Universidade Trás-os-Montes e Alto Douro

Cytogenetic and biochemical characterization of the progeny of plants of bread wheat 'Jordão' biofortified with Iron and/or Zinc

Dissertação de Mestrado em Genética Molecular Comparativa e Tecnológica

Miguel Ângelo Dias Baltazar

Orientador: Professor Doutor José Eduardo Lima-Brito
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Vila Real, 2020

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This thesis is dedicated to
my mom, my dad, my sister and my niece.

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Cytogenetic and biochemical characterization of the progeny of plants of bread wheat 'Jordão' biofortified with Iron and/or Zinc

Abstract

Seed priming with micronutrients (nutripriming) has several agronomic advantages if performed with suitable dosages. Nutripriming has been commonly used in the biofortification of crops such as bread wheat (*Triticum aestivum* L. em Thell.). However, the usage of excessive amounts of micronutrients like Iron (Fe) and Zinc (Zn) can induce cytotoxicity (traceable by both cytogenetic and biochemical analyses) and phytotoxicity (detectable upon the characterization of yield-related components). Nutripriming is performed in each generation, but the benefits and/or the cytotoxicity-related stress memory of nutripriming in the next unprimed generation/offspring is unknown. Therefore, this work evaluated how hydropriming and nutripriming performed in the parental S0 seeds influenced the germination, mitosis, biochemical profile and yield-related components of the first generation of unprimed bread wheat cv. 'Jordão' seeds (S1 seeds). The S0 seeds were previously primed with distilled water (hydropriming) and nutriprimed with 4 $mg.L^{-1}$ and/or 8 $mg.L^{-1}$ of Fe and/or Zn. These concentrations induced cytotoxicity, nucleolar stress and increased the total soluble protein content. Unprimed S1 seeds were used as control (control S1).

In the whole wheat flour samples of each S1 offspring, sixteen free amino acids (a.a.) and five soluble sugars were identified and quantified using HPLC-FLD and HPLC-PAD, respectively. In most of the S1, the content of each a.a. increased relative to the control being glutamic acid and glutamine, proline and glycine the most abundant. Glucose, ash content and crude protein (CP) of the S1 offspring increased relative to the control. However, only the ash content was significantly different ($p < 0.05$) among the control and remaining offspring. Sucrose, fructose, raffinose, maltose and total starch showed a significant decrease ($p < 0.05$) in few S1 offspring relative to the control. The protein amount previously determined in the whole wheat flour of S0 seeds was significantly higher ($p < 0.05$) in those primed with 4 $mg.L^{-1}$ or 8 $mg.L^{-1}$ of Fe + Zn. Similar results were observed in their S1. The overall biochemical data revealed that the Fe and/or Zn nutripriming improved the grain nutritional

status and the stress tolerance in the S0 seeds and plants, and these advantages were transmitted to the unprimed S1 offspring.

The cytotoxicity generated by nutripriming with 4 $mg.L^{-1}$ and/or 8 $mg.L^{-1}$ of Fe and/or Zn in the S0 seeds resulted in longer mean germination time (MT) values and high frequencies of cell cycle and chromosomal anomalies. However, their respective S1 offspring showed a higher germination rate, shorter MT values and higher mitotic index (MI). Nonetheless, despite the higher percentages of dividing cells with anomalies (%DCA) observed in the S1, revealing the inheritance of a stress memory, a lower number of anomalies was detected in this offspring. In sum, the germination and cytogenetic data of the S1 suggested an attenuation of the cytotoxicity detected in the S0. Furthermore, the S1 plants surpassed the average values of seven yield-related components characterized previously in the S0 plants.

The biochemical, cytogenetic and the yield-related components characterizations performed in this work evidenced the transmission of an attenuated cytotoxicity-related stress memory from the S0 to the S1. A higher intergenerational inheritance of benefits at the biochemical, germination, mitotic and yield level was also detected.

Our results demonstrated that the repetition of nutripriming in each generation is unnecessary converting this method on an even more affordable biofortification approach.

Keywords: Biochemical profile; cell cycle; cytotoxicity; intergenerational effects; nutripriming; stress memory; yield-related components.

Caracterização citogenética e bioquímica da descendência de plantas de trigo mole 'Jordão' biofortificadas com Ferro e/ou Zinco

Resumo

O *priming* de sementes com micronutrientes (*nutripriming*) tem vantagens agronómicas quando realizado com dosagens adequadas e é frequentemente usado na biofortificação de trigo mole (*Triticum aestivum* L. em Thell.). Porém, dosagens excessivas de micronutrientes como o Ferro (Fe) e Zinco (Zn) induzem citotoxidade (detetável através de análises citogenéticas e bioquímicas) e fitotoxicidade (observável pela caracterização de componentes relacionados com o rendimento). Como o *priming* de sementes é realizado em cada geração, desconhece-se se ocorre transmissão de benefícios e/ou memória de stress relacionada com a citotoxicidade para a descendência não-tratada. Assim, este trabalho avaliou a influência do *priming* de sementes na germinação, mitose, perfil bioquímico e componentes relacionados com o rendimento da primeira geração de sementes não-tratadas da cultivar 'Jordão' de trigo mole (sementes S1). Esta descendência teve origem em sementes S0 tratadas com água destilada (*hydropriming*) e com 4 $mg.L^{-1}$ e/ou 8 $mg.L^{-1}$ de Fe e/ou Zn. Estas concentrações induziram citotoxicidade, stress nucleolar e aumento do conteúdo proteico. Uma descendência S1 de sementes não-tratadas foi usada como controlo (controlo S1).

Nas amostras de farinha de trigo integral de cada descendência S1, identificaram-se e quantificaram-se dezasseis aminoácidos (a.a.) e cinco açúcares solúveis usando HPLC-FLD e HPLC-PAD, respetivamente. Na maioria das descendências S1, o conteúdo de cada a.a. aumentou relativamente ao controlo. Os mais abundantes foram o ácido glutâmico + glutamina, prolina e glicina. A glucose, o conteúdo de cinza e a proteína bruta (PB) das descendências S1 aumentaram relativamente ao controlo. Contudo, apenas o conteúdo de cinza foi significativamente diferente ($p < 0.05$) entre o controlo e as restantes descendências. A concentração de sacarose, frutose, rafinose, maltose e amido apresentaram uma diminuição significativa ($p < 0.05$) num reduzido número de descendências relativamente ao controlo. O conteúdo proteico previamente determinado em farinha integral das sementes S0 foi significativamente ($p < 0.05$) mais elevado nos tratamentos com 4 $mg.L^{-1}$ ou 8 $mg.L^{-1}$ de Fe + Zn. Resultados semelhantes foram encontrados nas suas descendências S1. Os dados bioquímicos revelaram que o nutripriming com Fe e/ou Zn terá melhorado o valor nutricional

e a tolerância ao stress nas sementes e plantas S0, e que estas vantagens foram transmitidas à descendência S1 não-tratada.

Sementes S0 tratadas com 4 $mg.L^{-1}$ e/ou 8 $mg.L^{-1}$ de Fe e/ou Zn apresentaram tempos médios de germinação (TM) longos e elevadas frequências de anomalias no ciclo celular. Contudo, as respetivas descendências S1 apresentaram uma taxa de germinação superior, menores valores de TM, e índice mitótico (IM) superior à S0. Embora na S1 se tenham observado percentagens de células em divisão com anomalias (%CDA) superiores às da S0, revelando a transmissão de uma memória de stress, o número de anomalias foi mais reduzido. Globalmente, os dados de germinação e citogenéticos da S1 sugeriram uma atenuação da citotoxicidade detetada na S0. Adicionalmente, as plantas S1 ultrapassaram as médias relativas a sete componentes relacionados com o rendimento previamente caracterizados nas plantas S0.

As caracterizações realizadas neste trabalho evidenciaram a transmissão da S0 para a S1 de uma memória de stress relacionada com a citotoxicidade, embora atenuada, e de benefícios ao nível bioquímico, de germinação, mitótico e de rendimento.

Este trabalho demonstrou que não é necessário repetir o *priming* de sementes em cada geração, tornando este método de biofortificação ainda mais económico.

Palavras-chave: Ciclo celular; citotoxicidade; componentes relacionados com o rendimento; efeitos intergeracionais; memória de stress; *nutripriming*; perfil bioquímico.

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List of abbreviations

%DCA – Percentage of dividing cells with anomalies

a.a. – Amino acid

AQC - 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate

CP – Crude protein

cv. – Cultivar

DCA – Dividing cells with anomalies

DM – Dry matter

EDTA - Ethylenediaminetetraacetic acid

FAO – Food and Agriculture Organization

FAOSTAT - Food and Agriculture Organization Corporate Statistical Database

HPLC – High performance liquid chromatography

HPLC-FLD – High performance liquid chromatography with fluorescence detection

HPLC-PAD - High performance liquid chromatography by pulsed amperometric detection

IGC – International Grains Council

IPCC - Intergovernmental Panel on Climate Change

MI – Mitotic index

MT – Mean germination time

NSkMS - Number of spikelets of the main spike

NSMS - Number of seeds of the main spike

NSperSk - Number of seeds per spikelet

NSSS - Number of seeds of the secondary spikes

WSSS - Weight of seeds of the secondary spikes

ROS - Reactive oxygen species

RT - Room temperature

S0 - Parental seeds generation

S1 – First seeds generation

NT – Number of tillers with spike;

TNS – Total number of seeds

WSMS - Weight of seeds of the main spike

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1. General literature review

1.1. Introduction

This literature review will focus on: (i) the economic and nutritional value of wheat; (ii) the importance of the micronutrients Iron (Fe) and Zinc (Zn) in plants and humans; (iii) the strategies that have been used to ameliorate the Fe and Zn concentration in crops; (iv) the cytotoxic effects of micronutrient excess in the plants' biochemical composition, seed germination, early stages of plant development and yield-related characters; and (v) the transmission of beneficial effects and stress memory to the next generation(s).

1.2. Bread wheat and its global importance

Cereals like maize, wheat and rice, and their products, constitute the main source of feed and food worldwide, providing a significant number of calories and protein in the human diet (Qayyum *et al*. 2011; Henry *et al.,* 2016; [1]).

In 2017, the global production of wheat (*Triticum* spp.) was 771 million tonnes, being ranked in second among all cereals [1]. Wheat represents about 72% of the human consumption of cereals [2], supplying one-third of the world population with more than half of their daily calorie intake (Amiri *et al.*, 2018; [1]).

Bread wheat (*Triticum aestivum* L. em Thell; AABBDD; 2n = 6× = 42) is the most produced wheat species throughout the world (Shewry and Hey, 2015). It is mainly consumed in the form of baked products, and its nutritional quality is tied to the milling process it undergoes.

The wheat grain (also known as kernel or caryopsis) is composed of several different tissues which can be partitioned into three parts: bran, germ or embryo, and endosperm (Fig. 1).

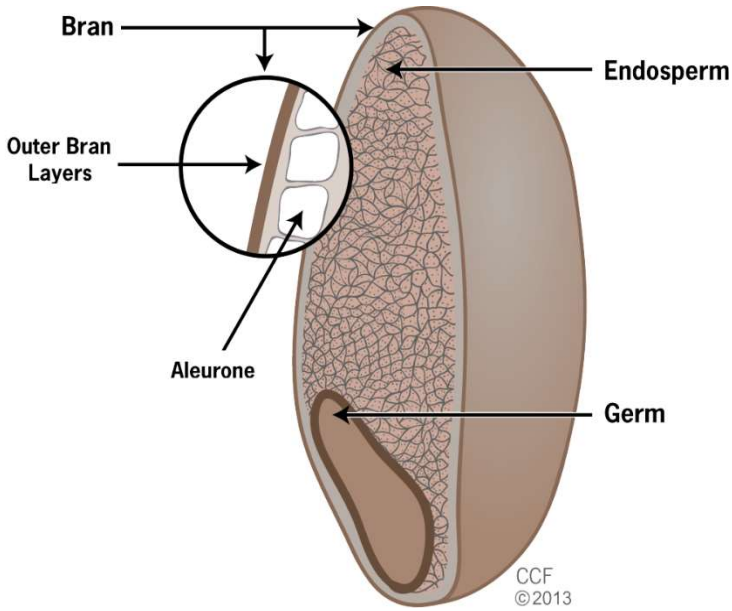

Figure 1. Whole-wheat grain main sections. Adapted from Bernstein *et al.* (2013)

The endosperm is rich in starch and constitutes 80 to 85% of the grain weight, followed by the bran with 13 to 17% and the germ with 2 to 3%.

Most of the minerals, vitamins, dietary fibre and bioactive compounds reside in the bran (Curti *et al*., 2013) that has been considered an excellent ingredient in the food (Prückler *et al.,* 2014; Babu *et al.*, 2018). Due to its high content of micronutrients, the wheat bran has been gradually included in the human diet over the years (Prückler *et al.,* 2014; Babu *et al.*, 2018). Moreover, several studies have linked the consumption of wheat bran and whole-wheat products to the prevention of several diseases and the improvement of the quality of life (see review by Călinoiu and Vodnar, 2018). However, during the milling process, the endosperm is separated from the other parts of the wheat kernel to obtain white flour containing mostly starch, storage proteins, amino acids and sugars (Shewry and Halford, 2002; Wrigley *et al*., 2016). As a consequence, most of the minerals present in the bran are lost during the milling process (Sarwar *et al*., 2013; Borrill *et al*., 2014). Therefore, in developing countries where wheat constitutes the main source of food, breeding programs have been focusing on the improvement of its nutritional quality (Myers *et al*., 2004; Soares *et al*., 2019). This quality is also highly influenced by the bioavailability of micronutrients in the soil, and their effects in plant growth and development (Nagajyoti *et al.*, 2010; Cakmak and Kutman, 2018).

1.3. The importance of micronutrients

The essential minerals are crucial for the plant's growth, development and reproduction (DalCorso *et al*., 2014) and can be classified into macronutrients or micronutrients depending on the concentration that they are required by plants (Barker and Pilbeam, 2015).

Boron (B), Chlorine (Cl), Copper (Cu), Iron (Fe), Manganese (Mn), Molybdenum (Mo), Nickel (Ni) and Zinc (Zn) are considered micronutrients since they are required in reduced amount by plants (Barker and Pilbeam, 2015). These are involved in organelle synthesis, structural integrity, cell division and elongation, photosynthesis, respiration, protein synthesis, hormone activation, oxidation-reduction reactions and nitrogen metabolism (Page and Feller, 2015).

The uptake of micronutrients by plants depends on their available forms and physical-chemical properties of the soil (e.g. pH) which are greatly influenced by natural processes and anthropogenic activities (Noulas *et al*., 2018). In addition, micronutrient deficiency or excess can seriously affect the life cycle of plants, causing various symptoms and ultimately leading to plant death if not treated. This not only influences the production of the crops and nutritional quality of the seeds but also affects human health (Bouis, 2007; Mayer *et al*., 2008).

A large portion of the world's population have diets that consist mostly of starchy cereals, roots and tubers grown in nutrient-poor soils (Dimkpa and Bindraban, 2016) that lead to health problems related to micronutrient deficiency (Fig. 2; Samoraj *et al.*, 2018).

