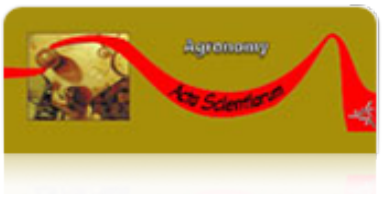

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Toxicogenetic effects are involved in the occurrence of imbibitional damage in soybean seeds

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ABSTRACT. Soybean represents a valuable source of food for humans and animals and the quality of the seeds has great importance for the establishment and high productivity of this crop. Soybean seeds require continuous improvement, which is dependent on a better understanding of the regulatory mechanisms that coordinate seed germination. To investigate whether the method of water absorption into soybean seeds could lead to abnormal seedlings, and if this could be associated with cytogenetical consequences, we premoistened the seeds of three cultivars; M 6410 IPRO, M6210 IPRO, and BMX Potência RR by direct immersion (fast method), use of a wet substrate (intermediate method), and by moist atmosphere (slow method) with distilled water at 25ºC for 24 hours. We investigated the normal and abnormal seedlings, electrical conductivity, mitosis, cell death, and the chromosomal abnormalities index. The comet assay was applied to investigate DNA fragmentation. Direct immersion in water induced seedling growth inhibition and caused cytological alterations associated with genotoxicity effects in the studied soybean genotypes. Slow premoistening of the seeds increased seedling performance as a result of higher final germination percentage (above 85%), reduced abnormal seedlings (below 5%, on average), and reduced the electrical conductivity of seeds. All three genotypes of soybean seeds lost their ability to withstand the imbibitional damage induced by direct immersion as abnormal seedlings increased. We concluded that the fast water absorption by seeds poses a threat to genomic integrity owing to its potential for genotoxicity to DNA, manifesting as breaks or loss of whole chromosomes. Slow premoistening of the seeds resulted in a longer time period to deal with damage. Stabilized seedling growth was provided by altering cytogenetic responses during uptake of water by soybean seeds through the maintenance of cell viability.

Keywords: chromosomal alterations; cytogenetical consequence; comet assay; seed imbibition.

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Introduction

Seed is of great importance to agricultural because it contains genetic information that is crucial to the performance of the cultivar, and its quality is responsible for establishing an adequate population of plants, which is the basis for profitability (Marcos Filho, 2015; Finch-Savage & Bassel, 2016). Also, seeds provide a highly effective strategy for transmission of genetic information from the mother plant to the next generation (Waterworth, Bray, & West, 2015). Seed quality is the most important parameter for yield improvement and in this way, special attention is given to soybean seed quality. This is one of the most important aspects in the search for elevated grain or seed production, given that quality directly interferes with soybean crop development (Silva et al., 2017).

Germination is one of the most unreliable periods in the life of a plant, and the uniformity and emergence percentage can deeply affect the production and quality of the crop (Nonogaki, 2006). Seed germination, *sensu stricto*, can be defined as the sequence of physiological events occurring before radicle emergence in imbibed non-dormant seeds. The term 'germination' is often used for seedling establishment which is, in a strict sense, a pos-germinative event (Nonogaki, 2006). Here, we focus strictly on the mechanisms of germination, which involve this critical phase of plant life that might determine seedling establishment and subsequent soybean persistence, all of which are influenced by the early imbibition stage.

Early cytological studies identified DNA and membrane damage and disruption of organelles in seeds stressed by abiotic factors (Dekkers et al., 2015). These correlated with a loss in seed germination. To the

best of our knowledge, cell maintenance and genome integrity are critically important to prevent mutation prior to the re-initiation of cell cycle activity in the embryonic meristems (Dresch, Masetto, & Scalon, 2015; Waterworth et al., 2015; Masetto & Faria, 2019).

Although the fastest water uptake is often indicative of the membrane degradation and cellular deterioration typical of nonviable seeds which are metabolically inactive (Kranner, Minibayeva, Beckett, & Seal, 2010; Macovei et al., 2017), recently, seed analysts have shown that soybean seed lots belonging to certain cultivars and submitted to premoistening (i.e. slow hydration before the germination test) might have their real germination potential evaluated by increasing the germination of soybean seed lots. These soybean seed cultivars were not deteriorated but should be pre-moistened through slow hydration before execution of the germination test. This is in agreement with the Rules for Seed Analysis (Brasil, 2009) which recommends the use of slow imbibition for premoistening before the germination test to avoid imbibitional damage of seeds.

