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Soluble amino acid profile, mineral nutrient and carbohydrate content of maize kernels harvested from plants submitted to ascorbic acid seed priming*

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ABSTRACT

Both the scientific community and society have shown interest in improving the content of amino acids, carbohydrates and mineral nutrients in maize because it represents an important staple food in many developing countries. Earlier studies demonstrated that the treatment of seeds using ascorbic acid (AsA-seed priming) enhanced soluble carbohydrates, proteins and soluble amino acids for other species. AsA seed priming in maize showed the potential for reducing abiotic stresses. The effects on grain quality have not been previously demonstrated. This study investigated the impacts of AsA seed priming on maize kernel quality of seeds produced by the plants generated from the primed seeds, based on the amino acid profile and carbohydrate and mineral nutrient contents. AsA seed priming improved the maize kernel quality with respect to the ascorbate content, boron allocation, total carbohydrate content and increased soluble amino acid levels, including serine, tyrosine, alanine, valine, glutamate, arginine, proline, aspartate, lysine and isoleucine, whereas soluble methionine was decreased. Therefore, AsA seed priming can represent a potential technique for improving maize grain quality.

Key words: vitamin C, grain quality, boron, sugar, amino acids.

INTRODUCTION

The importance of seed plants to humans is unquestionable and they have been studied for a wide range of aspects (Azevedo et al. 1997, Carvalho et al. 2011, Bevilaqua et al. 2015, Dresch et al. 2015, Sneideris et al. 2015, Soares et al. 2015), but in particular to their nutritional quality due to their consumption by animals and humans (Azevedo et al. 1997). Cereals and legume seeds are significant plant protein sources in the human diet (Azevedo et al. 1990). Maize is a staple food in developing countries worldwide, including North and South American countries (FAO 1995). For example, Brazil is third largest maize producer in the world (55.6 million tons per year). Most Brazilian maize production is oriented toward animal feed, especially to aviculture and swine (FAO 2011, ABIMILHO 2013). Brazilians consume an average of 18 kg of maize *per capita* per year, while Mexicans consume an average of 63 kg of maize per year (EMBRAPA 2005). However,

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maize protein quality is fairly poor because it contains very low amounts of the essential amino acids lysine (Lys) and threonine (Thr) (Azevedo et al. 1997).

Twenty amino acids are fundamental building blocks of proteins and play crucial roles in metabolism (Zeng et al. 2013, Vilhena et al. 2015). Humans can synthesize 11 amino acids, while the others, known as essential amino acids, must be acquired from the diet (Azevedo et al. 1997). Plants can produce all twenty amino acids, three of which, tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) are derived from the Shikimate pathway (Herrmann 1995), while most are derived from the Krebs tricarboxylic acid cycle in the mitochondria (Wang and Larkins 2001, Azevedo et al. 2006).

Lys and Thr originate from the aspartate metabolic pathway, which is also responsible for the synthesis of methionine (Met) and isoleucine (Ile) (Azevedo et al. 2003, 2004a, b). Humans and some animals are unable to synthesize a number of essential amino acids and must obtain them from the diet (Azevedo et al. 1997). Lys is the most limiting essential amino acid in cereals, reducing the nutritional value (Azevedo et al. 2006). More amino acids must be made available in maize to offset the low protein consumption in developing countries (Azevedo et al. 1997).

In addition to amino acids, plant foods contain almost all of the mineral nutrients that are essential for animal and human nutrition. Mineral nutrients are also essential to every form of life (Gupta and Gupta 2014). For example, humans require large amounts of calcium (1200 mg/day), potassium (1600-2000 mg/day), phosphorous (800-1200 mg/day), sodium (500-635 mg/day), magnesium (280-350 mg/day), iron (10-30 mg/day), zinc (12-16 mg/day) and manganese (2-5 mg/day), as well as low concentrations of *trace* elements (McDowell 1992). Based on the average mineral nutrient concentrations in maize (McDowell 1992) and Brazilian consumption statistics, maize accounts for approximately 1.2% of calcium, 11.6-17.5% of phosphorous, 9-11.2% of potassium, 5.6-17% of iron, 5-12.5% of manganese and 4.3-5.7% of zinc consumed daily. The lack of mineral elements negatively impacts bone and teeth hardness and affects metabolic parameters, such as enzyme systems and organic molecules (Gupta and Gupta 2014). Therefore, mineral deficiencies are less likely when a variety of food or feeds are available for both humans and animals (McDowell 1992). Increasing the amount of mineral nutrients in staple food crops could be an interesting approach to aid the reduction of mineral deficiencies in humans and animals.