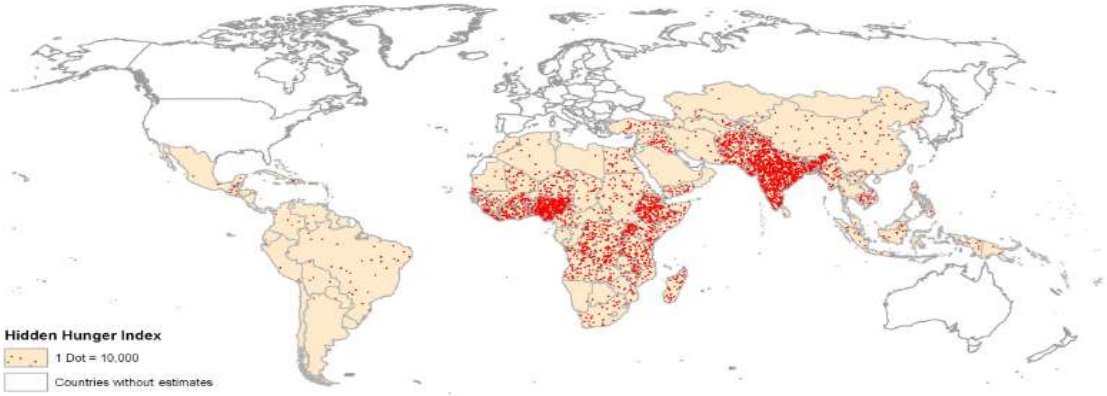

Figure 2. World map representing the population affected by hidden hunger associated with Fe, Zn and vitamin A deficiencies. (Adapted from Muthayya *et al*., 2013).

It is estimated that two billion lives are affected by hidden hunger, a chronic deficiency of essential micronutrients and vitamins. As the name implies, the symptoms of hidden hunger are usually not visible. However, it is linked to higher disease and mortality rates (Muthayya *et al.*, 2013). Fe and Zn are two of the main culprits in a hidden hunger. Among the 7 billion of people in the world, it is estimated that over 60% have diseases related to Fe deficiency while around 30% have problems of Zn deficiency (Samoraj *et al.*, 2018).

The most severe Fe-deficient population are children and women, causing them to be debilitated in both physical and mental work capacity. Severe cases of this deficiency also lead to complications in childbirth and mortality for both mothers and children (Graham *et al*., 2012). It is believed human Fe deficiency is the most common cause for anaemia worldwide, as it is essential in red cells (Milto *et al*. 2016). Other common clinical problems include fatigue, angular stomatitis, painful glossitis, dysphagia and restless leg syndrome; and more importantly, long-term cognitive impairment and behavioural problems during infancy (Moll and Davis, 2017). Zn deficiency is also correlated to a variety of physiological issues: increased susceptibility to diseases, reduced physical performance, growth retardation, impaired brain development and birthing problems in women (Gibson, 2012; Terrin *et al.*, 2015). Inadequate zinc intake is especially important in children under the five years of age, as this leads to a stunt in growth but can ultimately lead to death (Wessells and Brown, 2012). Nonetheless, this deficiency leads to DNA damage and has been previously linked to an increase in the risk of cancer (Ho, 2004).

1.3.1. Iron and Zinc

As previously mentioned, Fe and Zn are fundamental for plant growth and development, playing an important role in various biochemical and physiological processes (Barker and Pilbeam, 2015).

In plants, Fe is essential in many metabolic processes such as photosynthesis, respiration, nitrogen assimilation, hormone biosynthesis, production and scavenging of reactive oxygen species (ROS), osmoprotection, and pathogen defence (Kim and Guerinot, 2007; Hänsch and Mendel, 2009; Rout and Sahoo, 2015). Most of the cellular Fe is found in the chloroplasts, being consistent with its major function in photosynthesis (Hänsch and

Mendel 2009). Another hotspot for Fe is the mitochondria, where it is a major constituent in clusters involved in the respiration process (Connorton *et al.*, 2017).

Zn is a functional, structural and regulatory cofactor of various essential enzymes and proteins in plants (Sinclair and Krämer, 2012). Over 1200 proteins are predicted to bind, contain or transport Zn (Hänsch and Mendel, 2009). These include transcriptions factors, oxireductases, hydrolytic enzymes, and a large number of zinc-finger proteins (Krämer and Clemens, 2005; Sinclair and Krämer, 2012). Moreover, DNA and RNA synthesis and maintenance are performed by Zn dependent enzymes such as DNA-polymerases, RNA-polymerases, histone deacetylases, splicing factors and RNA-editing enzymes present in the mitochondria and chloroplasts (Krämer and Clemens, 2005). Zn also plays an important role in seed development, and Zn-deficient plants show a delayed maturity (Cakmak and Kutman, 2018).

The deficit but also the excess of micronutrients affect the plant yield, nutritional quality, morphology, biomass and photosynthesis negatively.

Despite being fundamental to plants, Fe and Zn also integrate the list of hazardous heavy metals (Vodyanitskii, 2016). The excess of micronutrients or heavy metals derived from natural sources or anthropogenic activities induces plant osmotic stress and consequent redox imbalance due to the accumulation of ROS (Truta *et al*., 2013; Anand *et al.*, 2017; Noulas *et al*., 2018). Osmotic stress leads to changes in different compounds such as total soluble sugars, a.a., protein, phenolics, hormones, and lipids (Gzik, 1996; Anand *et al.,* 2017). Moreover, their accumulation also causes problems at the molecular level such as DNA damage and disruption of normal enzyme activity, affecting the cell cycle (Sinclair and Krämer, 2012; Kobayashi and Nishizawa, 2014; Briat *et al.*, 2015; Taranath *et al.*, 2015).

On the other hand, intensive cropping systems, high-yielding cultivars lacking nutritional content and acid soils, are increasing the micronutrient deficiency worldwide (Alloway, 2008; Imtiaz *et al.*, 2010). In this sense, increasing the nutritional quality and producing micronutrient enriched cereals has been one of the major priorities for researchers and breeders (Zeidan *et al.*, 2010; Gao *et al.*, 2012; Bharti *et al*., 2013; Trijatmiko *et al.*, 2016; FAO 2017; Soares *et al*., 2019).

1.4. Biofortification – an overview

Reducing micronutrient malnutrition has been the focus of agronomists, nutritionists and researchers. Thus, strategies such as food diversity, food supplements, food fortification and biofortification have been intensively studied (Wakeel *et al.*, 2018).

Despite food diversity being considered the most obvious choice to remediate malnutrition, access to healthy food is limited in developing countries which are the most affected. Food supplementation and food fortification, the addition of micronutrients to a person diet or processed foods, respectively, are also expensive and difficult to sustain (Wakeel *et al.*, 2018).

Biofortification is the process of increasing the concentration of nutrients in food crops (Singh *et al*., 2016). It poses as an excellent alternative to the approaches mentioned above, being more cost-effective, sustainable, and a suited long-term solution (Samoraj *et al.*, 2018). Furthermore, biofortification aims the reduction of human mortality and morbidity rates related to micronutrients deficiency, while increasing the crops yield and resistance (Samoraj *et al.*, 2018). Although its efficiency is still not comparable to food fortification, biofortified crops have been shown to impact the human diet significantly (Bouis, 2018). Two main strategies of biofortification have been used to increase the nutritional value of crops: genetic biofortification and agronomic biofortification (Singh *et al.*, 2016; Ali *et al.*, 2018).

Genetic biofortification is achieved either through traditional breeding of crops or through genetic engineering. Conventional breeding of crops uses several varieties of the same plant with different nutritional characteristics to develop an improved food crop (Bouis, 2007). Although it is overall more accepted than genetic engineering, traditional breeding is very limited. Furthermore, a focus on yield despite other traits; complexities of the gene × environment interaction, and the difficulty in introducing the desired traits, have been limiting the success of this approach (Tester and Bacic, 2005). Breeders also found other adversities in traditional breeding since they must combine quantity and quality, grain yield with flour quality, or the use of varieties or lines with restricted genetic variability whose success is highly dependent on the availability of minerals in the soil (Beyer, 2010; Bouis *et al.*, 2011; Vodyanitskii, 2016; Venske *et al.*, 2019).

Genetic engineering relies on the unlimited genetic pool of different species, allowing the transfer and expression of desirable genes independently of their evolutionary and taxonomic relations (Garg *et al.*, 2018). The development of transgenic crops involves

substantial efforts and investment during their development phase (Hefferon, 2016). Although genetic engineering seems promising, and has become easier and faster to perform nowadays, it is still limited by problems of approval and public acceptance of genetically modified crops (Wakeel *et al.*, 2018).

Agronomic biofortification could be more cost-effective in increasing micronutrients in the edible parts of crops. Evidence suggested that this approach can increase both the crops yield and nutritional food quality (de Valença *et al.*, 2017). Strategies of agronomic biofortification include the micronutrient application to the soil, the exogenous application of micronutrients enriched solutions to the leaves; or seed treatments such as seed priming. The soil fertilization and foliar application are an immediate solution to the lack of micronutrients in the soil and/or plants, respectively (White and Broadley, 2009; Zhang *et al.*, 2012). However, the success of these approaches is limited by the mobility of the minerals through the phloem. For instance, Zn has lower mobility in the phloem than Fe, restricting the accumulation of the former in the edible parts of the plant (Broadley *et al.*, 2007).

Seed priming is considered one of the most accessible, successful and cost-effective agronomic biofortification methods and has been extensively studied and widely used in the past years (Harris *et al*., 2008; Farooq *et al.*, 2012; Ali *et al.*, 2018).

1.4.1. Seed priming

Seed priming consists of the control of the hydration level of the seed to trigger metabolic and enzymatic processes usually occurring during the early germination phase. It is considered one of the best approaches to enhance seed quality (Paparella *et al.,* 2015). This biofortification technique leads to an increased germination rate, greater tolerance to biotic and abiotic stresses, higher grain yield and a more vigorous plant (Farooq *et al.*, 2008; Afzal *et al*., 2008; Rajjou *et al.*, 2012; Reis *et al.*, 2018; Sundaria *et al*., 2019; among others).

Seed priming can be performed with various agents, and each technique is named accordingly. Table 1 presents different priming methods and summarizes their benefits and applications in recent wheat research.

Table 1. Summary of different priming techniques and recent applications in wheat. Adapted from Bhowmick (2018) and Choudhary *et al*. (2019).

Priming technique	Priming medium	Advantages	Examples of recent research in wheat
Hydropriming	Distilled water	Enhancement of seed germination, seedling emergence and enzyme activity.	Anwar *et al*. (2018); Arif *et al*. (2019); Saddiq *et al*. (2019).
Halopriming	Organic salt solutions (e.g. NaCl, KNO_3, $CaCl_2$)	Enhancement of seed germination, seedling emergence and crop yield under adverse conditions.	Kumar *et al.* (2017); Arif *et al.* (2019); Saddiq *et al* (2019).
Osmopriming	Osmotic solutions (e.g sugars, polyethylene glycol)	Enhancement of seed germination and crop performance.	Hakeem *et al.* (2017); Abid *et al.* (2018)
Hormopriming	Hormone solutions (e.g. kinetin, abscisic acid, salicylic acid)	Enhancement of vegetative growth and photosynthetic activity.	Sher *et al.* (2017); Ulfat *et al*. (2017); Bagheri *et al*. (2018)
Solid matrix priming	Water and a solid material (e.g. vermiculite, expanded calcined clay)	Enhancement of seed germination, seedling emergence and antioxidant enzymes activity.	Ahmed *et al.* (2016)
Biopriming	Solutions containing beneficial microorganisms (e.g. *Pseudomonas* spp., *Trichoderma* spp.)	Enhancement of seed germination, crop establishment, quality and yield.	Meena *et al.*, (2017); Bagheri *et al*. (2018)
Nutripriming	Solutions containing macro- or micronutrients (e.g. Fe, Zn, B.)	Improves seed germination, nutrient content, quality, seedling establishment, plant growth, yield-related components and protein content.	Rehman *et al.* (2015); Reis *et al.* (2018); Carvalho *et al.* (2019)
Nanopriming	Solutions containing engineered nanoparticles (NPs) (e.g. ZnNPs, FeNPs, etc.)	Increases germination, nutrient concentration in the grain, improves seedling dry weight and vigour.	Hatami *et al.* (2018); Medina-Velo *et al.* (2018); Sundaria *et al*. (2019)

As sustainable agriculture becomes more relevant due to climate change and world resource management, seed priming poses as one of the best alternatives to maintain the quality and production of crops (Raj and Raj, 2019).

Nutripriming constitutes a promising method for increasing the micronutrient amount in the crop seeds (like Fe and Zn) or edible portions of the plant, to overcome their insufficiency in the soil or cultivars (see review by Farooq *et al.*, 2012). This affordable method can also enhance yield and/or protein amount in various crops, including wheat (Farooq *et al.*, 2012*;* Prom-u-thai *et al.,* 2012; Ali *et al*., 2018; Cakmak and Kutman, 2018; Reis *et al*., 2018; Carvalho *et al.*, 2019; Nadeem *et al*., 2019). In addition to the biochemical advantages, nutripriming also benefits morpho-physiological and molecular aspects of the plants, and in most of the cases, it seems to function better than the other biofortification strategies (Harris

et al. 2008; Farooq *et al.,* 2012; Mondal and Bose, 2019). Nonetheless, nutripriming is only beneficial when suited micronutrient doses are used, since their excess may cause abiotic stress, leading to cyto- and phytotoxicity (Moussa and El-Gamal, 2010; Iqbal *et al*., 2012; Nawaz *et al.* 2013; Rehman *et al*. 2015; Reis *et al*., 2018).

The analysis of yield-related components can monitor the toxicity effects of nutripriming, germination rate and time (Prom-u-thai *et al*., 2012; Rehman *et al*., 2015; Ali *et al*., 2018; Sundaria *et al*., 2019) and/or through the occurrence of anomalies in the mitotic cell cycle and chromosomes (Kumari *et al*., 2011; Taranath *et al*., 2015; Reis *et al*., 2018).

Seed nutripriming presents many agronomic advantages if performed with proper dosages of micronutrients. Although the excessive amounts of micronutrients like Fe and Zn are tightly regulated in plants, the use of high dosages in seed priming can cause cyto- and phytotoxicity (Palmgren *et al*., 2008; Reis *et al*., 2018). In this sense, seed nutripriming can be helpful to unravel and contribute for the understanding of mechanisms underlying plant stress responses (Sharma and Dietz, 2006; Emamverdian *et al.*, 2015; Sheteiwy *et al.*, 2016).

1.5. Abiotic stress due to micronutrients excess

In plants, stress is a disruption in homeostasis that produces changes at the physiological, biochemical, cellular and molecular level (Gaspar *et al*., 2002).

The plant stress can be biotic if including attacks from herbivores and pathogens or abiotic like exposure to extreme temperatures, reduced water availability, nutrient deficiency, heavy metals and salinity (Gill *et al*., 2016), among others.

Plants are sessile organisms, being continuously subjected to environmental fluctuations which affect their growth, development and productivity (Gaspar *et al*., 2002).

Improving plant stress tolerance is crucial for adaptation to environmental fluctuations and sustainable production since susceptible crops require high water availability and fertilizer amounts and present lower nutritional quality (Soares *et al*., 2019).

Cereals are moderately sensitive to a variety of abiotic stresses causing significant reductions in crop production and quality (Dolferus *et al.*, 2011; Bowne *et al.*, 2018). Abiotic stresses caused by extreme weather episodes are predicted to increase in frequency and severity due to climate change (IPCC 2014), influencing the micronutrient availability (Fedoroff *et al*., 2010; Soares *et al*., 2019).

