8.3±1.0	9.1±0.9	8.7±0.8	9.4±0.7	