It is assumed that the premoistening of seeds involves partial imbibition (as opposed to full hydration) of seeds by various strategies such as shortening imbibition duration or exposing seeds to relatively low external water potential (Chen & Arora, 2013). According to Varier, Vari, and Dadlani (2010), premoistened seeds have a longer period of time to repair metabolic lesions before germinating. The process involves exposing the seeds to a moist atmosphere that impairs the total water uptake into the seeds, while allowing slow imbibition. Thus, DNA damage resulting from reactive oxygen species (ROS) accumulated throughout the prolonged storage period must be repaired during seed imbibition and prior to the initiation of cell division to improve growth and avoid genotoxic stress (Kranner et al., 2010).

The method of premoistening seeds as it relates to improved germination and stress tolerance is not fully understood, nor has gene expression associated with the process leading to radicle emergence been fully elucidated (Nonogaki, 2006; Chen & Arora, 2013). It was universally accepted that the development of a multicellular organism, such as a plant, is directly related to the processes of cell division, growth, and differentiation (Harashima & Schnittger, 2010). Indeed, recent evidence shows that in early-imbibing seeds, damage to the embryo genome must be repaired prior to the initiation of cell division to minimize growth inhibition, mutation of genetic information (Waterworth et al., 2015), and passive cell death (Masetto & Faria, 2019). DNA damage is caused during replication processes, but also by various endogenous and exogenous factors under stressful conditions. DNA double-strand breaks (DSBs) are the most severe type of DNA damage and lead to a loss of chromosomal fragments (Umeda, Aki, & Takahashi, 2019).

The present work focuses on the physiological significance of soybean seeds that were submitted to premoistening methods characterized by the fast or slow water uptake. Fast hydration was achieved by the direct contact of the seeds with water, and slow hydration was achieved through exposure to a moist atmosphere.

In addition, the contrasting effects exerted by the fast or slow uptake of water by the seeds were evaluated at the toxicogenetic levels intended to improve seed germination. These methods represent a valuable approach for the seed biology and industries.

Material and methods

Experiment assembly

The experiment was performed using a completely randomized design (CRD) consisting of three cultivars of soybean (*Glycine max* L.) seeds; BMX Potência RR, M 6210 IPRO RR, and M6410 IPRO RR. Three premoistening methods were used (rapid water uptake, wet substrate, and moist atmosphere), each with four repetitions. The control was considered the wet substrate. Each repetition was represented by 50 seeds for each genotype of *Glycine max* L. seeds.

Plant material and premoistening methods

Five lots of three types of transgenic soybean seeds (BMX Potência RR, M 6210 IPRO RR, and M6410 IPRO RR) were used in order to understand the physiological and cytogenetic events during the early seed germination phase. The soybean seeds of each cultivar were hydrated by one of three techniques: (1) rapidly, by direct complete immersion in distilled water (75 mL water, 25ºC, 24 hours), (2) slowly, by exposure to a moist atmosphere wherein a single layer of seeds was placed on a stainless-steel screen and suspended over 40 mL of water inside a plastic box (11,0 x 11,0 x 3,5 cm; 25ºC, 24 hours), and (3)

intermediate, by placing seeds on wet substrate. In this case, the substrate consisted of rolls of germination paper moistened with water in an amount equivalent to 2.5 times the weight of the dry substrate (25ºC, 24 hours). This method was considered the control treatment because it is the technique most commonly used by the seed industry and laboratories. Both initially, and after each premoistening period, the water content of the seeds was determined, and the following seed characteristics were evaluated.

Water content

Water content was measured using the oven method (103 ± 2ºC for 17 hours) (International Seed Testing Association [ISTA], 2016), with two replications of 5.0 g of seeds; the results were expressed as a percentage (wet basis).

Effect of water dynamics during seed premoistening

The measurements of the water dynamics during premoistening of the seeds were analyzed in independent samples of 200 seeds (n = 200) and replicated in four subsamples of 50 seeds each (r = 4, a = 50). We maintained the samples in a germination chamber (Biochemical Oxygen Demand, Tecnal, state of São Paulo, Brazil) at 25ºC under constant white light.

The mass over time was recorded with a scale balance (precision: 0.001 g) and plotted as a mass increment curve. The water content (ISTA, 2016) of the seeds was recorded over time to plot water content curves.

Germination test

Seed asepsis was performed with 2% sodium hypochlorite solution for 2 min., and then the seeds were washed with distilled water. Seeds were then distributed in rolls of germination paper and moistened with water in an amount equivalent to 2.5 times the weight of the dry substrate (ISTA, 2016). For each treatment, 50 seeds were sown, in quadruplicate, and were kept in a Mangelsdorf germinator. The results were expressed as a mean percentage of 'normal' and 'abnormal' seedlings, according to young plant morphoanatomical characters based on survival in optimum field conditions.