The high concentrations of heavy metals like Fe and Zn in soils result naturally from the geochemical material but also from the anthropogenic activity, such as pollution, the use of pesticides and fertilizers in agriculture, mining, metalwork, industrial practices and construction (Broadley *et al*., 2007; Nagajyoti *et al*., 2010; Truta *et al.*, 2013). These micronutrients, despite essential to plants in a reduced amount, when present in excess, cause cyto-, geno- and phytotoxicity (Manara, 2012; Truta *et al*., 2013). Additionally, the micronutrients excess induces several adverse effects like the accumulation of ROS, cytotoxicity, lower germination rate and delay in seedlings production as well as reduced yield (Shafi *et al.*, 2009; Sheteiwy *et al.*, 2016; Reis *et al.*, 2018).

1.5.1. Influence on germination, cell cycle and early stages of development

Previously, it was demonstrated that seed priming with highly concentrated solutions of Fe is cytotoxic and lead to a decrease in the germination rate and seedling growth (Mirshekari, 2010). More recently, Reis *et al*. (2018) observed that concentrations of Zn and Fe higher than 2 $mg.L^{-1}$ significantly increased the mean germination time, decreased the germination rate and mitotic index, and increased the number of cells with anomalies in bread wheat.

Seed priming performed with concentrated solutions of Zn were also toxic to the germination and seedling growth of bread wheat (Harris *et al*., 2008; Rehman *et al.*, 2015; Hassan *et al*., 2019; Shilpie and Mishra, 2019).

Cytogenetic studies performed in bread wheat and other species have demonstrated that the abiotic stress generated by the excess of micronutrients causes cytotoxicity which affect the seed germination, seedling growth, and the regularity of the cell cycle (El-Shahaby *et al.*, 2003; Rout and Das, 2003; Li *et al.*, 2005; Street *et al.*, 2007; Kranner and Colville, 2011; Reis *et al*., 2018).

The evaluation of the mitotic index (MI) is a measure of cytotoxicity (Smaka-Kincl *et al.*, 1996). Moreover, the frequency of chromosomal anomalies such as breaks, chromatin stickiness, metaphase arrest (C-mitosis), chromatin bridges, among many others, also indicates the level of toxicity at which the plant is subjected (Pekol *et al.*, 2016).

Studies of the effects of seed priming with Fe or Zn in different crops have been previously performed (Prasad *et al.*, 1999; Harris *et al*., 2008; Truta *et al*., 2013; Rehman *et*

al., 2015; Reis *et al*., 2018). A high concentration of Zn is known to affect cell division, and in turn, reduce root growth (Prasad *et al.*, 1999; El-Ghamery *et al*., 2003).

1.5.2. Influence on yield components

The analysis of yield components is crucial in the determination of the productivity of crops such as wheat (Singh and Diwivedi, 2002). Despite the genetic properties of each cultivar, the habitat and the agronomic factors also affect productivity. These conditions are translated into quantitative parameters at each different stage of development (Khan *et al.*, 2003; Sainis *et al.*, 2006). Hence, it is essential to understand the relationships between grain yield and grain weight per spike (Harasim *et al.*, 2016).

Interactions between genetic and environmental factors play an important role in crop development. Furthermore, abiotic stresses can significantly influence the crops yield, and researchers emphasize that yield components are much dependent on environmental conditions (Harasim *et al.*, 2016). Even mild abiotic stresses can affect grain yield despite the vegetative parts suffering no alterations (Dolferus *et al.*, 2011). Extreme temperatures and drought are of the most studied abiotic stresses, with results indicating that heat or cold, and water scarcity reduce the crop production, even in tolerant varieties of the same species (Mirbahar *et al*., 2009; Pimentel *et al*., 2015; Kaur *et al.*, 2016; among others).

A major threat in agriculture productivity is the contamination of soils with heavy metals. Heavy metal toxicity, such as those resulting from a high concentration of cadmium, copper and chromium in the soil, cause yield reduction (Wani *et al.*, 2007; Wani *et al.*, 2008; Ma *et al.*, 2015).

Seed priming performed with Fe and/or Zn solutions with concentrations above 2 $mg.L^{-1}$ reduced yield-related components in bread wheat (Reis *et al*., 2018).

Therefore, to overcome crop yield losses due to various abiotic stresses, one of the major goals of plant breeding is to improve stress tolerance (Lämke and Bäurle, 2017).

1.5.3. Influence on the biochemical profile

Plants have developed sophisticated adaptation and defence mechanisms to mitigate the impacts of abiotic stress (Lämke and Bäurle, 2017).

Physical barriers, heavy metal storage organs, proteins, cellular exudates, a.a. and hormones are all part of a first defensive mechanism against heavy metal toxicity (Emamverdian *et al.*, 2015). Thus, the accumulation of osmolytes in the cytoplasm, such as sugars, a.a. and organic acids is a plant stress response (Parida and Das, 2005).

The synthesis of a.a. is essential as they are crucial constituents of proteins. Moreover, they are necessary for cellular reactions, influencing several physiological processes such as plant growth, development, generation of metabolic energy, and resistance to stress (Anjum *et al.*, 2014; Hildebrandt *et al.* 2015). Furthermore, abiotic stress, such as heavy metal concentrations, increase the biosynthesis of some a.a. (Sharma and Dietz, 2006).

Asparagine is crucial in the regular transport and storage of nitrogen (Lea *et al.*, 2007). It is known to accumulate in cereals grains in response to abiotic stress, such as deficiency of micronutrients (Lea *et al.*, 2007; Gao *et al.*, 2016).

Serine is required for the biosynthesis of biomolecules necessary for cell proliferation and along with arginine are constituents of proteins involved in plant development and rapid reprogramming of the transcriptome during stress (Reddy and Shad Ali, 2011).

Lignin, a central structural component of the cell wall, has phenylalanine as a vital component. Moreover, phenylalanine, along with tyrosine are precursors of specialized compounds related to plant fitness (Yoo *et al.,* 2013).

Histidine is a known component of metal chelator enzymes in cells and has been observed to increase during heavy metal stress (Sharma and Dietz, 2006).

Threonine, methionine and glycine are essential in the seed development (Jander *et al.*, 2004; Joshi *et al*., 2010). The catabolism of isoleucine leads to the production of cellular energy (Mooney *et al.*, 2002). The above a.a. are usually accumulated during abiotic stress, providing an alternative carbon source (Joshi *et al.*, 2010).

Though several amino acids are major components used for cellular growth and differentiation, proline is distinct. Proline is vital in the recovery of plants from various abiotic stresses, and it is accumulated during adverse environmental conditions (Verslues and Sharma, 2010; Kaur and Asthir, 2015). This a.a. can act as ROS scavenger, osmolyte, metal chelator and signalling molecule, protecting cells from damage originated from abiotic stress

(Szabados and Savouré 2010). Abiotic stresses can cause osmotic stress, increasing the production of ROS beyond normal. Also, antioxidant mechanisms have limited action during stress (Baxter *et al*., 2013). Proline brings ROS concentration to normal ranges by modulating mitochondrial functions, triggering specific gene expression and stabilizing membranes (Kaur and Asthir, 2015). Moreover, the production of proline also enhances the hydrolysis of storage proteins during germination (Copeland and McDonald, 2001).

Proteins are macro-biomolecules composed of a large number of a.a.. These biomolecules can also contain hydrogen, oxygen, carbon, and nitrogen and are involved in a series of metabolic pathways in both humans and plants (Young and Pellett, 1994). Some of these pathways are essential such as the DNA replication and the transport of molecules. Besides, proteins are the building blocks of cell membranes. The biosynthesis of proteins plays a critical role in the energy requirement of plant growth. Plants produce most of its protein content during the day, while at night, the starch degradation restores the low availability of carbon and energy (Amthor, 2000). Abiotic stresses like salinity, heat, cold and drought, lead to the production of different types of proteins to mitigate the adverse effects of these factors (Joshi, 2018). It is known that abiotic stress cause damage to cellular components such as membrane lipids, proteins and nucleic acids (Zhu ,2016). Previous studies reported that proteins could accumulate during heat stress (Gurusinghe *et al.*, 2002). Also, the protein residues can be affected by chemical processes ongoing during stress leading to some proteins being irreversibly damaged, and consequently degraded by proteases, increasing the amount of free a.a. (Joshi *et al.*, 2010). For example, under water stress, proteins accumulate due to abscisic acid stimulating their synthesis (Iqbal *et al.*, 2010). The expression of heat-shock proteins also increases the tolerance to subsequent rises in temperature as sudden differences can lead to cell death (Wahid *et al.*, 2007). Regarding the effects of seed priming in protein content, several authors observed that this treatment increased the total amount of protein (Nouman*, et al.* 2014; Sheteiwy *et al.* 2017; Cao *et al.* 2019; Carvalho *et al.*, 2019). In particular, the priming of bread wheat seeds with 4 mg L^{-1} and 8 mg L^{-1} of Fe and/or Zn allowed an increase of the total soluble protein content in bread the whole wheat flour despite the observation of toxicity at the nucleolar level.

Sugar molecules act as regulators of the plant metabolism, growth and stress responses (Rolland and Sheen, 2005; Rolland *et al.*, 2006; O'Hara *et al.*, 2013). Soluble sugars are involved in the photosynthesis, metabolism of carbohydrates and lipids, protein synthesis and gene expression during abiotic stress (Rosa *et al.*, 2009). Furthermore, during abiotic or biotic

stress, the concentration of soluble sugars increases in plant tissues affected by the accumulation of ROS (Roitsch, 1999, Couée *et al*., 2006). Glucose has been observed to increase proline concentration under salinity (Gadallah, 1999; Hayat *et al*., 2012). Fructose and sucrose are also known to provide membrane protection and scavenge ROS under oxidative-stress (Keunen *et al*., 2013; Singh *et al.*, 2015).

Starch is a simple molecule composed of glucose residues. It functions as the carbohydrate storage in plants, being associated with storage organs such as roots, rhizomes, tubers, stems and seeds (Zeeman *et al*., 2010). Besides acting as a storage molecule, starch is also linked to the mediation of plant abiotic stress responses, improving stress tolerance correlated to starch degradation. When photosynthesis is limited, plants remobilize starch to provide energy and carbon. The degradation of this molecule releases sugars and other metabolites that support plant growth and act as osmoprotectants, mitigating the effects of stress (Krasensky and Jonak, 2012). Additionally, under stress conditions, plants increase starch degradation in the vegetative tissues and remobilize sugars to the seed (Cuellar-Ortiz *et al.*, 2008).

1.6. Intergenerational effects and stress memory

The effects of the environmental stress on a plant offspring have been well known for several years (see Roach and Wulff, 1987; Rossiter, 1996). Under stress, the parental generation alters specific traits in its offspring to enhance their growth, development and success under the same conditions (Herman and Sultan, 2011). The influence of the environmental experiences of a parental generation in its offspring can be defined as an intergenerational effect if extending to the first stress-free generation, or as transgenerational effect if transmitted to at least two stress-free generations (Herman and Sultan, 2011; Lämke and Bäurle, 2017; Wang *et al.*, 2018).

Stress memory is the information retained by a plant after being submitted to a first biotic or abiotic cue, leading to a modified or sustained response upon recurring stress (Fig. 3; Lämke and Bäurle, 2017). This memory usually acts at the phenotypic level and does not introduce changes in DNA sequence in the stressed plant (Hilker *et al.*, 2016). In the past few years, attempts to understand how the stress memory developed by plants exposed to different

abiotic or biotic stimuli affect the next stress-free generation or offspring have been made (Lämke and Bäurle, 2017; Mauch-Mani *et al.*, 2017).

Abiotic stress has effects in the morphological, physiological and metabolic characteristics of plants, influencing traits such as germination, growth, biochemical profile and overall production. However, how these factors influence the same traits in the offspring is not entirely known. Hence, in the last decade, efforts have been made to understand the inter- and transgenerational effects caused by abiotic stress (Lämke and Bäurle, 2017, and references therein; Mauch-Mani *et al.*, 2017).

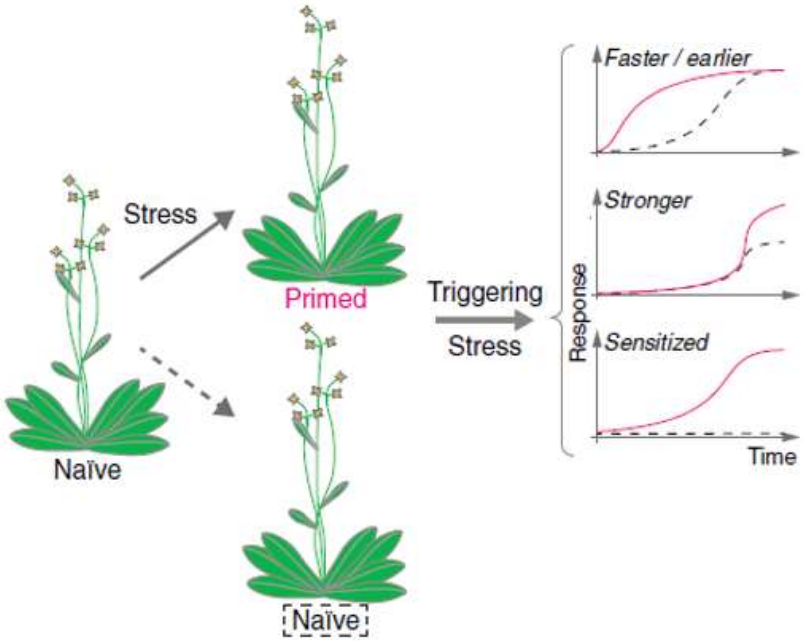

Figure 3. Priming modifies responses to a triggering stress cue. Adapted from Lämke and Bäurle (2017)

The offspring of *Aegilops triuncialis* grown under drought, nutrient stress and exposure to high heavy metal concentrations in the soil has been shown to germinate faster and to flower earlier in comparison with the offspring of plants under normal conditions (Dyer *et al.*, 2010). Similar results concerning nutrient concentration were obtained by Latzel *et al.*, (2010) in other species. The stress caused by drought and extreme temperatures in the parental generation also appears to benefit the development of the next stress-free generation (Mondoni *et al.*, 2014; Lu *et al.*, 2016; Wang *et al.*, 2018). The effects of stress memory have also been related to increases in yield and improvements in germination and seedling growth (Whittle *et al.* 2009; Rajjou *et al.* 2012).

There appears to be a correlation between the stress experienced by the parental generation and the phenotype of the resulting stress-free offspring (Herman and Sultan, 2011).

Probably during the seed maturation, the parental generation prepares the next offspring to the adverse conditions that it is experiencing (Herman and Sultan, 2011).

The maternal plant mobilizes carbohydrates, proteins and nutrients to the developing seed (Aguirre *et al.*, 2018). In certain species, maternal plants can maintain or increase seed provisioning under stress conditions despite producing fewer offspring (Metz *et al.*, 2010; Larios and Venable, 2015). Thus, inter- and transgenerational effects mediate the seed provisioning and can promote offspring success (Herman and Sultan, 2011).

Since seed priming is usually performed in each generation, during the literature review, works related to the knowledge of the effects of nutripriming in the next unprimed offspring were not found.