In addition to representing an important food source, maize is also a significant source of biofuel in some countries. For example, the total maize production of the United States has been stimulated by the industrial use of alcohol for fuel (USDA 2011). According to Bothast and Schlicher (2005), fuel alcohol is an alternative energy source that is becoming increasingly popular in the United States because of the country's dependence on foreign petroleum (approximately 62% is imported) and necessity for renewable energy resources. Maize starch is a major carbohydrate storage product in United States and can be readily converted to glucose (Bothast and Schlicher 2005). Individual units of glucose are linked together in chains by alpha 1–4 and alpha 1–6 linkages (amylose and amylopectin, respectively), forming a complex insoluble crystalline starch structure (Tester et al. 2004) that is broken down prior to the fermentation process to produce ethanol (Bothast and Schlicher 2005).

Some studies have tested pre-sowing seed treatments (seed priming) using hormones (Carvalho et al. 2011, Sneideris et al. 2015) and vitamins such as ascorbic acid (AsA) to improve the stress tolerance (Farooq et al. 2013, Alcântara et al. 2015). AsA treatment increased the amount of soluble carbohydrates, proteins and soluble amino

acids in beans (Azooz et al. 2013), and increased the proline content in wheat (Farooq et al. 2013), however these analyses were performed at the vegetative stage. A recent elegant study verified that seed priming using zinc-amino acid quelates improved wheat grain quality when the content of Zn and Fe are concerned (Seddigh et al. 2016). In view of the potential of this technique, the aim of the present study was to test AsA seed priming on maize to verify possible nutritional characteristic variations on the seeds produced by the plants generated from the primed seeds.

MATERIALS AND METHODS

PLANT MATERIAL AND FIELD CONDITIONS

Maize seeds (*Zea mays* L.) from Dow AgroSciences (DA) (2B587PW variety) were primed with dry AsA at a ratio of 3.8 g of AsA per 25 seeds (Alcântara et al. 2015). Following the treatment, the seeds were sieved to remove excess acidic powder. Unprimed and AsA-primed seeds were germinated in the Anhumas District (Lat. 22°45' - 22°50' S; Long. 48°00' - 48°05' W; 460 m altitude), Brazil, in Yellow Red Latosol with the following characteristics: pH 4.6, 27 $mmol_c$ kg^{-1} Al, 18 mg kg^{-1} phosphorus, 2.6 $mmol_c$ kg^{-1} potassium, 5 $mmol_c$ kg^{-1} calcium, 3 $mmol_c$ kg^{-1} magnesium, 19% base saturation and 72% changeable aluminum. The soil was supplemented with nitrogen, phosphorus and potassium using 250 kg ha^{-1} of NPK 08:28:16 prior to sowing.

The experiment was conducted using a randomized block design with five repetitions over a total area of 690 m^2. Each block was composed of 3 rows (8 plants per row). The seeds were sown during the summer (rainy season) and harvested during the winter (dry season). All maize ears in each block were harvested and 35 cobs from each treatment were obtained via random sampling.

ASCORBATE CONTENT

The ascorbate content in maize kernels was determined as described in Arakawa et al. (1981) with modifications. Kernels powders (400 mg) were homogenized in 5% (w/v) TCA. The homogenate was centrifuged at 15,000 × *g* for 15 min at 4 °C. Following centrifugation, 50 µL of supernatant was collected and diluted in 70 µL of TCA (5%), 125 µL of pure ethanol and 125 µL of Na_2HPO_4 (0.2 M, pH 8.0). This mixture was incubated at 25 °C for 10 min. Subsequently, the following reagents (all dissolved in pure ethanol) were added to the same tube: 125 µL of 0.24% (w/v) N-ethylmaleimide, 125 µL of pure ethanol, 125 µL of 4% (v/v) H_3PO_4, 250 µL of 0.5% (w/v) bathophenanthroline and 150 µL of 0.03% (w/v) $FeCl_3$. The mixture was incubated at 30 °C for 90 min. The readings were taken at 534 nm. Three independent replicates were used.

