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Effects of glyphosate on the morphology and cell cycle of soybean seedlings tolerant and sensitive to the herbicide

Abstract – The objective of this work was to evaluate the phytotoxic and cytogenotoxic effects of glyphosate on the direct development of soybean seedlings tolerant and sensitive to this herbicide. Seeds of one cultivar sensitive to and of two tolerant to glyphosate were placed to germinate in contact with distilled water (control) and two herbicide solutions (distilled water + 0.06 or 0.12% glyphosate), at 25°C, for seven days. Seedling responses to glyphosate were evaluated through the germination test, the seedling growth test, and the cell-cycle analysis (mitotic index and rate of abnormalities), carried out on the third, fourth, fifth, and seventh days after sowing. In the germination test under the herbicide treatments, no normal seedlings were observed for the sensitive cultivar, only for the tolerant ones. The herbicide treatments reduced the length of the seedlings and altered their morphology, making the radicle smaller than the hypocotyl. Regarding cytogenotoxic effects, glyphosate had a mitodepressive action on sensitive and tolerant seedlings, while the rate of abnormalities increased only in the sensitive cultivar. Glyphosate negatively affects the development of soybean seedlings at a macroscopic and cellular level, especially in cultivars sensitive to this herbicide.

Index terms: *Glycine max*, cytotoxic effects, germination, mitotic index, seedling development.

Efeitos do glifosato na morfologia e no ciclo celular de plântulas de soja tolerantes e sensíveis ao herbicida

Resumo – O objetivo deste trabalho foi avaliar os efeitos fitotóxicos e citogenotóxicos do glifosato no desenvolvimento direto de plântulas de soja tolerantes e sensíveis a este herbicida. Sementes de uma cultivar sensível e de duas tolerantes ao glifosato foram colocadas para germinar em contato com água destilada (controle) e duas soluções do herbicida (água destilada + 0,06 ou 0,12% de glifosato), a 25°C, por sete dias. As respostas das plântulas ao glifosato foram avaliadas por meio do teste de germinação, do teste de comprimento de plântulas e da análise do ciclo celular (índice mitótico e taxa de anormalidades), realizados no terceiro, no quarto, no quinto e no sétimo dia após a semeadura. No teste de germinação sob os tratamentos com o herbicida, não foram observadas plântulas normais da cultivar sensível, apenas das tolerantes. Os tratamentos com o herbicida reduziram o comprimento das plântulas e alteraram a sua morfologia, tornando a radícula menor que o hipocótilo. Em relação aos efeitos citogenotóxicos, o glifosato teve ação mitodepressiva sobre as plântulas sensíveis e tolerantes, enquanto a taxa de anormalidades aumentou apenas na cultivar sensível. O glifosato afeta negativamente o desenvolvimento de plântulas de soja em nível macroscópico e celular, principalmente em cultivares sensíveis a este herbicida.

Termos para indexação: *Glycine max*, efeitos citotóxicos, germinação, índice mitótico, desenvolvimento de plântulas.

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Introduction

Soybean [*Glycine max* (L.) Merr.] plays a crucial role in global agriculture as it represents one of the main sources of vegetable oil and protein (Islam et al., 2019). Currently, genetically modified (GM) soybean cultivars are protagonists in agricultural areas, especially due to the expectation of an increased productivity, reduced production costs (Kumar et al., 2020), flexibility in weed control (Duke, 2018), and applicability of the no-tillage system. However, the adoption of GM cultivars may have contributed to the increase of 55.8% observed in the use of herbicides (Seixas et al., 2022). The use of glyphosate, for example, increased almost 15 times since the launch of the first Roundup Ready cultivar in 1996 (Benbrook, 2016), highlighting the importance of evaluating the possible effects of this herbicide.