1.7. Objectives

This works aims to evaluate the effects of seed priming with water (hydropriming) and different concentrations of Fe and/or Zn on the germination, mitosis, biochemical profile and yield-related components of the untreated S1 offspring of the bread wheat cv. ‘Jordão’ by comparison with an untreated offspring (control).

2. Materials and methods

2.1. Plant material and whole wheat flour samples preparation

The 'parental' seeds generation (S0) of bread wheat (*T. aestivum*; AABBDD; 2n=6×=42) cv. 'Jordão' were primed with distilled water (hydropriming) and aqueous solutions with different concentrations of Fe [Iron (II) sulphate heptahydrate, $FeSO_4.7H_2O$] and/or Zn [Zinc sulphate heptahydrate, $ZnSO_4.7H_2O$]. The resulting plants were potted and followed till their maturity by Reis *et al*. (2018).

In this work, we used the first seed generation (S1 seeds) of those plants, particularly, those that resulted from plants whose S0 seeds were hydroprimed and primed with 4 $mg.L^{-1}$ and/or 8 $mg.L^{-1}$ of Fe and/or Zn. The first seed generation of seeds (S1 seeds) derived from the different seed priming treatments will be named through this work as S1 offspring (Table 2). We included an unprimed S1 offspring as control.

Table 2. Plant material used in this work: S1 offspring of unprimed S0 seeds (control) and S1 offspring of S0 seeds that were hydroprimed and nutriprimed with Fe and/or Zn by Reis *et al*. (2018).

S1 offspring of S0 seeds that were:	
Unprimed	Control
Soaked in distilled water	Hydropriming (0 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn)
Soaked in single micronutrient solutions	4 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn
	8 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn
	0 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn
	0 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn
Soaked in double micronutrient solutions	4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn
	8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn

For the analyses of germination (see item 2.4.1) and mitotic cell cycle in chromosome spreads obtained from root-tips (see items 2.4.2 and 2.4.3), a total of 80 S1 seeds (10 seeds per S1 offspring) were used. The resulting plantlets were potted and followed until the plant maturity for the characterization of the yield-related components.

For the biochemical analyses, 24 groups of S1 seeds were prepared, corresponding to three repetitions (n = 3) of seeds of three to five plants from each S1 offspring (Table 2). The overall weight of each group or repetition of S1 seeds ranged from 5 to 19 g.

All S1 seeds were lyophilised at -80ºC and 200mT for 4 days, milled to achieve a fine whole wheat flour and then protected from humidity until the analysis.

2.2. Solvents and chemicals

All chemicals and reagents were of analytical grade unless specified otherwise. Amino acids standards (L-alanine, L-arginine, L-aspartic acid, glycine, L-glutamic acid, L-histidine, L-isoleucine, L-leucine, L-lysine, L-norvaline, L-phenylalanine, L-serine, L-threonine, L-tyrosine, L-tryptophan and L-valine) were purchased from Sigma-Aldrich (Steinheim Germany). 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was acquired from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Acetonitrile (UPLC grade), calcium disodium ethylenediaminetetraacetic acid (EDTA), sodium borate, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Panreac (Castelar del Vallés, Barcelona, Spain). Sodium acetate (anhydrous), triethylamine (TEA), absolute ethanol, glacial acetic acid, carmine and phosphoric acid were purchased from Sigma-Aldrich.

2.3. Biochemical analysis

2.3.1. Analysis of the free amino acids profile by high-performance liquid chromatography with fluorescence detection (HPLC-FLD)

For determination of the free a.a. concentration (except for tryptophan and tyrosine) 25 mg of each whole wheat flour sample was added to 5 ml of 6M HCl and tightly sealed with a cap. The hydrolysis was carried out at 110ºC for 24h, after which the samples were left to cool at room temperature (RT) and adjusted to pH 2.0 with NaOH. To each sample, 1 ml of 5mM of the internal standard L-norvaline solution was added and completed with distilled water till a final volume of 50 ml in a volumetric flask. An aliquot of 1 ml was then filtered through a 0.22 µm filter and stored at 4ºC until initiating the analysis.

For tryptophan and tyrosine, 25 mg of each sample was added to 5 ml of 5M NaOH, and the hydrolysed at 120 ºC for 12 h. The samples were left to cool at RT, and the pH was

adjusted to 2.0 with HCl. To each sample were added 100 µl of tramadol hydrochloride at 500 $\mu g.ml^{-1}$ and distilled water till a final volume of 50 ml within a volumetric flask. An aliquot of 500 µl was filtered through a 0.22 µm filter into an HPLC vial and stored at 4ºC until analysis.

Processed samples and calibration curve standards were prepared according to the pre-column derivatization procedure using AQC, as described by Cohen (2001) with slight modifications. In an HPLC vial insert, 5 µl of sample or standard along with 35 µl of borate buffer mixture (0.2M of sodium borate and 5mM of Calcium disodium EDTA, pH 8.8) and 10 µl of AQC (3 $mg.ml^{-1}$ in acetonitrile) were added and immediately mixed for 30 sec. Vials were tightly capped and stored at 50ºC for 10 min and placed in the autosampler system maintained at 10ºC.

For tyrosine and tryptophan, no derivatization procedure was necessary, and processed samples were directly injected into the HPLC system.

The a.a. separation was carried out on an ACE 5 C 18 column (5 µm, 150 x 4.6 mm i.d., Advanced Chromatography Technologies Ltd., Aberdeen, Scotland). In all a.a., except for tyrosine and tryptophan, a ternary gradient program was employed with the mobile phases being 140mM of Sodium acetate, 17mM of triethylamine, 1mM of EDTA in water, pH 4.95 (phase A), acetonitrile/distilled water (60:40, v/v) (phase B) and water (phase C). The program started at 100% phase A, increasing to 33% phase B and 7% phase C during 40 min; following an increase to 40% phase B and reduction of phase C to 0% during 8 min; and finally increasing to 100% phase B in 0.5 min, and maintained for 5.5 min. The column was re-equilibrated for 10 min among injections. Fluorescence detection occurred at excitation 250 nm and emission 395 nm. For tyrosine and tryptophan, a gradient program consisting of 50 mM NaH_2PO_4 (phase A) and acetonitrile (phase B) was used. The program started at 5% phase B, increasing to 60% phase B during 8 min and maintained for 1 min. The program then returned to the initial conditions in 0.5 min, which were kept for another 3.5 min for column re-equilibration. The injection volume was 5 µl with the column oven set to 40ºC. Fluorescence detection was performed according to a timetable: excitation at 274 nm, emission at 304 nm; changing to excitation at 280 nm and emission at 340 nm at 3 min; changing to excitation at 202 nm and emission at 296 nm at the 5 min mark of the chromatographic run.

The chromatographic analyses were performed on a Thermo Scientific Dionex UltiMate 3000 Series system (Thermo Fisher Scientific, Inc., Waltham, USA), composed by a RS

quaternary pump, a WPS-3000RS autosampler (maintained at 4 ºC), a TCC-3000RS column compartment (maintained at 35 ºC), and a FLD-3400RS fluorescence detector (excitation and emission wavelength were set to 250 and 395 nm, respectively).

Results were interpreted on the Chromeleon software version 7.2 (Thermo Fisher Scientific, Inc., Waltham, USA). The a.a. identification was performed by comparison with authentic standards and quantification according to calibration curves prepared and analysed anew during every day of analysis.

2.3.2. Analysis of soluble sugars by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD)

An amount of 100 mg of each whole wheat flour sample was added to 6 ml of distilled water, and the extraction was performed at 80 ºC for 30 min. Each solution was transferred to a volumetric flask, and the volume was completed to 50 ml with distilled water. An aliquot of 1 ml was filtered through a 0.22 µm filter and stored at 4ºC until analysis.

The soluble sugars separation was achieved in a Dionex CarboPac PA200 (3 x 250 mm i.d.) analytical column (Thermo Fisher Scientific, Inc., Waltham, USA), performed with 30 mM NaOH. To all mobile phases, 2 mM of Barium hydroxide was added to prevent the formation of carbonates, filtered through a 0.22 µm membrane filter and kept under a helium atmosphere (0.3-0.5 bar) during the entire time of analysis. The column oven was kept at 28 ºC, and the injection volume was 5 µl. The soluble sugars were detected by pulsed amperometric detection using a 6041RS amperometric cell with gold working electrode through a quadrupole waveform: +200 mV (500 ms), -2000 mV (10 ms), +600 mV (10 ms) and -100 mV (10 ms). The chromatographic analyses occurred on a Thermo Scientific Dionex UltiMate 3000 Series system (Thermo Fisher Scientific, Inc., Waltham, USA), composed by an LPG-3400RS quaternary pump, a WPS-3000TRS autosampler (maintained at 4 ºC) and an ECD-3000RS electrochemical detector with an incorporated column compartment. Results were interpreted using the Chromeleon software version 7.2 (Thermo Fisher Scientific, Inc., Waltham, USA).

2.3.3. Quantification of ash, crude protein and total starch

The lyophilised whole wheat flour samples were analysed using the official procedures described by the Association of Official Analytical Chemists (AOAC, 1990) for ash (#942.05), crude protein (#954.01) and total starch (#996.11).

For the quantification of ash content (#942.05), porcelain capsules were previously identified and incinerated at 550 ºC for 30 min, following 30 min in an oven at 105 ºC. After cooling in an exsiccator, each capsule weight was registered with and without 1 g of whole wheat flour and left at 105 ºC overnight. Capsules with the sample were weighted the next day to obtain the dry matter (DM) content (g) and submitted to 550 ºC for 3h to register the ash content ($g.Kg^{-1}$ DM) following the equation [Ashes weight (g) / DM (kg)].

Crude protein (CP) was determined following the Kjedahl method, procedure #954.01 of the AOAC (1990). In sum, 0.2 g of the milled sample was dried at 65 ºC and placed in test tubes. A Selenium tablet (99.9% Potassium sulphate and 0.1% Selenium) was added to each tube followed by 5 ml of sulfuric acid. Digestion occurred in a lab digester for 1h at 420 ºC, and test tubes cooled to RT. Distillation was performed by an automatic Kjedahl distiller (UDK 149 Automatic Kjeldahl Distillation Unit, VELP Scientifica, Italy) by adding water, 40% Sodium hydroxide and 4% Boric acid for 4 min and 20 s. The titration process was conducted by an automatic titrator (TITROLINE EASY K automatic titrator, VELP Scientifica, Italy) using sodium tetraborate decahydrate. Crude protein content ($g.Kg^{-1}$ DM) was obtained based on the following equation [(Titration volume × (acid titration value – blank value))*1.4*6.25/ DM (kg)]

For the determination of the total starch content, a Megazyme Total Starch Assay Kit (Product code: K-TSTA-100A, Megazyme) was used with accordance to the procedure described by the AOAC (1990) (#996.11). Briefly, 100 mg of whole wheat flour were weighted to a glass test tube and added 0.2 mL of aqueous ethanol (80% v/v) to wet and aid the dispersion of the sample. There were then stirred, and 3 mL of thermostable α-amylase was added. Incubation took place in a water bath for 12 min, and shaken after 4, 8, and 12 min to ensure complete homogeneity of the slurry, as well as ensuring sample was deposited. After, 0.1mL amyloglucosidase was added tubes stirred and placed in a bath at 50 ºC for 30 min. Contents of each tube were transferred to a 100 mL volumetric flask and a wash bottle used to rinse the whole contents of the tube. Volume was adjusted with distilled water and mixed thoroughly. Aliquots of each sample were centrifuged at 3.000 rpm for 10 min, and

0.1mL of the clear undiluted filtrate was transferred to glass test tubes. 3.0mL of glucose oxidase/peroxidase reagent (GOPOD) was added to each tube and to the D-glucose controls and reagent blanks, followed by incubation at 50 ºC for 20 min.

The absorbance of each sample was read at 510 nm in a spectrophotometer, and the starch concentration obtained by comparison with the D-glucose calibration curve obtained.

2.4. Germination and cytogenetic analyses

2.4.1. Germination, collection and fixation of root-tips

A total of 80 S1 seeds (including the control) were allowed to germinate in Petri dishes containing filter paper moistened with distilled water. The germination occurred at 25 ºC in the dark.

Seeds were monitored daily for a total of 8 days and considered germinated when the radicle presented around 2 mm of length. The percentage of germination (number of germinated seeds/ total number of seeds placed in Petri dishes × 100%) and the mean germination time (MT) (in days) were evaluated.

Root-tips with 1 to 1.5 cm in length were collected and immediately fixed in a solution of absolute ethanol and glacial acetic acid (3:1, v/v) freshly prepared. The fixed root-tips were stored at -20 ºC till the preparation of chromosome spreads.

2.4.2. Preparation of mitotic chromosome spreads

The fixed root tips were stained with 2% of aceto-carmine for 48h at 25 ºC and used for the preparation of chromosome spreads following Lima-Brito *et al.* (1996). After the removal of the glass coverslip, the slides were air-dried and mounted with a drop of VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, Peterborough, UK) and a glass coverslip of 24 × 50 mm.

2.4.3. Cells scoring and images capturing

Three chromosomal preparations of each S1 offspring were observed and scored on the optical microscope. Fifty fields were observed per preparation, and all the cells (interphase nuclei, normal and abnormal cells) of each field were scored. For the dividing cells, all the mitotic phases were also identified. The mitotic index (MI) [number of diving cells/number of counted cells × 100%] was calculated based on the observed results, where the number of counted cells corresponds to the sum of interphase and mitotic cells. The percentage of dividing cells with anomalies (%DCA) [number of dividing cells with anomalies/number of diving cells × 100%] was also determined.

The cell images were captured using an Olympus BX41 microscope (Olympus America, Inc., New York, USA) with a CCD digital camera XC10 (Olympus America, Inc., New York, USA) with the software cellSens (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.5. Characterization of yield-related components

In total, 80 plants (including the control) of the S1 generation were potted and installed at UTAD and further characterized for the following seven yield-related components: (i) number of spikelets of the main spike (NSkMS); (ii) number of seeds of the main spike (NSMS); (iii) number of seeds per spikelet (NSperSk); (iv) weight of the seeds of the main spike (WSMS); (v) tiller number with spike (TN); (vi) number of seeds of the secondary spikes (NSSS); and (vii) weight of seeds of the secondary spikes (WSSS). The same yield-related components were characterized in the S0 plants by Reis *et al*. (2018).

2.6. Statistical analyses

All results are shown as mean ± standard error (S.E.) values per offspring. For the statistical analyses of the cell counting, each preparation was considered a repetition.

For the biochemical analyses, namely for the identification and/or quantification of the free a.a. and soluble sugars, ash, crude protein (CP) and total starch, nine replicates of whole wheat flour samples were used.

One-way analyses of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were performed with the software IBM SPSS Statistics for Windows, Version 20 (IBM Corp., Armonk, NY., USA). The *p*-value significance was set for probabilities lower than 5% ($p < 0.05$).

3. Results

3.1. Identification and quantification of free amino acids

Most of the (untreated) S1 offspring of bread wheat plants whose S0 seeds were primed with distilled water (hydropriming) and solutions with 4 and 8 $mg.L^{-1}$ of Fe and/or Zn showed higher concentrations of each free a.a, when compared to the control offspring (unprimed seeds) (Table 3).