AMINO ACID QUANTIFICATION

The soluble amino acids were extracted from the maize following the method of (Bieleski and Turner 1966 with some modifications as described by Gaziola et al. 1999) via three technical replicates and three repetitions. Kernel powder was sifted through 60-mesh sieves, and 200 mg of the flour was homogenized in 2 mL of MCW (chloroform:methanol:water, 12:5:3). The mixture was incubated overnight at 4 °C and centrifuged at 10,000 g for 20 minutes. The supernatant was removed and 0.5 mL of pure chloroform and 0.75 mL of Milli-Q water were added for each 2 mL of MCW. Another centrifugation step was performed and the aqueous phase was carefully removed. The tubes containing the aqueous phase were placed in a water bath and incubated at 38 °C for 1h. The lids were left open to allow the alcohol to evaporate. The remaining phase was lyophilized, and the amino acids were resuspended in 200 µL of Milli-Q water. The soluble amino acids analysis was then

performed using an Ultra Performance Liquid Chromatography (UPLC) Acquity system (Waters) with a BEH C18 column (2.1 x 100 mm, 17 μm) at 43 °C. Derivatization was performed using 70 μL of borate buffer, 10 μL of sample and 20 μL of AccQ-fluor reagent (Waters). The mixture was placed in a water bath at 55 °C for 10 minutes. The injection volume was 1 μL, and the wavelength used for amino acid detection was 260 nm. The gradient changed the proportion of AccQ-Tag Eluent A (A) (Waters), 10% Acetonitrile (B), Milli-Q Water (C) and 100% Acetonitrile (D). A was changed from 10 to 9.9% between 0 and 0.29 min, from 9.9 to 9% between 0.29 and 5.49 min, from 9 to 8% between 5.49 and 7.3 min, from 8 to 7.8% between 7.1 and 7.69 min, from 7.8 to 4% between 7.69 and 8.59 min, and from 4 to 10% between 8.59 and 10.2 min. B was changed from 0 to 80% between 0 and 5.49 min, from 80 to 15.6% between 7.1 and 7.3 min, and from 15.6 to 0% between 7.3 and 10.2 min. C was changed from 90 to 90.1% between 0 and 0.29 min, from 90.1 to 11% between 0.29 and 5.49 min, from 11 to 57.9% between 5.49 and 7.3 min, from 57.9 to 70.9% between 7.3 and 7.69 min, from 70.9 to 36.3% between 7.69 and 8.59 min, and from 36.3 to 90% between 8.59 and 10.2 min. D was changed from 0 to 18.5% between 0 and 7.1 min, from 18.5 to 21.3% between 7.1 and 7.69 min, from 21.3 to 59.7% between 7.69 and 8.59 min, and from 59.7 to 0% between 8.59 and 10.2 min.

CARBOHYDRATE QUANTIFICATION

Carbohydrate quantification was conducted using the method of Tappi (1991) with some modifications. Maize kernels were ground using a mill knife and passed through a 60-mesh sieve. The results were expressed relative to 1 g of dried material.

The mass equivalent to 1 g of dried material was placed into filter paper bags (gramature 70) to obtain the extracts. The extraction was performed using 700 mL of a toluol:ethanol solution (2:1) over a period of 8 h and in 700 mL of pure ethanol for another 8 h. The samples were then placed into Erlenmeyer flasks with 100 mL of Milli-Q water (22 °C) and incubated with constant agitation (160 rpm) over a period of 48h. The starch and water was filtered using porous glass, and the filtrate residue (pure starch) was dried overnight in an oven at 107 °C.