Electrical conductivity

After each treatment, four replications of 50 seeds per treatment were individually weighed (0.01 g precision) and soaked in 75 mL deionized water at 25ºC for 24 hours. Electrical conductivity was then measured on a Digimed (state of São Paulo, Brazil); results were expressed in $\mu S\ cm^{-1}\ g^{-1}$ (ISTA, 2016).

Cytogenetoxic evaluation

After exposure to the control and each treatment, the roots were collected and fixed in an ethanol-acetic acid (3:1) solution for at least 24 hours. Next, they were washed and hydrolyzed in 1 N HCl at 60ºC. Slides were prepared by the squashing technique and stained by Diff-Quick® (Panótico Rápido®, Laborclin, Brazil; adapted from Meneguetti, Silva, Zan, & Ramos, 2012). A total of 1000 cells were counted per slide, amounting to five slides per treatment and 5000 cells analyzed per treatment. Samples were evaluated with a light electronic microscope (ZEISS® Primo Star, Germany, 100 X).

The parameters evaluated were as follows: mitotic index (MI), calculated by the ratio between the number of dividing cells and the total of observed cells; cell death index (CDI), evaluated by dividing the total number of dead cells (reduced size and dark cell nuclei) by the total number of cells analyzed; chromosome alterations, expressed by the frequency of cells presenting abnormalities (chromosome bridges, breakage, c-metaphase, stickiness, lost and laggard chromosomes) divided by the total number of cells counted. Micronuclei were counted to evaluate the Mutagenicity Index (IMT).

The comet assay was performed following the methodology proposed by Souza et al. (2019). Briefly, 25 radicles/roots were collected from each treatment and diluted in 500 µL of phosphate-buffered saline (PBS) solution. Subsequently, this material was centrifuged at 14,000 RPM for 10 s and 200 µL of the supernatant was discarded.

Slides were prepared in duplicate for each treatment, and each slide contained 60 µL of cell suspension and 240 µL of 0.5% ($v\ v^{-1}$) low melting point agarose at 37ºC. Slides were incubated in lysis solution for 4 hours and then in 0.3 $moL\ L^{-1}$ NaOH and 0.001 $moL\ L^{-1}$ EDTA (pH > 13) buffer solution for 20 min. to denature the DNA. Then, they were electrophoresed at 37 V, 300 mA, for 25 min. Slides were neutralized with 0.4 $moL\ L^{-1}$ Tris, fixed with ethanol PA, and stored at 4ºC until counts were performed. They were

stained with 30 µL of ethidium bromide (1.6 mg mL^{-1}) and 100 nucleoids per slide were observed under a fluorescence microscope (Labmed, Lx 400) on the 40 × objective. Next, slides were photographed and analyzed using the software Lucia comet assay (LIM, Prague, CZ) using the parameters tail length and tail DNA %.

Statistical test

The experimental design was a completely randomized one; for each of the three soybean genotypes, three techniques of premoistening were carried out with four replications, each using 50 seeds. For the physiological quality (normal seedlings, abnormal seedlings, and electrical conductivity), cytological, and comet assay evaluations, ANOVA analyses (represented by critical difference at 0.05 probability level) were computed to determine significant variations, if any, between and among techniques of preconditioning (including controls). Data were submitted to analysis of variance by F test ($p < 0.01$), and means were compared by the Tukey test ($p \leq 0.05$) with the software Sisvar.

Results and discussion

Water uptake rate, normal and abnormal seedlings, and electrical conductivity

A deep connection between the soybean genotypes, rate of water uptake, and DNA damage existed at all stages during the absorption of water by the seeds. The gain in seed fresh weight of the three genotypes occurring during the imbibition period was also measured (Figure 1). Seeds soaked directly in water during phase I of imbibition (from 0 to 8 hours) were characterized by a rapid increase in fresh weight (Figure 1a). On the other hand, a slower gain in fresh weight was evidenced when imbibition occurred with wet substrate and the moist atmosphere (Figure 1b and c, respectively). Finally, it is worth noting that a further significant increase in fresh weight was observed both after 10 hours of imbibition, and during the subsequent period (Figure 1).