In all S1 offspring, including the control, the most abundant a.a. were the combination of glutamic acid and glutamine that ranged from 0.163 to 0.351 $mmol.g^{-1}$, proline (varying from 0.108 to 0.199 $mmol.g^{-1}$) and glycine (0.105 to 0.135 $mmol.g^{-1}$) (Table 3). On the other hand, a.a. with the lowest average concentrations in all offspring was tryptophan which ranged from 0.008 to 0.010 $mmol.g^{-1}$ (Table 3).

Apart from threonine, the average concentration of all a.a. showed significant differences ($p < 0.05$) among the S1 offspring (Table 3). In particular, the offspring of S0 seeds primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn treatment showed the highest mean values for nine of the sixteen analysed a.a. Six of them (histidine, threonine, valine, isoleucine, leucine and phenylalanine) (Table 3) considered as essential for the human diet (Tessari *et al.*, 2016; Garg *et al.*, 2018).

The S1 offspring of S0 seeds primed with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn showed higher mean values of aspartic acid + asparagine, alanine and lysine when compared to the remaining offspring. In contrast, the offspring of treatments performed just with Fe showed the highest serine concentrations (Table 3). Histidine content was higher in the S1 offspring of the: 8 $mg.L^{-1}$ Zn, 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn, and 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ treatments (Table 3). Tryptophan concentration (0.01 $mmol.g^{-1}$) was higher in the S1 offspring of 4 $mg.L^{-1}$ Zn treatment (Table 3)

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Table 3. Mean (± standard error, SE) concentration of free amino acids (mmol.g^{-1}) determined per S1 (unprimed) offspring. The mean values resulted from nine replicates. Values followed by different lowercase letters are statistically significant different ($p < 0.05$) among the S1 offspring.

S1 offspring of:	Concentration (mmol.g^{-1}) of the amino acids:								
	Aspartic acid + Asparagine	**Serine**	**Glutamic acid + Glutamine**	**Histidine**	**Glycine**	**Arginine**	**Threonine**	**Alanine**	**Proline**
Control	0.068 ± 0.010 a	0.086 ± 0.004 a	0.163 ± 0.003 a	0.017 ± 0.001 a	0.107 ± 0.008 a	0.033 ± 0.002 a	0.040 ± 0.004	0.059 ± 0.003 a	0.108 ± 0.002 a
Hydropriming	0.072 ± 0.011 a	0.089 ± 0.003 a,b	0.161 ± 0.003 a	0.017 ± 0.001 a	0.110 ± 0.009 a,b	0.034 ± 0.002 a	0.042 ± 0.004	0.060 ± 0.003 a,b	0.108 ± 0.002 a
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	0.073 ± 0.005 a,b	0.112 ± 0.006 c	0.233 ± 0.019 b	0.022 ± 0.001 b,c	0.114 ± 0.005 a,b	0.036 ± 0.003 a	0.038 ± 0.002	0.067 ± 0.004 a,b,c	0.142 ± 0.009 b
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	0.078 ± 0.007 a,b	0.117 ± 0.005 c	0.240 ± 0.018 b	0.018 ± 0.001 a,b	0.105 ± 0.004 a	0.034 ± 0.003 a	0.037 ± 0.002	0.072 ± 0.004 a,b,c,d	0.141 ± 0.01 b
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.113 ± 0.014 b,c	0.099 ± 0.003 a,b,c	0.310 ± 0.007 c	0.023 ± 0.001 c,d	0.123 ± 0.004 a,b	0.046 ± 0.001 b	0.042 ± 0.002	0.068 ± 0.003 a,b,c	0.189 ± 0.008 c
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	0.139 ± 0.005 c,d	0.106 ± 0.005 a,b,c	0.351 ± 0.01 c	0.027 ± 0.001 c	0.126 ± 0.005 a,b	0.050 ± 0.002 b	0.041 ± 0.001	0.079 ± 0.003 b,c,d	0.189 ± 0.004 c
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.162 ± 0.012 d	0.111 ± 0.005 c	0.323 ± 0.009 c	0.024 ± 0.001 c,d,e	0.124 ± 0.004 a,b	0.048 ± 0.002 b	0.042 ± 0.002	0.090 ± 0.004 c	0.176 ± 0.001 c
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	0.106 ± 0.007 a,b,c	0.108 ± 0.005 b,c	0.314 ± 0.009 c	0.027 ± 0.001 d,c	0.135 ± 0.004 b	0.051 ± 0.001 b	0.047 ± 0.002	0.081 ± 0.006 c,d	0.199 ± 0.002 c
***p*-value**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.005**	**< 0.001**	**> 0.05**	**< 0.001**	**< 0.001**

Table 3. (continued). Mean (± standard error, SE) concentration of free amino acids (mmol.g^{-1}) determined per S1 (unprimed) offspring. The mean values resulted from nine replicates. Values followed by different lowercase letters are statistically significant different ($p < 0.05$) among the S1 offspring.

S1 offspring of:	Concentration (mmol.g^{-1}) of the amino acids:						
	Valine	**Lysine**	**Isoleucine**	**Leucine**	**Phenylalanine**	**Tyrosine**	**Tryptophan**
Control	0.043 ± 0.002 a	0.029 ± 0.001 a	0.030 ± 0.002 a	0.061 ± 0.003 a	0.037 ± 0.001 a	0.019 ± 0.00 a,b	0.008 ± 0.00 a
Hydropriming	0.044 ± 0.002 a	0.030 ± 0.002 a	0.030 ± 0.002 a	0.062 ± 0.003 a	0.038 ± 0.002 a	0.032 ± 0.001 b,c	0.009 ± 0.00 c
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	0.049 ± 0.003 c,b	0.035 ± 0.003 a,b	0.033 ± 0.002 a,b	0.071 ± 0.005 a,b	0.045 ± 0.003 a	0.025 ± 0.002 a	0.008 ± 0.00 b
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	0.047 ± 0.003 c,b	0.036 ± 0.002 c,b	0.032 ± 0.002 c,b	0.070 ± 0.005 c,b	0.040 ± 0.003 a	0.026 ± 0.002 a,b	0.008 ± 0.00 a
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.051 ± 0.001 a,b	0.038 ± 0.002 a,b,c	0.037 ± 0.001 a,b	0.085 ± 0.003 b,c	0.064 ± 0.003 c	0.034 ± 0.001 c	0.010 ± 0.00 c
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	0.054 ± 0.002 b	0.045 ± 0.002 c,d	0.039 ± 0.001 b	0.086 ± 0.002 c	0.059 ± 0.003 b.c	0.033 ± 0.00 a,b,c	0.009 ± 0.00 c
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.052 ± 0.002 a,b	0.047 ± 0.001 d	0.035 ± 0.001 a,b	0.079 ± 0.002 b,c	0.048 ± 0.001 a,b	0.032 ± 0.001 a,b	0.009 ± 0.00 c
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	0.056 ± 0.002 b	0.040 ± 0.002 b,c,d	0.039 ± 0.001 b	0.087 ± 0.001 c	0.067 ± 0.003 c	0.032 ± 0.001 a,b,c	0.009 ± 0.00 c
***p*-value**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**

3.2. Identification and quantification of soluble sugars

The average concentrations of total sugars showed statistically significant differences ($p < 0.05$) among the different S1 offspring (Table 4).

The highest concentration of total sugars (102.47 $mg.g^{-1}$) was found in the control offspring (Table 4). On the other hand, the lowest total sugars content (69.89 $mg.g^{-1}$) was detected in the S1 offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Zn (Table 4).

Table 4. Mean (± SE) concentration of soluble sugars ($mg.g^{-1}$ sample) determined per S1 (unprimed) offspring. The mean values resulted from nine replicates. Values followed by different lower-case letters are statistically significant different ($p < 0.05$) among the S1 offspring.

S1 offspring of:	Concentration ($mg.g^{-1}$ sample) of the soluble sugars:					
	Glucose	**Sucrose**	**Fructose**	**Raffinose**	**Maltose**	**Total sugars**
Control	1.25 ± 0.11 a	13.99 ± 0.34 c	0.90 ± 0.11 b	3.00 ± 0.08 b,c,d	83.34 ± 2.02 c	102.47 ± 2.18 c
Hydropriming	1.62 ± 0.09 a,b	9.77 ± 0.3 a	0.70 ± 0.04 a,b	2.22 ± 0.11 a	62.2 ± 1.36 a,b,c	76.5 ± 1.62 a,b
4 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn	1.49 ± 0.06 a,b	11.93 ± 0.66 a,b,c	0.71 ± 0.06 a,b	2.66 ± 0.1 a,b,c	76.67 ± 9.32 b,c	93.45 ± 9.78 b,c
8 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn	1.57 ± 0.09 a,b	10.37 ± 0.18 a,b	0.90 ± 0.05 b	3.15 ± 0.09 c,d	67.86 ± 2.41 a,b,c	83.85 ± 2.57 a,b,c
0 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn	1.43 ± 0.08 a,b	10.96 ± 0.63 a,b	0.54 ± 0.03 a	2.47 ± 0.09 a,b	58.37 ± 3.86 a,b	73.76 ± 4.24 a,b
0 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn	1.72 ± 0.10 a,b	11.02 ± 0.67 a,b	0.53 ± 0.04 a	2.61 ± 0.25 a,b,c	54.02 ± 3.86 a	69.89 ± 4.01 a
4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn	1.91 ± 0.27 b	11.11 ± 0.52 a,b	0.77 ± 0.15 a,b	2.63 ± 0.22 a,b,c	68.86 ± 7.46 a,b,c	85.26 ± 7.3 a,b,c
8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn	1.66 ± 0.07 a,b	12.18 ± 0.41 b,c	0.69 ± 0.02 a,b	3.35 ± 0.15 d	53.3 ± 3.77 a	71.18 ± 3.91 a,b
***p*-value**	**< 0.05**	**< 0.001**	**< 0.05**	**< 0.001**	**< 0.05**	**< 0.001**

Glucose increased in all S1 offspring relative to control, being significantly higher ($p < 0.05$) than the control in the S1 offspring of 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn (1.91 $mg.g^{-1}$) (Table 4). Relative to the other sugars, in few S1 offspring was detected a significant decrease ($p < 0.05$) in comparison with the control (Table 4). The lowest mean values of sucrose and raffinose were observed in the S1 offspring of hydropriming (9.77 $mg.g^{-1}$ and 2.22 $mg.g^{-1}$, respectively) (Table 4). The lowest average value of fructose was observed in the S1 offspring of 8 $mg.L^{-1}$

Zn (0.53 mg.g^{-1}) and the lowest of maltose in the offspring of 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn (53.3 mg.g^{-1}) (Table 4). Although the S1 offspring of 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn and 8 mg.L^{-1} Fe presented higher average concentrations of raffinose (3.35 mg.g^{-1} and 3.15 mg.g^{-1}, respectively) than the control, no statistically significant differences ($p > 0.05$) among these offspring were found (Table 4).

3.3. Determination and quantification of ash, crude protein and total starch

The average concentration of ash, crude protein (CP) and total starch showed significant differences ($p < 0.05$) among the studied S1 offspring (Table 4).

For ash, the lowest average concentration was observed in the control offspring (18.0 g.kg^{-1}) and the highest one in the S1 of 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn (25.2 g.kg^{-1}) (Table 5).

The lowest average CP content was found in the control offspring (120.6 g.kg^{-1}) whereas the highest was detected in the S1 of 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn (209.7 g.kg^{-1}), and 8 mg.l^{-1} Zn (206.7 g.kg^{-1}) (Table 5).

Table 5. Mean (± SE) concentration (g.Kg-1 dry matter, DM) of ash, crude protein and total starch determined per S1 (unprimed) offspring. The mean values resulted from nine replicates. Values followed by different lowercase letters are statistically significant different ($p < 0.05$) among the S1 offspring.

S1 offspring of:	**Concentration (g.Kg^{-1} DM) of:**		
	Ash	**Crude protein**	**Total starch**
Control	18.00 ±0.77 a	120.60 ±0.70 a	628.40 ±5.50 d
Hydropriming	22.90 ±0.65 b	188.80 ±11.80 b,c,d	546.60 ±6.20 a,b
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	23.10 ±1.10 b	145.50 ±10.30 a,b	594.60 ±13.30 c,d
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	22.60 ±1.45 b	157.70 ±13.10 a,b,c	630.10 ±15.90 d
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	24.40 ±0.42 b	201.40 ±5.40 c,d	560.90 ±7.90 b,c
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	24.90 ±0.24 b	206.70 ±8.80 d	537.90 ±13.70 a,b
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	22.50 ±0.36 b	187.90 ±10.60 b,c,d	537.40 ±8.00 a,b
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	25.20 ±0.23 b	209.70 ±11.20 d	512.80 ±7.80 a
***p*-value**	**< 0.001**	**< 0.001**	**< 0.001**

The highest average concentration of total starch was found in the S1 offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Fe (630.1 $g.kg^{-1}$) and in control (628.4 $g.kg^{-1}$) (Table 5). The lowest average concentration of total starch was found in the S1 offspring of plants resulting from seed priming with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (512.8 $g.kg^{-1}$) (Table 5).

3.4. Analysis of the mean germination time

A germination percentage of 100% was observed in all S1 offspring seeds.

The S1 offspring of plants whose seeds were primed with 4 $mg.L^{-1}$ Fe, 4 $mg.L^{-1}$ Zn, 8 $mg.L^{-1}$ Zn and 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn showed the lowest mean germination time (MT = 2.0 days) (Table 6).

Table 6. Mean germination time (MT) determined per S1 (unprimed) offspring that resulted from different priming treatments performed in the S0 seeds (indicated in table) and unprimed seeds (control). The mean values are representative of 10 seeds. Values followed by different lower-case letters are statistically significant different ($p < 0.05$) among offspring

S1 offspring of:	**MT (days) (Mean ± SE)**
Control	2.70 ± 0.38 a
Hydropriming	2.40 ± 0.29 a
4 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn	2.00 ± 0.00 a
8 $mg.L^{-1}$ Fe + 0 $mg.L^{-1}$ Zn	2.10 ± 0.09 a
0 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn	2.00 ± 0.00 a
0 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn	2.00 ± 0.00 a
4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn	2.00 ± 0.00 a
8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn	7.20 ± 1.08 b
***p* value**	**< 0.001**

Although the hydroprimed and unprimed seeds (control) revealed higher MT values (2.4 and 2.7 days, respectively) than the previous ones, only the S1 offspring of 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn showed a significantly higher MT value ($p < 0.001$) that showed statistically significant differences relative to the remaining offspring, including the control (Table 6).

3.5. Evaluation of the mitotic cell cycle

A total of 25,742 cells were counted in this work, with an average of 3,218 cells per S1 offspring. Normal and abnormal dividing cells in different mitotic phases were scored in all S1 descendants (Tables 7 and 8).

Table 7. Total number of counted (interphase + mitotic) cells and normal dividing cells, values of mitotic index (MI) presented as mean (± SE) and percentage of normal dividing cells in different mitotic phases determined per S1 (unprimed) offspring that resulted from various priming treatments performed in the S0 seeds (indicated in table) and unprimed seeds (control). The mean values resulted from the score of three chromosomal preparations per S1 offspring. Values followed by different lower-case letters are statistically significant different ($p < 0.05$) among offspring.