The starch hydrolyses were conducted by adding 3 mL of 72% H_2SO_4 to 300 mg of dried pure starch. This mixture was incubated in a water bath at 30 °C for 1h. Next, 84 mL of Milli-Q were added to this mixture and autoclaved at 120 °C for 1h. The acid-hydrolyzed starch was then filtered using glass microfiber (GF-1; D 47 mm). The sugars were quantified using liquid chromatography (Dionex, model DX 500). Two technical replicates and four biological replicates were used. Figure 1 shows the starch hydrolysis steps.

MINERAL NUTRIENT QUANTIFICATION

Maize kernels were ground using a mill knife and passed through a 60-mesh sieve. The powder was dried at 60 °C and sent to the Laboratório de Análise de Tecido Vegetal at Departamento de Ciências do Solo of "Escola Superior de Agricultura Luiz de Queiroz – Universidade de São Paulo" and the nutrient content was measured according to Malavolta et al. (1997).

STATISTICAL ANALYSIS

A statistical analysis was performed for all of the data using StatSoft software (version 7.0, Tulsa, OK, USA). The significant differences between the means of the treatments were determined at a confidence level of 95% using the Duncan test.

RESULTS

In this research we obtained data that can help on the understanding of the effect of AsA seed treatment on maize grain quality. Firstly, the modified method proposed by Arakawa (1981) was used to verify whether AsA priming had interfered