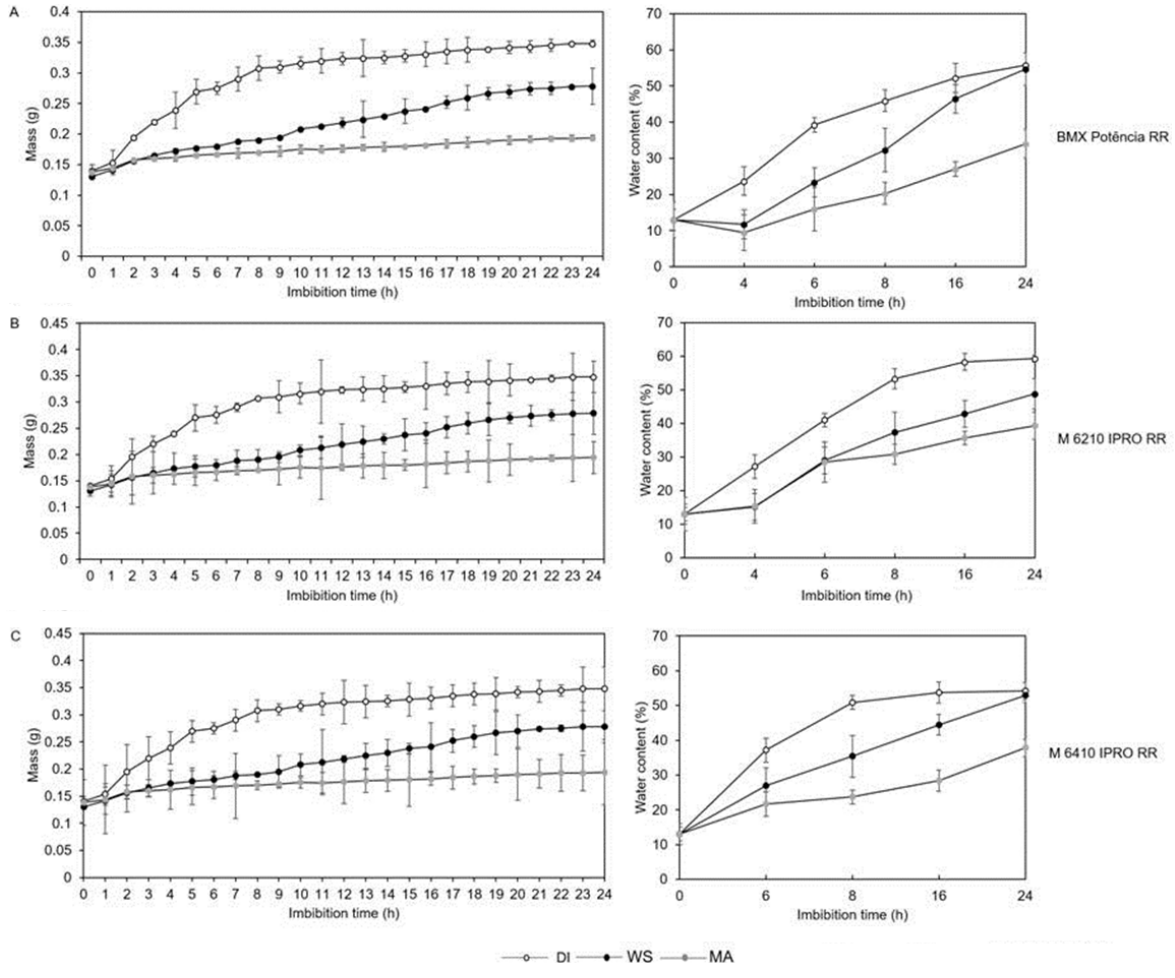

Figure 1. Mass increment and water content over time in three cultivars of soybean seeds (A - BMX Potência RR, B - M 6210 IPRO, C - M 6410 IPRO) during premoistening methods. DI, direct immersion; WS, wet substrate (control); MA, moist atmosphere. Bars are confidence intervals at $\alpha = 0.05$.

The analysis of variance (ANOVA) showed significant ($p < 0.01$) influence of preconditioning methods on the germination (characterized as normal seedlings) and electrical conductivity for all of the soybean seeds genotypes (Table 1). In general agreement with seeds submitted to slow hydration, a significant increase in normal seedlings was observed in the three genotypes (except for BMX Potência RR which did not show a difference from the seeds placed on the wet substrate) according to Tukey's test ($p = 0.05$; Table 1). However, the decline in the percentage of normal seedlings alone does not define the precise point of seedling resumption.

There is an indication of the genotype tolerance to imbibitional damage. When seeds were preconditioned slowly (moist atmosphere) and at an intermediate rate (wet substrate) this phenomenon became more pronounced (Table 1), especially for BMX Potência. Overall, all three genotypes of soybean seeds lost their ability to withstand the imbibitional damage induced by direct immersion as abnormal seedlings increased (Table 1). In agreement with the data from normal seedlings, slow hydration of the seeds showed a decline in seedling abnormalities.