S1 offspring of:	Counted cells	Normal dividing cells	MI (%) (Mean ± SE)	Mean percentage ± SE of normal dividing cells in:			
				Prophase	Metaphase	Anaphase	Telophase
Control	3041	1601	52.06 ± 1.39 b	89.24 ± 1.57 a,b	7.75 ± 1.40 b,c	1.58 ± 0.20 a	1.16 ± 0.08
Hydropriming	3246	1592	49.07 ± 0.42 a,b	87.9 ± 0.42 a,b	5.09 ± 0.03 a,b,c	4.1 ± 0.18 b	1.59 ± 0.58
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	2718	1277	46.98 ± 0.38 a,b	91.64 ± 0.82 b	3.44 ± 0.27 a,b	1.6 ± 0.25 a	1.29 ± 0.44
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	3911	1730	44.27 ± 1.02 a	88.93 ± 0.52 a,b	4.17 ± 0.18 a,b,c	2.32 ± 0.28 a,b	1.83 ± 0.37
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	3885	1871	48.16 ± 0.48 a,b	91.45 ± 1.03 b	4.05 ± 1.02 a,b,c	1.84 ± 0.17 a	0.91 ± 0.04
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	2744	1258	45.98 ± 1.48 a	87.37 ± 0.40 a,b	5.41 ± 0.64 a,b,c	2.38 ± 0.11 a,b	1.67 ± 0.12
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	3164	1529	48.35 ± 0.18 a,b	86.05 ± 1.00 a	9.48 ± 2.06 c	1.81 ± 0.68 a	1.24 ± 0.36
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	3033	1400	46.14 ± 0.64 a	90.53 ± 0.60 a,b	1.92 ± 0.22 a	2.77 ± 0.36 a,b	1.16 ± 0.28
***p* value**	-	-	**< 0.05**	**< 0.05**	**< 0.05**	**< 0.05**	**0.764**

The highest mitotic index (MI) value was found in the control offspring (52.06%) and the lowest one in the S1 offspring of plants whose S0 seeds were primed with 8 mg.L^{-1} Zn (44.27%) (Table 7). Statistically significant differences ($p < 0.05$) for the MI values were detected between the control and the S1 offspring of plants that resulted from seed priming with 8 mg.L^{-1} Fe, 8 mg.L^{-1} Zn and 8 mg.L^{-1} Zn + 8 mg.L^{-1} Fe (Table 7).

The average percentage of normal diving cells was determined according to the mitotic phase. Among the S1 offspring, we found statistically significant differences ($p< 0.05$) for prophase, metaphase and anaphase cells (Table 7).

In all S1 offspring, most of the normal dividing cells were in prophase (Fig. 4a). Its highest value was observed in the offspring of plants that resulted from seed priming with 4 $mg.L^{-1}$ Fe (91.64%) whereas the lowest one was detected in the S1 offspring of 4 $mg.L^{-1}$ Zn + 4 $mg.L^{-1}$ Fe (86.05%) (Table 7). The lowest average percentage of normal metaphase cells (Fig. 4c) was found in the S1 offspring of plants derived from seed priming with 8 $mg.L^{-1}$ Zn + 8 $mg.L^{-1}$ Fe (1.92%) and the highest value in the S1 offspring of S0 seeds primed with 4 $mg.L^{-1}$ Zn + 4 $mg.L^{-1}$ Fe (9.48%) (Table 7). The highest average percentage of normal anaphase cells (Fig. 4f) was found in the S1 offspring of hydropriming (4.1%), and the lowest value was observed in control (1.58%) (Table 7). The average percentage of normal telophases (Fig. 4i) show no statistically significant differences ($p = 0.764$) among S1 offspring. Its highest value was found in the offspring of S0 plants resulting from nutripriming with 8 $mg.L^{-1}$ Fe (1.83%) while the lowest was detected in the S1 offspring of plants treated with 4 $mg.L^{-1}$ Zn (0.91%) (Table 7).

Different irregularities were found in the dividing cells (Fig. 4b, d, e, g, h and j; Table 8).

The abnormal prophase cells showed a single type of irregularity which was chromatin stickiness (Fig. 4b). The irregular metaphases presented mitotic spindle disturbance (Fig. 4e) and chromatin stickiness (Fig. 4d). Chromatin stickiness was also observed in both anaphase (Fig. 4h) and telophase irregular cells (Fig. 4j). Some irregular anaphases showed chromatin bridges (Fig. 4g).

Most of the S1 offspring showed prophase, metaphase and anaphase cells with irregularities (Table 8). However, irregular telophase cells were only found in the S1 offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (Table 8).

The average percentage of irregular cells in prophase and metaphase showed statistically significant differences ($p < 0.001$) among the S1 offspring (Table 8). However, no significant differences ($p > 0.05$) were found for the average percentage of anaphase and telophase cells among the S1 offspring (Table 8).

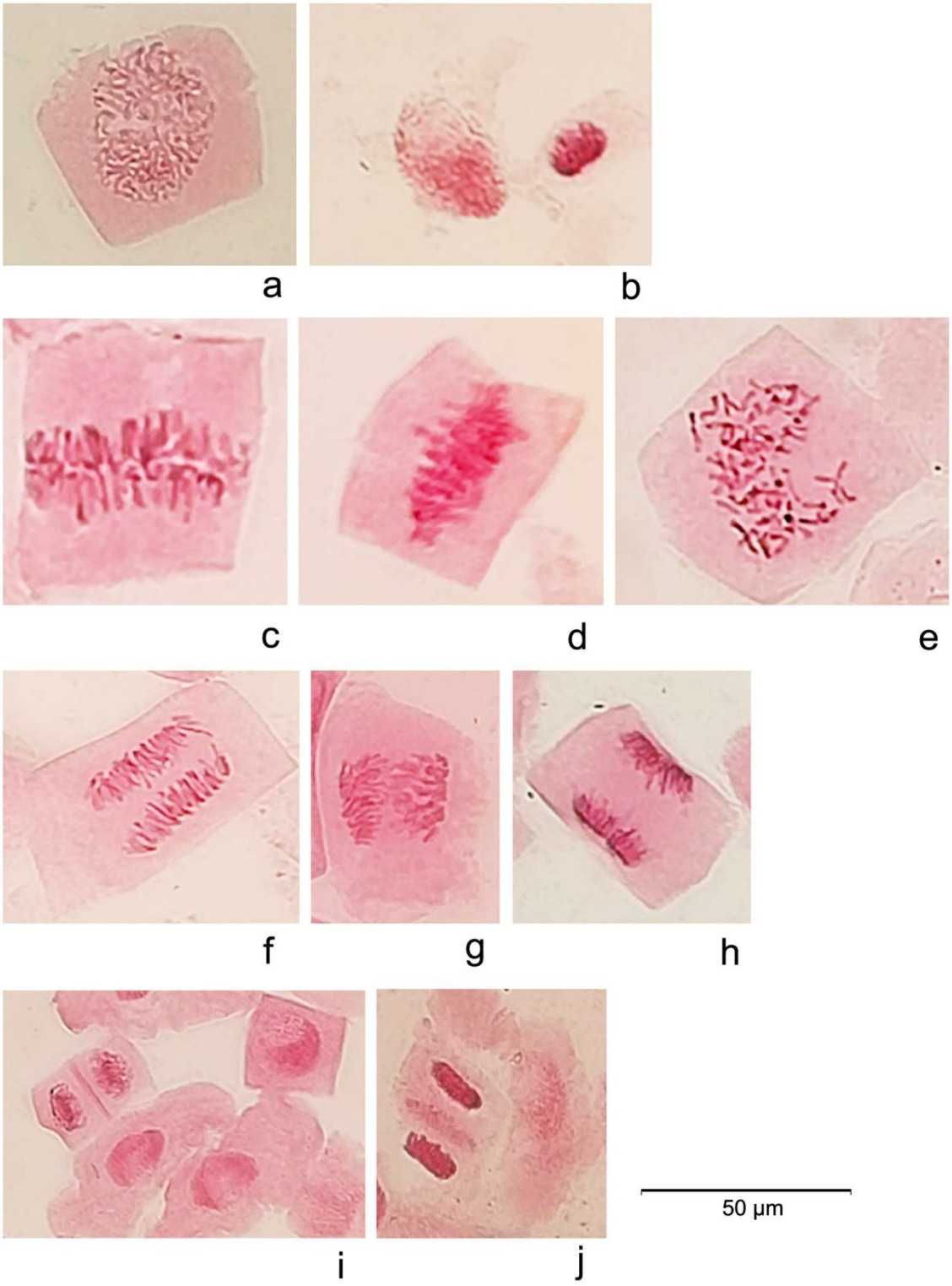

Figure 4. Normal (a, c, f, i) and abnormal (b, d, e, g, h, j) dividing cells in different mitotic phases observed in one or various S1 offspring. (a) Normal (late) prophase; (b) early prophase (at left) and sticky prophase (at right); (c) normal metaphase; (d) sticky metaphase; (e) C-metaphase; (f) normal anaphase; (g) anaphase with chromatin bridges; (h) sticky anaphase; (i) normal telophase and (j) sticky telophase.

Chromatin stickiness was detected in irregular prophase and metaphase cells except for the control S1 offspring that did not show sticky prophases (Table 8). Sticky anaphases and telophases were only found in the S1 offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (Table 8). The chromatin stickiness was the only type of irregularity found in both prophase and telophase (Table 8). The average percentage of sticky prophase cells in the S1 offspring analysed here increased with the augment of Fe, Zn, and Fe + Zn concentrations (Table 8). Hence, the highest average percentage of sticky prophases was found in the S1 offspring of plants resulting from seed priming with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (2.51%) (Table 8).

The irregular metaphase cells showed chromatin stickiness as well as mitotic spindle disturbance that generated C-metaphases (Table 8). The lowest average of sticky metaphases was observed in the control S1 offspring (0.71%) whereas the highest value was registered in the S1 offspring of plants resulting from S0 primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (31.75%) (Table 8). Therefore, the average of sticky and/or disturbed metaphases differed significantly ($p < 0.001$) among the S1 offspring (Table 8). The mean percentage of C-metaphases presented no statistically significant differences ($p = 0.225$) among the S1 offspring (Table 8). The highest average value of C-metaphases (30.16%) was detected in the S1 offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (Table 8).

The irregular anaphase cells showed chromatin bridges that were detected in six of the eight S1 offspring analysed here. Chromatin stickiness was only found in the offspring of plants whose S0 seeds were primed with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (Table 8). The average percentage of abnormal anaphase cells with stickiness or chromatin bridges did not present significant differences among the S1 offspring ($p > 0.05$) (Table 8). The same was verified for the sticky telophases (Table 8).

The lowest average percentage of dividing cells with anomalies (% DCA) were found in control (0.27%) whereas the highest one was detected in the S1 offspring of plants resulting from 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn (3.62%; Table 8). Moreover, the average % DCA determined per S1 offspring evidenced an increase with the augment of Fe, Zn, Fe + Zn (Table 8). The average % DCA showed statistically significant differences ($p < 0.001$) among the S1 offspring (Table 8).

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Table 8. Mean ± SE percentage values of abnormal cells in different mitotic phases, with particular irregularities per mitotic phase, and percentage of diving cells with anomalies (% DCA) per S1 (unprimed) offspring that resulted from various priming treatments performed in the S0 seeds (indicated in table) and unprimed seeds (control). The mean values resulted from the score of three chromosomal preparations per S1 offspring. Values followed by different lower-case letters are statistically significant different ($p < 0.05$) among offspring

S1 offspring of:	Mean percentage (± SE) of abnormal dividing cells in:				Mean percentage (± SE) of abnormal dividing cells with the following irregularities:						% DCA (Mean ± SE)
	Prophase	Metaphase	Anaphase	Telophase	Sticky prophase	Sticky and/or disturbed metaphase	C-metaphase	Sticky anaphase	Bridges in anaphase	Sticky telophase	
Control	0.00 ± 0.00 a	4.10 ± 1.25 a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 a	0.71 ± 0.58 a	3.39 ± 1.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.27 ± 0.06 a
Hydropriming	0.49 ± 0.11 a,b	17.34 ± 2.06 a	0.00 ± 0.00	0.00 ± 0.00	0.49 ± 0.11 a,b	9.73 ± 1.66 a,b	7.60 ± 3.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.32 ± 0.17 a,b
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	0.76 ± 0.40 a,b,c	30.32 ± 2.25 a,b	20.00 ± 9.43	0.00 ± 0.00	0.76 ± 0.4 a,b,c	18.57 ± 1.78 b	11.75 ± 2.07	0.00 ± 0.00	20.00 ± 9.43	0.00 ± 0.00	2.03 ± 0.43 b,c,d
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	1.95 ± 0.15 b,c,d	19.68 ± 1.85 a	8.63 ± 4.85	0.00 ± 0.00	1.95 ± 0.15 b,c,d	10.97 ± 1.84 a,b	8.71 ± 2.48	0.00 ± 0.00	8.63 ± 4.85	0.00 ± 0.00	2.75 ± 0.23 b,c,d
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.97 ± 0.29 a,b,c	23.69 ± 10.75 a	11.67 ± 2.08	0.00 ± 0.00	0.97 ± 0.29 a,b,c	10.33 ± 4.02 a,b	13.36 ± 6.91	0.00 ± 0.00	11.67 ± 2.08	0.00 ± 0.00	1.74 ± 0.18 a,b,c
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	2.09 ± 0.32 c,d	20.47 ± 5.25 a	6.73 ± 2.79	0.00 ± 0.00	2.09 ± 0.32 c,d	7.33 ± 0.95 a,b	13.15 ± 5.99	0.00 ± 0.00	6.73 ± 2.79	0.00 ± 0.00	3.01 ± 0.49 c,d
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	0.64 ± 0.14 a,b,c	7.85 ± 0.97 a	3.52 ± 2.03	0.00 ± 0.00	0.64 ± 0.14 a,b,c	3.59 ± 0.21 a	4.27 ± 0.76	0.00 ± 0.00	4.74 ± 2.02	0.00 ± 0.00	1.42 ± 0.14 a,b
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	2.51 ± 0.29 d	61.9 ± 9.72 b	5.66 ± 2.62	4.17 ± 3.40	2.51 ± 0.29 d	31.75 ± 4.14 c	30.16 ± 11.7	3.70 ± 3.02	1.96 ± 1.60	4.17 ± 3.40	3.62 ± 0.32 d
***p* value**	**< 0.001**	**< 0.001**	**0.175**	**0.466**	**< 0.001**	**< 0.001**	**0.225**	**0.466**	**0.142**	**0.466**	**< 0.001**

3.6. Yield-related components characterization

Seven yield-related components were characterized in the mature S1 offspring plants, including the control (Table 9).

Table 9. Mean ± SE values of yield-related components characterized per S1 offspring that resulted from various priming treatments performed in the S0 seeds (indicated in table) and unprimed seeds (control). The mean values resulted from the characterization of 10 adult plants per S1 offspring. Values followed by different lower-case letters are statistically significant different ($p < 0.05$) among offspring.
Note: NSkMS - number of spikelets of the main spike; NSMS - number of seeds of the main spike; NSperSk - number of seeds per spikelet; WSMS -weight of seeds of the main spike; NT – number of tillers with spike; NSSS - number of seeds of the secondary spikes; WSSS -weight of seeds of the secondary spikes; TNS – total number of seeds (sum of the main spike seeds with seeds of the secondary spikes).