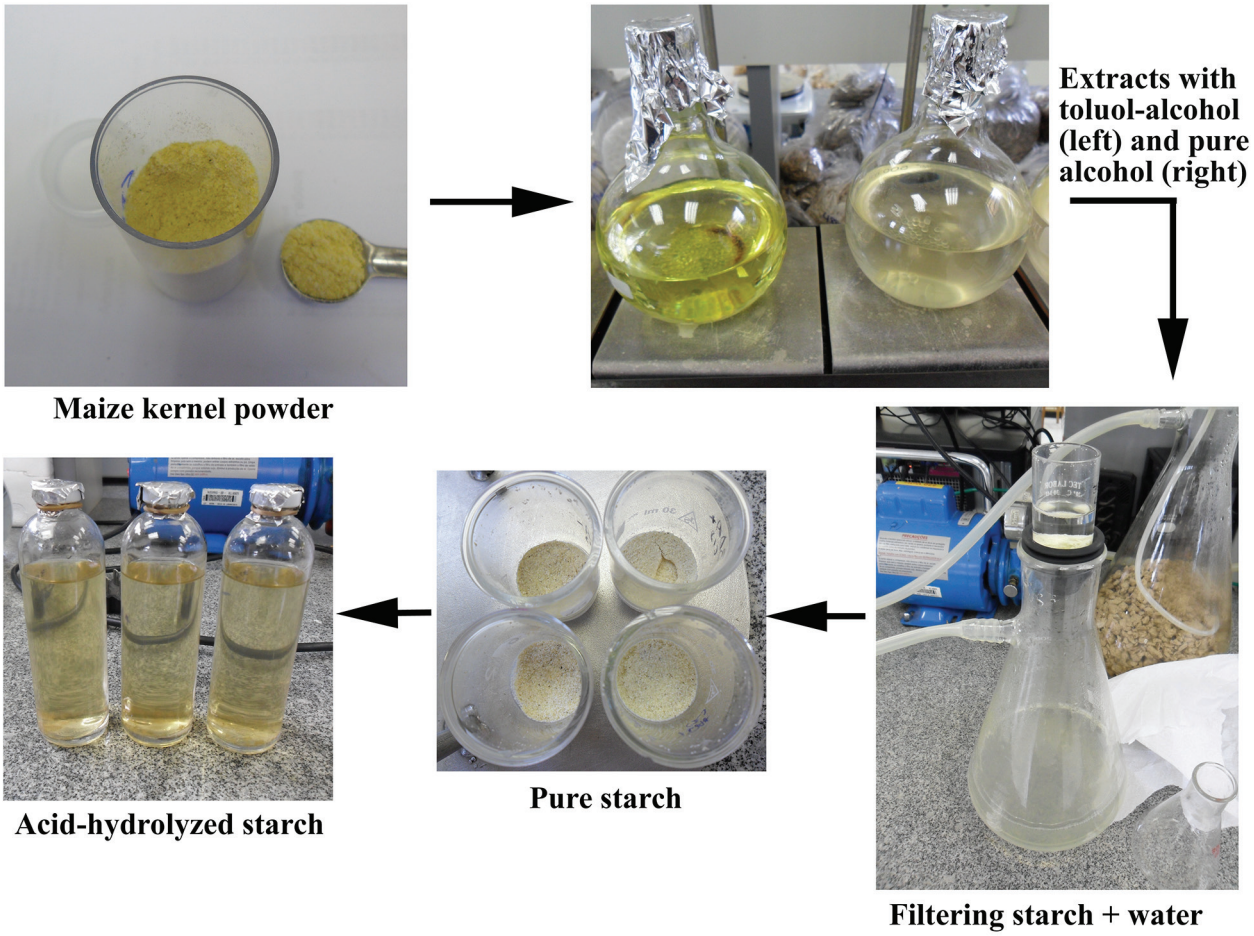

Figure 1 - Carbohydrate quantification steps in pure maize starch.

with the ascorbate content in the maize kernels. Seed priming significantly increased the ascorbate content in the DA variety from 62.4 to 86.2 µM of ascorbate.g^{-1} of kernels powder.

His, Gly, Thr, Leu and Phe contents of the DA maize kernels did not change (Table I). In contrast, the contents of the amino acids Ser (1.7x), Arg (1.4x), Asp (2.3x), Glu (2.2x), Ala (2.2x), Pro (2.7x), Lys (1.7x), Tyr (1.4x), Val (2.2x) and Ile (1.5x) increased, whilst AsA priming reduced the Met content by almost 50% (from 65.18 to 35.93 µg.g^{-1} dry matter; Table I).

To assess whether the increase of some amino acids was occurring simultaneously with mineral nutrient amelioration, the quantifications of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), boron (B), cupper (Cu), iron (Fe), manganese (Mn) and zinc (Zn) were performed. AsA seed priming significantly enhanced the allocation of B to the maize grains (from 1.41 to 2.80 mg.kg^{-1} dry matter; Table II), an increase of approximately 75%. Boron is element that may increase carbohydrates in reproductive tissues, as observed in a previous study (Pandey and Gupta 2013). In addition, AsA treatment also increased the total carbohydrate levels in maize kernels (from 936.25 to 974.97 mg of carbohydrates per gram of pure starch; Figure 2).

DISCUSSION

This study demonstrated the beneficial effects of AsA seed priming on maize kernel quality produced

TABLE I
Soluble amino acids in the DA variety maize kernels (μg.g^{-1} DM).

Treatment	His	Ser	Arg	Gly
Unprimed	155.98 ± 12.02 a	107.19 ± 4.85 a	123.44 ± 10.83 a	23.31 ± 5.73 a
Ascorbic acid	204.77 ± 17.43 a	178.51 ± 14.01 b	170.47 ± 5.62 b	39.83 ± 6.42 a
	Asp	**Glu**	**Thr**	**Ala**
Unprimed	148.21 ± 15.79 a	600.05 ± 28.48 a	57.03 ± 20.77 a	245.59 ± 7.13 a
Ascorbic acid	333.52 ± 46.03 b	1312.59 ± 186.37 b	51.69 ± 32.36 a	534.43 ± 81.1 b
	Pro	**Lys**	**Tyr**	**Met**
Unprimed	1484.92 ± 137.94 a	88.47 ± 5.38 a	431.82 ± 23.72 a	65.18 ± 2.5 a
Ascorbic acid	4022.09 ± 460.36 b	153.92 ± 16.96 b	606.40 ± 51.12 b	35.93 ± 4.68 b
	Val	**Ile**	**Leu**	**Phe**
Unprimed	100.98 ± 7.65 a	61.10 ± 3.78 a	72.43 ± 9.04 a	124.89 ± 9.19 a
Ascorbic acid	225.63 ± 18.65 b	89.79 ± 2.72 b	103.07 ± 9.23 a	170.55 ± 26.72 a

The means with different letters denote significant differences at a confidence level of 95%.

TABLE II
Mineral nutrients in the DA variety maize kernels.