One way of testing the hypothesis that an increase in the rate of water uptake triggers cell compartment disruption would be to perform a study of the electrical conductivity of the seeds. After 24 hours the cell leakage showed some minor differences between the premoistening techniques. They were, however, sufficient enough to detect the harm caused by direct immersion, thus corroborating the results of the analysis of seedling establishment (Table 1).

Table 1. The influence of premoistening techniques on the results of the germination test and electrical conductivity of three genotypes of soybean seeds.

Genotype	25ºC/24 hours	Normal seedlings (%)	Abnormal seedlings (%)	Electrical conductivity ($\mu S\ cm^{-1}\ g^{-1}$)
BMX Potência RR	DI	48 ^{B}	19 ^{A}	42,40 ^{A}
	WS	85 ^{A}	3 ^{B}	39,45 ^{AB}
	MA	86 ^{A}	5 ^{B}	37,54 ^{B}
M 6210 IPRO	DI	43 ^{C}	37 ^{A}	52,22 ^{A}
	WS	85 ^{B}	9 ^{B}	49,00 ^{AB}
	MA	97 ^{A}	2 ^{C}	47,42 ^{B}
M 6410 IPRO	DI	22 ^{C}	46 ^{A}	54,75 ^{A}
	WS	75 ^{B}	25 ^{B}	50,37 ^{A}
	MA	86 ^{A}	8 ^{C}	51,75 ^{A}

DI: direct immersion; WS: wet substrate; and MA: moist atmosphere. 'Normal' seedlings refer to individuals able to survive in field conditions and 'Abnormal' refer to those that are unable. Mean values followed by the same superscript in a column for each genotype do not differ among themselves according to the Tukey's test at 5% probability.

Cytogenetic analysis and comet assay

The micronucleus assay evaluates the presence of intracytoplasmic chromatin moieties, the induction of breaks, or the loss of whole chromosomes, thus characterizing the mutagenic potential of the studied agent. In the present study, the external agent was represented by the direct immersion of the seeds.

Corroborating the macroscopic data, the microscopic analysis revealed a statistically significant reduction in cell viability. There was a significant influence in the rate of water uptake, determined by preconditioning techniques, on cell death in soybean seeds and on the other cytogenetic characteristics evaluated (Table 2). According to the mitotic index (MI) of the cultivar M6410 IPRO, seeds submitted to the moist atmosphere presented a higher number of cells in cell division compared to the other water uptake rates (Table 2). For the other genotypes, none observed significant differences from the control, although for seeds of the three genotypes, as the rate of water uptake increased, the cell division decreased. This indicates the need for a reduction in the rate of water uptake (i.e. through slow hydration) to deal with the abiotic stress imposed by the water surrounding the seed that might inhibit the ability to complete normal seedling establishment.

The same pattern was observed for CDI; the highest rate of cell death (CD) was observed with rapid water uptake, indicating that the fast entry of water into the seeds caused irreversible cellular damage and determined the cell's death (Table 2). This was also evaluated through *in vivo* DNA breakage (Tail DNA and Tail lenght results). The cellular damages observed were characterized by the high micronuclei results due to nuclei injuries. These arose from direct immersion according to the mutagenicity index (MTI), as well as chromosome aberrations (CAI; Table 2) and physiological disturbances (Table 1).

The occurrence of nucleus injuries in the meristematic cells of the soybean radicles might avoid the continuity of the root growth, since they determined the reduction in the size and/or condensation of the nucleus, presence of micronuclei, cell shoots, disorders in the mitotic cycle and chromosomal delay (Figure 2) and might cause disturbance of the mechanisms involved in the preservation of the integrity of the genome.

Table 2. The influence of premoistening techniques on the mitotic index (MI), cell death index (CDI), chromosome alterations index (CAI), mutagenicity index (MTI), and *in vivo* DNA breakage (Tail DNA and Tail length).