S1 offspring of:	NSkMS	NSMS	NSperSk	WSMS (g)	NT	NSSS	WSSS (g)	TNS
Control	18.40 ± 0.38 a	54.60 ± 2.84 a	2.98 ± 0.18	1.48 ± 0.16 a	9.10 ± 1.20	283.80 ± 43.48	5.71 ± 1.08 a	338.4 ± 45.70 a
Hydropriming	20.00 ± 0.49 a,b	71.60 ± 3.97 b,c,d	3.58 ± 0.18	2.27 ± 0.18 a,b,c	9.40 ± 0.70	382.60 ± 35.69	10.56 ± 1.19 a,b	454.2 ± 39.50 a,b
4 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	20.80 ± 0.31 b	65.90 ± 3.87 a,b,c	3.15 ± 0.15	1.82 ± 0.26 a,b	10.10 ± 0.96	365.90 ± 41.13	6.87 ± 0.9 a,b	431.8 ± 39.50 a,b
8 mg.L^{-1} Fe + 0 mg.L^{-1} Zn	23.10 ± 0.54 c	76.20 ± 3.73 c,d	3.33 ± 0.20	2.86 ± 0.11 c	11.90 ± 0.97	516.10 ± 64.01	12.51 ± 1.51 b	592.3 ± 67.84 a,b
0 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	18.50 ± 0.25 a	61.80 ± 2.56 a,b,c	3.34 ± 0.13	2.12 ± 0.17 a,b,c	12.40 ± 1.10	450.40 ± 50.26	8.79 ± 1.17 a,b	512.2 ± 54.11 a,b
0 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	23.40 ± 0.49 c	83.40 ± 2.73 d	3.58 ± 0.15	2.87 ± 0.20 c	9.40 ± 1.29	470.80 ± 92.01	11.4 ± 2.32 a,b	554.2 ± 98.96 a,b
4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn	18.00 ± 0.28 a	58.80 ± 3.96 a,b	3.26 ± 0.21	2.03 ± 0.21 a,b,c	10.90 ± 0.93	376.50 ± 41.28	7.53 ± 0.89 a,b	435.3 ± 45.06 a,b
8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn	21.60 ± 0.62 b,c	76.60 ± 3.09 c,d	3.54 ± 0.11	2.65 ± 0.20 b,c	11.90 ± 1.11	544.60 ± 73.19	11.19 ± 1.65 a,b	621.2 ± 79.14 b
***p* value**	**< 0.001**	**< 0.001**	**0.141**	**< 0.001**	**0.214**	**0.064**	**< 0.05**	**< 0.05**

In all S1 offspring, the average values of the seven yield-related components were higher than the control except for the NSkMS (Table 9).

Most of the yield-related components, except NSperSK, NT and NSSS, showed statistically significant differences ($p < 0.05$) among the S1 offspring.

The S1 offspring of nutripriming with 8 mg.L^{-1} Zn showed the highest mean values of four yield-related components, namely, NSkMS, NSMS, NSperSK and WSMS (Table 9).

The highest mean values of NSkMS were found in the S1 offspring of S0 seeds primed with 8 mg.L^{-1} Zn (23.4) and 8 mg.L^{-1} Fe (23.1), while the lowest means were

observed in the 4 mg.L^{-1} Fe + 4 mg.L^{-1} Zn, and in control (Table 9). Regarding the yield-related component NSMS, the highest average value was detected in the S1 offspring of S0 seeds treated with 8 mg.L^{-1} Zn (83.4) and the lowest mean value was observed in control (54.6) (Table 9). For the yield-related component NSperSK, there were no significant differences among the S1 offspring (p = 0.141). All the S1 offspring showed higher values than the control. The highest mean values (3.58) for the NSperSK were found in the offspring of S0 seeds hydroprimed and primed with 8 mg.L^{-1} Zn while the lowest one was detected in control (Table 9).

Relatively to the WSMS parameter, the highest mean value was observed in the S1 offspring of 8 mg.L^{-1} Zn (2.87 g) whereas the lowest mean value (1.48 g) was found in control (Table 9). The lowest mean value of TN was found in control (9.1) while the highest average was observed in the S1 offspring of 4 mg.L^{-1} Zn (12.4) (Table 9). Concerning the NSSS, the lowest average value was detected in control (283.8), whereas the highest one was found in the S1 offspring of 8 mg.L^{-1} Zn + 8 mg.L^{-1} Fe (544.6) (Table 9). The lowest mean value of WSSS was detected in control (5.71 g) and the highest one in the S1 offspring of 8 mg.L^{-1} Fe (12.51 g) (Table 9).

The average of TNS was higher in all S1 offspring relative to the control (338.4), but it was only significantly higher ($p < 0.05$) in the offspring of 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn (621.2) (Table 9).

4. Discussion

Hidden hunger is a global issue caused by micronutrient deficiencies. Cereal crops, such as wheat are the most consumed food by humans [2]. However, some wheat cultivars have low contents of Fe and Zn, leading to several problems in human health, especially in developing countries (Dimkpa and Bindraban, 2016). To overcome this problem, the scientific community and plant breeders have been studying and developing approaches to improve the nutritional quality of wheat and other crops (Garg *et al*., 2018; Venske *et al*., 2019).

Agronomic biofortification aims not only at the increase of micronutrients availability in the soil but also to improve the concentration of essential micronutrients in the edible parts of the plant (Singh *et al*., 2016). Seed priming has been considered one of the most suited, easy and affordable agronomic biofortification strategies to overcome the micronutrients deficiency in crops (Harris *et al*., 2008; Rehman *et al*. 2018). However, there is an absence of information related to the seed priming effects in the offspring of the primed plants, as seed priming is commonly performed in each generation. Moreover, if done with excessive dosages, seed priming cause adverse effects in the germination, mitotic cell cycle, and yield-related components, acting as abiotic stress (Gill *et al*. 2016, Reis *et al.*, 2018). It is known that the environment surrounding plants influence their offspring, but the underlying mechanisms are far from being wholly understood (Herman and Sultan, 2011; Lämke and Bäurle, 2017; Wang *et al.*, 2018).

This work was performed with S1 unprimed offspring of S0 plants that resulted from hydropriming and nutripriming with 4 and 8 $mg.L^{-1}$ of Fe and/or Zn and which were characterized at the germination, cytogenetic, and yield-related levels (Reis *et al*., 2018). These authors verified that concentrations of Fe and/or Zn above 2 $mg.L^{-1}$ were cytotoxic and negatively affected the germination and yield-related components. Specifically, priming treatments with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn, and 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn showed the highest values of %DCA and allowed nucleolar stress (Reis *et al*., 2018; Carvalho *et al*., 2019). However, the whole wheat flour samples of the S0 seeds primed with the previously mentioned treatments presented a higher amount of total soluble proteins than that resulting from hydropriming (Carvalho *et al*., 2019). The determination of the total protein content was the single biochemical characterization performed in the S0 generation. Nonetheless,

regarding the benefits in terms of yield-related components observed in the S0 plants primed with Fe and/or Zn concentrations up to 2 $mg.L^{-1}$ (Reis *et al*., 2018), and the high total protein content in S0 seeds primed with higher concentrations, allowed us to deeply explore the biochemical benefits of seed priming by including in the present work, an unprimed S1 offspring as control. Hence, in this work along with the evaluation of the effects of hydropriming and nutripriming with 4 and/or 8 $mg.L^{-1}$ of Fe and/or Zn on germination, mitosis and yield-related components in their respective unprimed S1 offspring, we also characterized their biochemical profile in whole wheat flour samples in relation to free a.a., soluble sugars, ash, CP and total starch, by comparison with unprimed seeds (control), and the results achieved by Reis *et al.* (2018) in the S0 parental generation.

4.1. Free amino acids profile and protein content

The a.a. have different roles in plants, including their use as building blocks of proteins, involvement in signalling processes and plant stress response (Zafar *et al.*, 2014; Hildebrandt *et al.*, 2015; Parlak, 2016; Zemanová *et al*., 2017). Additionally, the a.a. influence cellular reactions, physiological and metabolic processes (Hildebrandt *et al.*, 2015). The essential a.a. are required in adequate amounts in the daily diet because they are not synthesised by animals (Anjum *et al.*, 2005).

In the whole wheat flour samples analysed in this work, 16 free a.a. were identified. Eight of them (histidine, threonine, valine, lysine, isoleucine, leucine, phenylalanine and tryptophan) are considered essential for the human diet (Tessari *et al.*, 2016; Garg *et al.*, 2018). Besides, the S1 offspring of plants derived from seed priming with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn presented high concentrations of six of this eight essential a.a., namely, histidine, threonine, valine, isoleucine, leucine and phenylalanine.

The free a.a. composition in the whole wheat flour or white flour differs among bread and durum wheat varieties, but the high content of glutamic acid and/or glutamine is common among them (McDermott and Pace, 1957; García del Moral *et al*., 2007; Zafar *et al.*, 2014). As demonstrated here, the cv. ‘Jordão’ is not an exception. The same feature was noticed in the control offspring. Despite a non-significant decrease in the S1 offspring of hydropriming, the combination of glutamic acid and glutamine increased in the remaining S1 offspring resulting from the nutripriming performed with Fe and/or Zn in the S0 generation.

Glutamic acid is synthesised from α-ketoglutarate and other a.a. like ornithine, proline, arginine and glutamine (Zafar *et al*., 2014). Hence, the high levels of proline and glutamine contributed to the high glutamic acid found in the whole wheat flour samples analysed in this work. Also, proline and glutamine are the functional a.a. for dough formation (Zafar *et al*., 2014). The bread wheat cv. 'Jordão' analysed here belongs to the Portuguese Catalogue of Varieties since 1996, and it has been reported as high-quality wheat for baking (Catálogo Nacional de Variedades, 2018).

An important feature related to the baking use of the wheat flour is the amount of free asparagine and its role in the formation of the carcinogenic acrylamide during high-temperature cooking and processing (Sofo *et al*. 2018). The concentration of free asparagine in plants may vary with micronutrient availability, environment, genotype, and crop management or can be accumulated to high concentrations (along with proline and glycine betaine) in response to abiotic (salinity and drought) and biotic stresses, influencing the crop yield (Curtis *et al.*, 2018; Wang *et al*., 2018; Sofo *et al*., 2018). In the present study, all S1 offspring showed an increase in the average concentration of the combination of aspartic acid and asparagine. Nevertheless, only those resulting from plants whose S0 seeds were primed with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn and with Zn alone differed from the control. Curtis *et al.* (2018) reported that asparagine could become the predominant a.a. in cereal grains under some stress conditions.

Nonetheless, in this work, the combination of aspartic acid and asparagine was not among the more abundant free a.a. quantified in any of the analysed whole wheat flour samples. Asparagine and aspartic acid also have beneficial effects being associated with nitrogen transport and recycling, storage and metabolism in plants (Herrera-Rodríguez *et al.*, 2007; Lea *et al*., 2007; Gaufichon *et al.*, 2010; Gao *et al.*, 2016).

Other a.a., like serine, threonine, arginine, glutamine, histidine, glycine, isoleucine, leucine and tryptophan are involved in crucial pathways. Furthermore, these a.a. are also constituents of proteins and enzymes that are upregulated during stress (País *et al*., 2009; Wang *et al.*, 2010; Joshi *et al.*, 2010; Reddy and Shad Ali, 2011; Zemanová *et al.*, 2014; Kumar and Verslues, 2015; Kan *et al.*, 2015).

Leucine is one of the essential branched-chain a.a., along with valine and isoleucine. It promotes energy metabolism (glucose uptake, mitochondrial biogenesis and fatty acids oxidation), improving protein synthesis and inhibiting protein degradation in mammals (Duan

et al., 2016). Leucine, isoleucine and valine significantly increased in all S1 offspring relative to the control.

The a.a. content highly determines the nutritional value and quality of the wheat grain profile and protein content, but environmental conditions significantly affect these features as well as the grain production (Zafar *et al*., 2014). High temperature and drought shorten the duration of grain filling period and influence the a.a. composition by increasing the content of phenylalanine, glutamine and proline, and decreasing other a.a. due to the accumulation of gliadins, albumins and globulins (Zafar *et al*., 2014). The storage proteins gliadins are rich in glutamine and proline. They are accumulated in detriment of albumins and globulins that have structural and metabolic roles and are rich in threonine, lysine, methionine, valine and histidine (Zafar *et al*., 2014). The albumins and globulins accumulate early during the grain growth when the endosperm cells are still dividing whereas the amount of storage proteins increases in a later stage when the cell division stops (Triboi *et al.*, 2003; Martre *et al.*, 2003). This assumption can also explain the higher amounts of glutamine and proline found in the whole wheat flour samples analysed here and which were obtained from mature grain.

Arginine, phenylalanine, glycine and aspartic acid are less prone to variation among environments than tyrosine, lysine, threonine and valine (Zafar *et al*., 2014).

Proline which is one of the most studied a.a. in plants under stress, was also among the most abundant free a.a. detected in the flour samples analysed in this work. Proline accumulates in plants under biotic and abiotic stresses (osmotic, high salt concentrations, heat, drought and exposure to heavy metals) to protect the cell by decreasing the lipid peroxidation, improving the stability of the membrane, proteins and enzymes, and rising the activity of proteases (Khan *et al.*, 2009; Verslues and Sharma, 2010; Hayat *et al*., 2012; Liang *et al*., 2013; Kaur and Asthir, 2015; Anand *et al*., 2017). Proline accumulation improves the plant tolerance to stress but may decline the protein content (Anand *et al.*, 2017). Other authors reported a negative correlation between the content of essential a.a. and the percentage of protein under abiotic stress (García del Moral *et al.*, 2007; Zafar *et al.*, 2014). Nevertheless, in this work, all S1 offspring showed higher content of all a.a. (including proline and nine essential ones) and CP than the control.

Previously, the S0 generation was only analysed in terms of total soluble proteins amount for integration with the nucleolar activity data, and an increase of protein content was detected in seeds primed with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn, 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn and 8 $mg.L^{-1}$ Zn, relative to the hydroprimed seeds (Carvalho *et al.*, 2019). Also, the lowest average

value of protein content was found in S0 seeds primed with 8 $mg.L^{-1}$ Fe but did not differ statistically from the hydroprimed seeds (Carvalho *et al.*, 2019). Similar results were found hereupon comparison with the S1 offspring of those hydroprimed seeds. In the present work, due to the inclusion of unprimed seeds as control, it was noticed that the S1 offspring of hydroprimed seeds presented higher CP content than the control.

Hydropriming induces the secretion of the gibberellin hormone by the embryo and its transference to the aleurone whose functions rely on storage and secretion of hydrolytic enzymes (Copeland and McDonald, 2001). The enhancement of protein synthesis contributes for the embryo growth and degradation of storage proteins, triggering genetic and epigenetic changes involved in DNA repair mechanisms that affect the nucleolar morphology, organization (Lutts *et al.*, 2016) and activity (Carvalho *et al*., 2019). The enzymes are transferred to other tissues, and the production of proline enhances the hydrolysis of storage proteins during germination (Copeland and McDonald, 2001). A variety of proteases allow the conversion of insoluble storage proteins into soluble peptides and free a.a. (Anand *et al.*, 2017). In this work, the average concentration of proline did not show significant differences between the S1 offspring of hydropriming and control. Nonetheless, the increased protein amount in hydroprimed seeds also justifies the shortening of mean germination time, the increased germination rate and seedlings emergence, as well as the improvement of specific yield-related components (Reis *et al*., 2018). Due to its advantages, 'on-farm' hydropriming before sowing has been widely adopted by lower economic strength farmers for a range of crops, including wheat (Harris *et al.*, 2008).