Treatment	Macronutrients (g.Kg^{-1} DM)					
	N	**P**	**K**	**Ca**	**Mg**	**S**
Unprimed	15.74 ± 0.53 a	2.98 ± 0.10 a	2.96 ± 0.17 a	0.04 ± 0.00 a	0.87 ± 0.07 a	0.64 ± 0.03 a
Ascorbic acid	15.12 ± 0.55 a	2.99 ± 0.29 a	2.91 ± 0.19 a	0.04 ± 0.01 a	0.83 ± 0.05 a	0.62 ± 0.04 a
Treatment	Micronutrients (mg.Kg^{-1} DM)					
	B	**Cu**	**Fe**	**Mn**	**Zn**	
Unprimed	1.41 ± 0.42 a	1.60 ± 0.10 a	29.0 ± 1.15 a	5.6 ± 0.37 a	23.2 ± 0.78 a	
Ascorbic acid	2.80 ± 0.40 b	1.90 ± 0.19 a	28.0 ± 3.12 a	6.1 ± 0.60 a	23.8 ± 0.97 a	

The means with different letters denote significant differences at a confidence level of 95%.

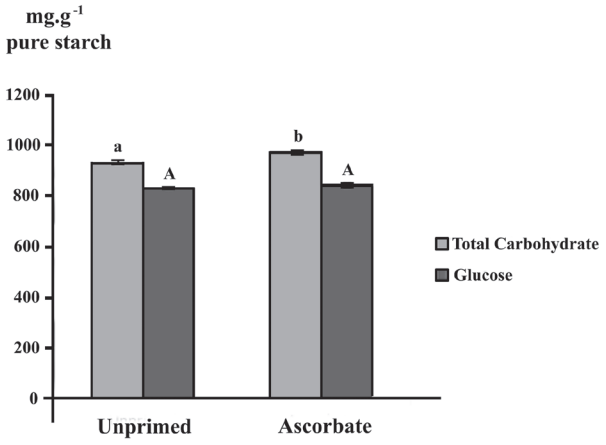

Figure 2 - Total carbohydrate and glucose contents in pure maize starch. Different letters denote significant differences at a confidence level of 95%.

by the plants derived from the primed seeds by increasing the contents of ascorbate and ten soluble amino acids, including Ser, Tyr, Ala, Val, Glu, Arg, Pro, Asp, Lys and Ile. Soluble amino acids are dissolved in MCW (methanol:chloroform:water) and are not incorporated into the proteins. AsA is a cofactor of 4-hydroxyphenylpyruvate dioxygenase, an enzyme involved in Tyr metabolism (Davey et al. 2000). Furthermore, it is possible that treatment with ascorbic acid redirects the flow of D-glucose-6-P to the amino acid metabolism because the same carbon source is used for ascorbic acid biosynthesis (Wheeler et al. 1998).

One key essential amino acid, Met, was decreased by the AsA treatment. Met shares the same biosynthetic route as Lys, Thr and Ile (Azevedo et al. 1997, 2006). The contents of Lys and Ile contents increased, whereas the Thr content was not affected. The Lys increase may to some extent result in feedback inhibition of specific

aspartate kinase (AK, EC 2.7.2.4.) isoenzymes that are controlling the carbons derived from Asp and are directed to the branches of the pathway, including the one leading to Met synthesis (Azevedo et al. 2006, Azevedo and Arruda 2010). Lys, Thr, Met and Ile are essential amino acids that share a common precursor, Asp, in a branched, complex and regulated pathway (Azevedo et al. 1997, 2006, Curien and Bastien 2009).

According to Azevedo et al. (1992), plants contain three forms of AK in maize, although the kinase activity is largely inhibited by Lys due to the presence of two Lys-sensitive isoenzymes, with only a 10-20% of AK activity being inhibited by Thr due to the feedback inhibition of the Thr-sensitive bifunctional AK-homoserine dehydrogenase isoenzyme (Azevedo et al. 1992). The AK sensitivity to feedback inhibition is a major limiting factor for Lys synthesis enhancement. Mutations conferring isoenzyme resistance to Lys may improve the amino acid content in maize kernels (Azevedo et al. 2003, 2004a, b). Met concentrations were increased in legume seeds by expressing a feedback-insensitive AK (Azevedo et al. 2006 and references there in). Wild-type plants growing in culture mediums containing Lys and Thr die following Met starvation due to inhibition of total AK activity (Azevedo et al. 1997). Therefore, the effects of AsA treatments expressing this type of AK enzyme mutation in maize may represent a potential future study that might bring some very interesting advances.