Genotype	25ºC/24 hours	MI	CDI	CAI	MTI	Tail DNA (%)	Tail length (µm)
BMX Potência RR	DI	12,07 ^{B}	8,12 ^{A}	0,00 ^{A}	0,28 ^{A}	45.48 ^{A}	39.04 ^{A}
	WS	21,60 ^{A}	2,98 ^{B}	0,04 ^{A}	0,10 ^{A}	39.89 ^{B}	36.79 ^{A}
	MA	22,00 ^{A}	2,26 ^{B}	0,06 ^{A}	0,20 ^{A}	34.52 ^{C}	30.40 ^{B}
M6210 IPRO	DI	15,6 ^{B}	17,10 ^{A}	0,02 ^{A}	1,02 ^{A}	42.58 ^{A}	32.43 ^{A}
	WS	26,44 ^{A}	7,4 ^{B}	0,001 ^{A}	0,60 ^{AB}	41.69 ^{B}	31.80 ^{A}
	MA	27,30 ^{A}	1,76 ^{C}	0,001 ^{A}	0,12 ^{B}	34.99 ^{C}	30.32 ^{B}
M6410 IPRO	DI	12,09 ^{B}	14,30 ^{A}	0,12 ^{A}	0,98 ^{A}	42.05 ^{A}	34.14 ^{A}
	WS	13,84 ^{B}	4,18 ^{B}	0,06 ^{AB}	0,22 ^{B}	36.56 ^{B}	33.30 ^{A}
	MA	16,30 ^{A}	2,06 ^{B}	0,00 ^{B}	0,34 ^{B}	34.07 ^{C}	27.35 ^{B}

DI: direct immersion; WS: wet substrate; and MA: moist atmosphere. Mean values followed by the same superscript in a column for each genotype do not differ among themselves by the Tukey's test at 0.05 probability.

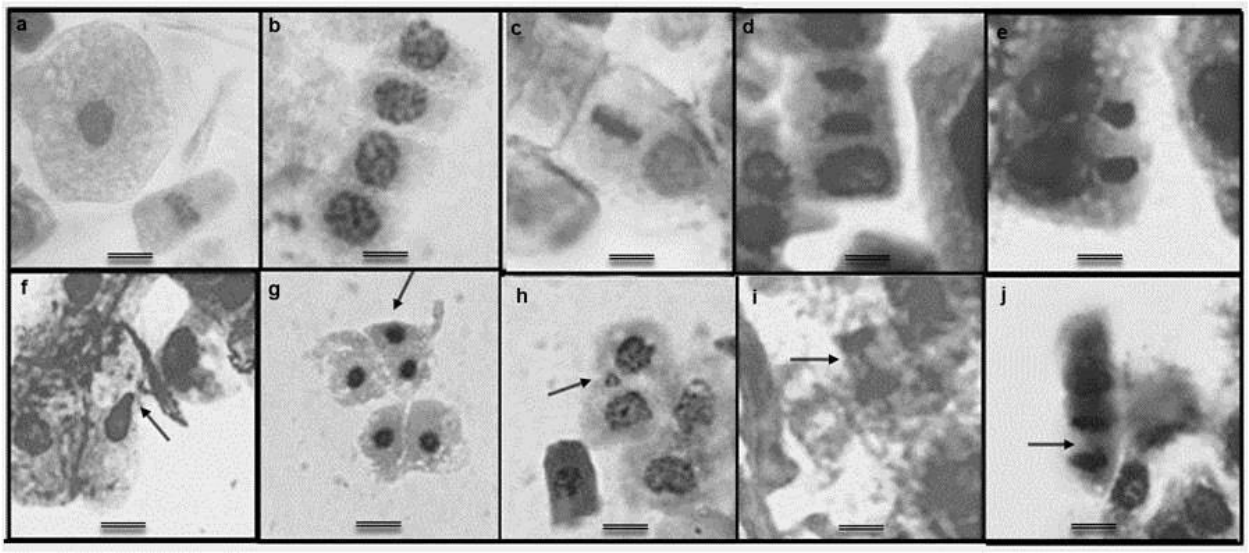

Figure 2. Meristematic soybean root cells in different mitosis phases and abnormalities observed after direct immersion into the water (25ºC, 24 hours). Normal cells in a. interphase and metaphase; b. prophase; c. metaphase and prophase; d. anaphase; e. telophase. Nuclear abnormalities: f. nuclear bud; g. small and shrunken nuclei; h. micronuclei; Chromosomal alterations: i. chromosome bridges; and j. chromosome laggards. Scale bar = 0,055 µm.

It is a common practice to rehydrate dehydrated tissue slowly in order to permit the re-establishment of functional membranes prior to placing the tissue into liquid water. Hence, we demonstrated that a further practice to pre-humidify the organelle structures prior to imbibition can prevent negative chromosomal alterations.

Chromosomal alterations have been widely used to evaluate the genotoxic effects of chemical agents and to evaluate their mechanisms of action on the genetic material of the exposed organisms. Thus, this procedure can be efficient for investigating the impacts of damages induced by early imbibition in soybean seeds. According to Cheng et al. (2010), water uptake is an important event in germination which acts as a switch for setting up discrete mechanisms for seed germination; switching the gene expression from a developmental mode to germinative mode.