The lowest CP content was shown by the control (unprimed seeds) offspring. Hence, independently of using distilled water or micronutrient-rich solutions, the seed priming performed in the S0 seeds by Reis *et al.* (2018) enhanced the protein content in that generation (Carvalho *et al*., 2019) as well as in the S1 offspring (this work). Like the a.a. composition, the wheat grain-protein amount fluctuates widely among genotypes and environments (Boila *et al*. 1996; Tanács *et al*., 1995; Zafar *et al*., 2014). Punia *et al.* (2019) classified wheat cultivars based on their protein content as following: those having protein content higher than 12% were classified as high protein cultivars; between 10 and 12% were considered medium protein cultivars and less than 10% protein content were categorized as low protein cultivars. The control offspring presented an average percentage of protein of 12.06% (120.60 $g.kg^{-1}$ dry matter) that places the bread wheat cv. 'Jordão' in the margin of the high and medium protein cultivar, according to Punia *et al*. (2019). Therefore, the

remaining S1 offspring can be considered in the category of high protein cultivar (protein content higher than 12%). Despite increased, the CP concentration in the S1 offspring of S0 seeds primed with Fe alone was not significantly different from the control. This result evidenced that seed priming with Zn alone or in combination with Fe contributed more for protein content improvement. This can be explained by the higher mobility of Zn in phloem than Fe (Raven *et al*., 2003) and also by the significant biological roles of Zn as activator or co-factor of metalloenzymes involved in carbohydrate metabolism and protein synthesis, among others (Anand *et al*., 2017; Noulas *et al*., 2018). During the early seed development, the increase of the abscisic acid amount contributes to the dormancy to avoid premature germination and stimulates the production of storage proteins (Raven *et al*., 2003).

4.2. Soluble sugars and total starch content

In this work, the total soluble sugar content decreased in all S1 offspring resulting from primed S0 seeds. Nevertheless, this decrease was only significantly different from the control offspring in the S1 offspring whose seeds were hydroprimed, primed just with Zn, and treated with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn. Among the five soluble sugars identified in the whole wheat flour samples of the different S1 offspring, only glucose showed an increase relative to the control. However, this value was only significant in the S1 offspring of plants whose S0 seeds were primed with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn. Bowne *et al.* (2012) reported that some wheat cultivars tolerant to drought might respond slowly with an increase of glucose and a.a. like proline, tryptophan, leucine, isoleucine and valine, since these biochemical molecules contribute to the osmotic adjustment and the synthesis of proteins involved in ROS scavenging. Though the periodical irrigation, the S0 plants probably experienced water deficit during the grain filling period, contributing to the increase of glucose in their offspring (S1 seeds). As suggested by Wang *et al.* (2018), the drought priming of parental wheat plants increased the drought tolerance of the offspring. Nevertheless, the high amount of glucose in mature grain is expected because, upon the disruption of the seed coat favoured by imbibition and in the presence of oxygen, glycolysis constitutes the first step of respiration needed for germination, though the possible occurrence of glucose breakdown under anaerobic conditions in early stages of germination (Raven *et al*., 2003).

The remaining sugars, sucrose, fructose, raffinose and maltose, demonstrated a significant decrease relative to the control. Seed priming also improves the seedlings dry weight, leaf area, leaf CO2 net assimilation, maximising the photochemical efficiency of photosystem II and activity of α-amylase under abiotic stress (Farooq *et al.*, 2017). Therefore, the more significant enzymatic activity of amylases during the grain maturation resulted in the degradation of oligosaccharides. This fact can explain the reduction of most of the soluble sugars and increase in the glucose relative to control, the latter being higher in the S1 offspring of S0 seeds primed with 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn, 8 $mg.L^{-1}$ Zn and 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn that also presented the highest amount of CP.

Odunlade *et al.* (2017) used green leafy powdered extracts to improve the nutritional value of wheat flour. The authors found that despite the increase in minerals (Mg, Fe, Na, Zn and Ca) with the augment of powder concentration there is a significant decrease in the antioxidant capacity and carbohydrates amount accompanied by a considerable increase of protein, fibre, ash and fat.

Punia *et al.* (2017) also found that the high protein content wheat cultivars (above 12% of protein content) showed a low content of carbohydrates. Similar results were found in the present study, namely the decrease of total sugars amount, as well as the increase of ash and CP concentration. The reduced antioxidant capacity may be explained by the decrease of the antioxidant glutathione pool (Anand *et al*., 2017).

Sucrose and starch are the major products of carbon assimilation pathways in most of the plants, and when carbon is fixed in excess during the day is exported to non-photosynthetic sink organs, including seeds (Koch, 2004). The assimilated carbon can also be transiently stored as starch in chloroplasts or as sucrose in vacuoles and then remobilised when sink demand exceeds photosynthetic carbon supply (during the night) (Durand *et al.*, 2018). Probably the enhancement of grain yield-related components reported by Reis *et al*. (2018) required higher demands of carbon explaining the decrease of sucrose and total starch in the S1 offspring resulting from the priming of the S0 seeds with Fe and/or Zn.

In this work, the total starch content significantly decreased in all S1 offspring relative to the control, except for the offspring of 8 $mg.L^{-1}$ Fe that showed a non-significant increase. The lowest amounts of total starch were detected in the S1 offspring of Fe + Zn. The activity of enzymes responsible for the starch breakdown, like α- amylase, β-amylase or α-glucosidase, can be inhibited by heavy metal stress. This fact can result in the accumulation of total carbohydrates (soluble saccharides and polysaccharides), impairing the mobilization of

storage protein and the translocation of soluble sugars and a.a., affecting the plant growth and development (Deef, 2007; Anand *et al*., 2017). All S1 offspring evaluated in this work showed a significant decrease of total starch amount relative to the control, demonstrating that the Fe and Zn dosages used for the priming of S0 seeds were not stressful enough to inhibit the enzymatic activity. The starch hydrolysis might be in the origin of the increased amounts of glucose detected in all offspring relative to the control.

4.3. Intergenerational effects of hydropriming and nutripriming in the next unprimed offspring

4.3.1. Impact on germination

The germination rate (100%) of all S1 offspring analysed here was improved relative to the values achieved in the S0 seeds by Reis *et al*. (2018).

Excluding the control, five out of the seven S1 offspring resulting from hydropriming and nutripriming with Fe and/or Zn presented lower mean germination time values than those reported by Reis *et al*. (2018) for their respective S0 seeds. Additionally, except for the S1 offspring of 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn that showed the highest mean germination time, the remaining S1 offspring presented lower mean germination time values than the control.

These results evidenced that the seed priming performed in the S0 generation probably contributed for a uniform and fast germination of the S1 seeds constituting intergenerational benefits. This fact can be explained by an improved nutritional status of the mother S0 plants, particularly regarding the availability of Fe, since its deficiency induces seed dormancy (Murgia and Morandini 2017). Hence, the improved Fe nutritional status of the S0 plants contributed to the amelioration of germination of their progeny seeds.

4.3.2. Mitotic cell cycle evaluation

Different abiotic stresses affect negatively the mitotic division and increase the number of cell cycle and chromosomal anomalies (Prom-u-thai *et al*., 2012; Rehman *et al.* 2015; Abdelsalam *et al*., 2018; Ali *et al.*, 2018; Carvalho *et al.*, 2018, 2019; Reis *et al.*, 2018). The

extent of the negative impacts of nutripriming with high dosages of Fe and/or Zn on the mitotic cell in the first stress-free generation is unknown.

Based on our observations, the lowest average MI values were found in the S1 offspring of seeds primed with 8 $mg.L^{-1}$ of Fe and/or Zn corroborating the data of Reis *et al.* (2018). Furthermore, the decrease in the average MI with Fe and/or Zn augment was also noticed in the S1 offspring. These results suggested an intergenerational stress memory related to the cytotoxicity that arose from the priming of S0 seeds with the highest concentration of Fe and/or Zn.

The S1 offspring of hydroprimed S0 seeds showed the second-highest average MI (49.07%). Hydropriming is widely used as a trigger to biochemical and enzymatic processes that enhance germination, plant growth and development (Paparella *et al.,* 2015). However, its effects on the next unprimed offspring were not studied. The average MI of the S1 offspring of hydroprimed seeds was similar to that reported for the S0 seeds (Reis *et al*. 2018). Thus, we can suggest that hydropriming did not have a significant influence on the MI of the first unprimed offspring.

In this work, most of the normal dividing cells of each S1 offspring were in prophase, but those average values were lower than the ones reported for the S0 generation (Reis *et al*., 2018). The high average number of normal and irregular prophases scored in plants during or after abiotic stress is indicative of cell cycle arresting (Pekol *et al*., 2016; Carvalho *et al*., 2018, 2019; Reis *et al*. 2018). Therefore, the decrease in the average number of normal prophases suggested that the dividing cells were able to proceed with the mitotic cycle. This fact is also confirmed by the higher number of normal metaphases, anaphases and telophases observed in most of the S1 offspring.

Additionally, most of the abnormal dividing cells were in metaphase and showed higher values than those reported by Reis *et al*. (2018). However, abnormal dividing cells in all mitotic phases and for the seven priming treatments (hydropriming and nutripriming) were found in the previous generation. Here, some S1 offspring did not show abnormal dividing cells in prophase, anaphase and/or telophase.

Only six types of cell cycle and chromosomal irregularities were found contrarily to the observed in the work of Reis *et al*. (2018). These results evidenced the inheritance of a stress memory related to the cytotoxicity, mostly evidenced in the S1 offspring of 8 $mg.L^{-1}$ Fe and/or 8 $mg.L^{-1}$ Zn. Nevertheless, some of those effects seem to be attenuated in the S1 offspring probably due to the occurrence of DNA repair mechanisms. Another argument for

the previous assumption is that in the S0 generation, chromatin stickiness was observed in all treatments and mitotic phases (Reis *et al*., 2018). However, in the present work, this anomaly was only detected in prophases and metaphases of S1 offspring, except in control for the case of sticky prophases. Also, sticky anaphases and telophases were only observed in the S1 offspring of 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn. Concerning that chromatin stickiness reflects high toxicity and may result in cell death upon the accumulation of chromosomal aberrations, and cell division anomalies (Liu *et al.*, 1996), its reduction in the S1 offspring indicates that the cytotoxicity induced by high dosages of micronutrients can become diluted throughout the next seed generations. In most of the cases studied so far, the stress memory is reset after one stress-free generation, and insights into the molecular conservation of stress memory in crops are scarce (Lämke and Bäurle, 2017). This study evidenced that the cytotoxicity-related stress memory was not reset in the S1 offspring as evidenced by the detection of cell cycle and chromosomal irregularities. These anomalies were previously reported in wheat and other plant species under abiotic stress (Liu *et al.*, 1996; Oladele *et al*., 2013; Pekol *et al*., 2016; Carvalho *et al.* 2018).

Overall the average values of %DCA decreased in the S1 offspring relative to the S0 generation (Reis *et al.* 2018), except for the offspring of hydropriming, 8 $mg.L^{-1}$ Zn, and 4 $mg.L^{-1}$ Fe + 4 $mg.L^{-1}$ Zn. The lowest average %DCA was observed in the control offspring.

4.3.3. Enhancement of yield-related components

Reis *et al.* (2018) observed that nutripriming with concentrations above 2 $mg.L^{-1}$ Fe + 2 $mg.L^{-1}$ Zn negatively affected most of the yield-related components when compared to hydropriming. Even though the S1 seeds were not primed, we intended to analyse how the nutripriming with Fe and/or Zn performed in the S0 seeds can affect yield-related parameters in the S1 descendants. Therefore, we characterized seven yield-related components in all S1 offspring, including the control which showed the lowest results for all yield-related components except for the number of spikelets of the main spike (NSkMS). The same yield-related components were analysed in the S0 plants (Reis *et al.* 2018), and the values reached in all S1 offspring surpassed those achieved in the previous generation. The present results evidenced that the seed priming performed in the S0 seeds improved the yield-related components of the S1 plants suggesting the transmission of intergenerational benefits. Similar

results were reported by other authors (Sultan *et al*., 2009; Wang *et al*., 2016; Tabassum *et al.*, 2017).

The S1 offspring of 8 mg.L^{-1} Fe, 8 mg.L^{-1} Zn, and 8 mg.L^{-1} Fe + 8 mg.L^{-1} Zn showed the highest values for most of the yield-related components analysed here. Additionally, the whole wheat flour of the S0 seeds primed with these treatments previously demonstrated high contents of total soluble proteins (Carvalho *et al.*, 2019). Similar results were also verified in the whole wheat flour of the respective unprimed S1. Therefore, the high protein content observed in both S0 and S1 seeds could be in the origin of the yield-related components enhancement observed in the S1 generation of unprimed seeds analysed in this work.

5. Conclusions

The present work constitutes the first approach in the evaluation of the effects of seed priming with different dosages of Fe and/or Zn on the biochemical profile, germination, mitotic cell cycle, and yield-related components of the respective unprimed S1 offspring.

We observed that the seed priming with concentrations of 4 $mg.L^{-1}$ and 8 $mg.L^{-1}$ of Zn alone and the combination of Fe and Zn performed in the S0 seeds resulted in the highest average concentration of most of the free a.a. identified in the respective S1 offspring.

These treatments also led to the highest levels of glucose, sucrose and/or raffinose, the highest CP and ash content, and the lowest amounts of total starch in the S1 offspring. Most of these biochemical molecules act during the plant stress response.

Accordingly, our results demonstrated that the nutripriming treatment performed in the S0 seeds improved the nutritional quality of the unprimed S1 seeds as revealed upon comparison with the control offspring.

The germination of the S1 offspring was improved relative to the S0 seeds since the former presented a higher germination rate (100%) and lower MT values. Moreover, all the S1 offspring except for those resulting from priming with 8 $mg.L^{-1}$ Fe + 8 $mg.L^{-1}$ Zn showed lower MT values than the control. Based on the biochemical and germination data achieved in the unprimed S1 offspring particularly in comparison with the control, it seems that the S0 “parental” generation provided the means for faster and uniform germination, due to the stress they endured.

The analysis of the mitotic cells of the S1 offspring suggested a partial inheritance of the stress memory endured by S0 seeds. Although the MI increased from one generation to the other, the trend of MI reduction with the increase of Fe and/or Zn concentration noticed in the S0 was maintained in the S1 offspring.

In our work, the proportionality between the number of anomalies and concentration of Fe and/or Zn was observed. However, the frequency of anomalies was low.

Despite the evidence of inheritance of a stress memory related to the cytotoxicity induced by the nutripriming with high dosages of Fe and/or Zn from S0 to the unprimed S1 offspring, some attenuation of cytotoxic effects on the mitotic cell cycle was observed. The attenuation of cytotoxicity observed in S1 offspring was also corroborated by the improvement of the yield-related parameters observed in the S1 plants.

Our work evidenced that the abiotic stress endured by the previous generation led to better results in the offspring it generated.

The data evidenced that despite the abiotic stress originated by nutripriming with high dosages of Fe and/or Zn in the parental generation, improved biochemical composition, germination and yield-related components was detected in its offspring. However, some cytotoxicity-related stress memory was also inherited as reflected by the MI and %DCA results.

In conclusion, the hydropriming and nutripriming performed in the S0 seeds benefited the germination and the yield-related parameters in the unprimed S1 offspring without the need to repeat the treatment in each generation.

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