Additionally, AsA may affect cystathionine synthesis during the step catalyzed by cystathionine ϒ-synthase because it has been previously identified as a major site for Met branch regulation (Giovanelli et al. 1989). Moreover, it is important to highlight that methionyl (Met)-tRNA^{Met} (the conventional representation for initiator tRNA) is required for protein synthesis (Kozak 1983). Therefore, it is possible that Met could be used for protein biosynthesis, reducing the soluble Met content. But yet, it is important to place into context the fact that once aspartate semialdehyde is formed in a two enzyme reaction steps involving AK and aspartate semialdehyde dehydrogenase, the carbons are divided into two branches, one leading to Lys synthesis, which appeared to be stimulated by AsA, whereas the other branch leads to Met and Thr synthesis (Azevedo et al. 1997, 2006). The latter is further divided into two branches, leading to Met synthesis and Thr synthesis. Although Thr content was not changed by AsA, the carbons entering this branch of the pathway were directed to Thr since this amino acid is later transformed into Ile, which was increased by the AsA treatment, but not to Met, which was decreased. Therefore, it seems that the AsA treatment directed the carbons of the aspartate pathway to Lys and Ile syntheses. These results are particularly important for cereal crops due to the low Lys and Thr contents in the seeds, which is one of the reasons for the low nutritional value of this plant group. It seemed clear that a proteomic approach (Arruda et al. 2013) was needed to further understand the changes observed. Analyses for protein amino acids and storage protein distribution in maize grains will be the focus of a separate report since a more detailed proteomic analysis using 2D-PAGE is being currently conducted in which distinct maize lines are being used.

The increase of total carbohydrate levels observed in this study may represent a side effect caused by the increased boron (B) in the grains because this element is involved in carbohydrate metabolism (Camacho-Cristóbal et al. 2008) and is important for reproduction and organogenesis (Huang et al. 2008, Brondani et al. 2012).

The sugar-borate complex transport through the cellular membranes was demonstrated by Gauch and Dugger (1953). According to these authors, B is initially transported by the xylem, and is then transported by the phloem together with sugar after reaching the leaves. During reproductive stages, the phloem exudates showed higher levels of B

than xylem sap, demonstrating the importance of this mineral nutrient during carbohydrate transport through the phloem (Huang et al. 2008, Takano et al. 2008).

Based on Blevins and Lukaszewski (1998) there is substantial evidence supporting an association between B and ascorbate metabolism. An interesting report showed that B deficiency can reduce root growth and ascorbate content in root tips of squash (*Curcubita pepo* L.) (Lukaszewski and Blevins 1996). These authors verified that AsA supplementation in medium lacking B rescued growth in these plants, suggesting that the lack of B affected vitamin C metabolism. Thus, AsA supplementation offset B deficiency and enabled root elongation. The novel observation in our study is that AsA seed priming induces B translocation during kernel production and therefore enhances total carbohydrate translocation that requires complexation with this mineral element. The enhancement in B translocation could be explained by the increase of AsA availability caused by AsA-seed priming.

In summary, this work contributes with information about the effect of AsA seed priming in the amelioration of maize kernel quality with respect to the ascorbate content, B allocation, total carbohydrate content and increased soluble amino acid levels, including Ser, Tyr, Ala, Val, Glu, Arg, Pro, Asp, Lys and Ile, although the soluble Met pool decreased. Maize is a staple food in many developing countries and the discoveries presented in this study may contribute in giving directions for enhancement of essential amino acids for the human diet, such as Lys and Ile. Moreover, a number of new methods and techniques are available that can be used to produce these new lines, for instance transgenic alterations as the ones produced for barley, another Lys deficient crop species, which have exhibited alterations in the concentrations of these key amino acids (Schmidt et al. 2015, 2016).

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