In this sense, the cytogenetic evaluation becomes fundamentally important, since such analyses provide better knowledge related to the action of agents in the DNA. The damages observed at cytogenetic levels can be due to the mechanism of water entrance into the seeds and are indicative of the occurrence of cell death (Figure 2). These cellular alterations indicate that soybean seedling abnormalities were determined mainly by the chromosomal alterations and micronuclei in the root meristem cell which were potentially cytotoxic.

It is widely accepted that the need for the genetic material must remain intact in order to continue vital actions in the cell, such as the resumption of embryo growth that will determine normal seedling development. Therefore, it seems conceivable that during slow hydration of seeds, a simultaneously capacity may have evolved to cope with the resulting cell damage. However, in the transition phase between fast water uptake by the seeds and visible germination (determined by the presence of the seedling), irreversible damage to the chromosomes occurred (Figure 2) and/or these were not sufficiently repaired.

Therefore, the continuity of the mitotic cycle to determine the adequate growth of the embryo structures was prevented. Accumulating genetic evidence is now revealing the importance of repair mechanisms to safeguard seed viability and germination performance (Waterworth et al., 2015; Macovei et al., 2017), especially prior to the germination process.

However, the abrupt water entry into the inner tissues of soybean seeds interfered negatively in one of these processes and became harmful to the normal seedling's development. Changes in DNA during the initial stages of imbibition were also verified in sunflower seeds, as determined by polymorphism (RAPD), suggesting the occurrence of mutation points and/or extensive DNA rearrangements (El-Maarouf-Bouteau, Mazuy, Corbineau, & Bailly, 2011). In order to cope with DNA damage, cells have evolved elaborate signaling cascades, collectively known as the DNA damage response (DDR), that detect DNA damage, coordinate DNA repair and, in proliferating cells, halt cell cycle progression (Ciccia & Elledge, 2010). Nevertheless, cytogenetic characterization of the damages resulting from the dynamic of rapid water uptake has not been observed in previous studies, especially for soybean seeds.

Chromosomal alterations and the presence of micronuclei observed in meristematic cells are related to genotoxicity and mutagenicity, respectively (Leme & Marin-Morales, 2009), as a result of water uptake which promote irreversible and toxic genetic effects. DNA damage poses a constant threat to genomic integrity owing to its mutagenic potential. Under normal physiological conditions, cells undergo the formation of many DNA lesions every day. The vast majority of these lesions are repaired without further consequences, but mutations resulting from incorrect repair can contribute to cellular dysfunction (Shaltiel, Krenning, Bruinsma, & Medema, 2015). Our results showed that the previous slow hydration of the seeds may at least partially prevent the genetic material from being irreversibly damaged. In contrast, genetic damages occurred with abrupt water entrance and became toxic, impairing the resumption of the cell cycle and the normal seedling's growth. An alternative or additional effect could be that the slow rehydration techniques used did not permit free access of oxygen to the tissue (unlike complete immersion), thereby avoiding the rate of production of damaging reactive oxygen species (ROS).

Corroborating the importance of the course of time during imbibition, replication of DNA is a relatively late event in germination; it does not usually occur until some hours after a seed imbibes water, when DNA damage sustained during developmental drying, or imbibition, or both, has been repaired and considerable protein synthesis has occurred (for a review see Sliwinska, 2009). Most new information has come from the study of animal cells. According to the model proposed by Pantelias and Terzoudi (2010), after DNA damage is induced, the chromatin structure may not necessarily be broken, but instead it unfolds into a conformation that is more accessible to repair enzymes at the sites of the lesions. As functional chromatin conformation changes occur at various stages of the cell cycle, at the chromosomal level, their dynamics will affect repair processes. As a result, chromosome structure may collapse at some of these sites, giving rise to chromatid breaks that may not be reinstated or be mis-rejoined, thus affecting the formation of chromosomal alterations.

There are indications that very high levels of chromosomal breaks accumulating in seeds is strongly related to seed vigor, viability, and chromosomal break frequency (Osborne & Boubriak, 2002; Weitbrecht, Müller, & Leubner-Metzger, 2011; Waterworth et al., 2015). The reduction of mitotic activity in soybean meristematic cells may be due to a decrease in cell division as a result of DNA breakage (Table 2). Once we demonstrated that chromosomal alterations arise from the fast water uptake stressful including anaphase bridges (fused chromosomes), micronucleus and chromosomal fragments (Figure 1) comprising genotoxic damages. At this point, the course of time during seed hydration (i.e. slow hydration) can be preponderant to effective conformation changes at the chromosomal level to allow the reestablishment of DNA.

It is worth noting that the data serves as a resource for evaluating the predictivity of genotoxicity when considered alone or in association with *in vivo* DNA breakage (comet assay) and for a better characterization of cell death that impairs normal seedling establishment. Therefore, a prevailing problem during germination tests and seed quality evaluations is rapid water uptake. In this way, preconditioned (slow hydration) seeds can germinate and establish faster to cope with this problem. Several studies performed in primed seeds have shown that when seed imbibition has previously been carried out in the presence of an osmotic agent, the rate of water uptake is reduced, and the level of oxidative DNA damage strongly increases. In this case, changes are observed in the expression profiles of DNA repair genes since their up-regulation is temporally delayed (Balestrazzi, Confalonieri, Macovei, & Carbonera, 2011; Ventura et al., 2012), especially in chilling-sensitive soybean seeds (Yu, Li, & Li, 2015).

Overall, an implicit assumption is that slow rehydration, if not beneficial, is at least not damaging to the tissue. In contrast, Perán, Pammenter, Naicker, & Berjak (2004) hypothesized that a similar phenomenon may occur upon rehydration; i.e. slow rehydration could lead to the further accumulation of damage in embryonic the axes of recalcitrant seeds. The authors observed that slow rehydration in a saturated atmosphere resulted in lower germination than rapid rehydration by direct immersion. Irrespective of the confounding influences of the rehydration technique, there is an effect depending on the external characteristics of the seed, the water content, and especially, the genotype of the soybean seeds.

This is corroborated by the earlier studies about the water uptake of coated soybean seeds analyzed by UV light microscope (Meyer, Steudle, & Peterson, 2007) and by micro-magnetic resonance imaging (Koizumi et al., 2008). The authors showed that the testa, making contact with the outer water, plays a role in controlling the rate of water uptake. There was a lag during first phase imbibition, which mainly represented the need to first hydrate the seed coat; a process beginning on the dorsal side, followed by the hilum side, and eventually extending around the whole seed coat. This suggested that the hydrated coat, now more permeable than it was initially, was still limiting the rate of water uptake, rather than the progressive hydration of the embryo. Therefore, it seems that there is a genetic character (determined by the differences between cultivars of the same species) to control the toxic damages that occur during imbibition (Waterworth et al., 2015). In this sense, it proved the possible influence of genotype in the initial water imbibition process in soybean seeds. Here, we demonstrated the same results according to the course of water uptake of soybean seeds, as seen by the differences between the three genotypes (BMX Potência RR, M 6210 IPRO RR, and M 6410 IPRO RR) in the present study.

Cellular genotoxic events determined by water immersion (an abiotic stress) commonly occur during the early imbibition stage. According to Cheng et al. (2010) different levels of two water stress-related proteins have already been observed in soybean seeds during imbibition (ADH and RAB21); such protein expression might have benefited soybean seeds during the anoxia stress caused by water uptake. Since the high rate of water entry into the seed that takes place during imbibition can damage the seed; imbibition is therefore also a critical phase during which irreversible damage to cell membranes may occur. As membranes change from a gel to a liquid crystalline state (Corbineau, 2012), there are a high number of mitochondria with poorly developed cristae and a limited distinction between the internal and external membranes, in addition to non-uniform cell walls with signs of degradation and disruption of the sugar-containing storage vacuoles (Gimenez, Amaro, Machado, & Ferreira, 2017).

The study of the cytogenotoxic processes activated in the nuclear compartment during seed imbibition, particularly the possible effects of the preconditioned seeds on DNA integrity and the transcriptional profiles of genes involved in DNA repair, might provide useful information to define optimized preconditioning protocols, as requested by seed laboratories and breeding technologies. The results shown here allow for the advancement of a model to understand the cytogenetic changes occurring in soybean seeds and their hydration-rehydration cycles during rapid water uptake.

Although the regulation of recovery competence is well-understood, little is known about factors that determine whether a cell will remain recovery competent or exit the cell cycle upon DNA damage (Shaltiel et al., 2015). Our results clearly delineate that seeds imbibed before germination not only suffered from water uptake, but also from a fast absorption time. Despite the ability, if the repair mechanisms are not efficient and/or are absent, cytotoxic and genotoxic damages that occur in the initial stages of water entrance are irreversible and impair the complete development of the normal seedling. In soybean, however, this behavior can be enhanced throughout the hydration time by ensuring slow hydration of the seeds.

Conclusion

Fast uptake of water by soybean seeds caused irreversible cellular damage and determined the death of cells. This was evaluated through *in vivo* DNA breakage. The cellular damage was characterized by micronuclei, due to nuclei injuries that arose from direct immersion according to the mutagenicity index, as well as chromosome aberrations and with physiological disturbances.

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