Glyphosate is a broad-spectrum herbicide with systemic action (Silva et al., 2018), which inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Duke, 2018). Such inhibition interrupts the synthesis of aromatic amino acids and other important metabolites for the plant (Ruszkowski & Forlani, 2022), whose consequent metabolic disorders lead to its senescence and death (Martinez et al., 2018).

Still regarding the effects of glyphosate, in germination tests in substrate moistened with the herbicide, considered an effective bioassay to discriminate seeds of GM and conventional genotypes, sensitive seedlings presented a lower development speed, stunted primary roots, and absence of secondary roots (Pereira et al., 2009; Melo et al., 2013). According to Bertagnolli et al. (2006), these results may be related to the interruption of the mitotic process.

In this line, other studies reported the cytogenotoxicity of glyphosate and its impact on the plant-cell cycle. Genotoxic effects, for example, were observed in spiderwort (*Tradescantia* L.) by Alvarez-Moya et al. (2011), as well as a reduction in the mitotic index and an increase in chromosomal aberrations in onion (*Allium cepa* L.), barley (*Hordeum vulgare* L.), and black gram [*Vigna mungo* (L.) Hepper] by Çavuşoğlu et al. (2011), Truta et al. (2011), and Khan et al. (2021), respectively. However, information about the cytotoxic and genotoxic effects on soybean is still lacking.

Monitoring the effects of glyphosate at the cellular level on sensitive and tolerant GM soybean seedlings could provide a more detailed knowledge and understanding of the potential risks inherent to the use of this herbicide, especially considering that its half-life can range from days to several months (Henderson et al., 2010). In addition, glyphosate and its derivatives have been found in soil samples (Karasali et al., 2019; Pelosi et al., 2022), which raises concerns about its possible adverse effects, including for GM cultivars that present tolerance mechanisms. There are also some reports that this herbicide negatively affects both the germination (Gomes et al., 2017) and initial development of seedlings (Santos et al., 2007; Pereira et al., 2018; Costa et al., 2023), also decreasing the physiological quality of the seeds and reducing the yield components of the plants treated with it (Albrecht et al., 2014). This explains the current concerns related to the consequences of the intensive and potentially indiscriminate use of glyphosate, given its constant presence in the cultural practices of GM crops and its possible persistence in soil samples, which could cause damage to diverse living organisms.

The objective of this work was to evaluate the phytotoxic and cytogenotoxic effects of glyphosate on the direct development of soybean seedlings tolerant and sensitive to this herbicide.

Materials and Methods

Three soybean cultivars were selected for the study: one sensitive [Capinópolis (UFV-16)] and two tolerant (P98Y11 and NS 6906 IPRO) to glyphosate, obtained from the germplasm collection of the Soybean Breeding Program at Universidade Federal de Lavras, located in the municipality of Lavras, in the state of Minas Gerais, Brazil.

The germination test was conducted following the recommendations of Regras para Análise de Sementes (Brasil, 2009), with modifications. Germinating paper was moistened with either water (control) or solutions (treatments) with different concentrations of the Roundup Original commercial herbicide (Monsanto do Brasil Ltda., São Paulo, SP, Brazil), composed mainly of 48% g L^{-1} isopropylamine salt of N-(phosphonomethyl) glycine and 35.6% acid equivalent of N-(phosphonomethyl) glycine. The applied concentrations of the herbicide were 0.06

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and 0.12%, obtained by dilution with distilled water. These percentages were chosen because they were investigated previously by other researchers, who observed negative effects on seedlings subjected to germination tests in substrate moistened with glyphosate (Pádua et al., 2012; Melo et al., 2013; Pereira et al., 2018; Costa et al., 2023), as done in the present study.

After prepared, the germination rolls were placed in a biochemical oxygen demand (BOD) incubator, at 25°C, for seven days. Evaluations were performed on the fifth and seventh days after sowing to determine the percentages of normal and abnormal seedlings for both the initial and final counts of germination (Brasil, 2009). The experimental design was completely randomized in a 3x3 factorial arrangement, with three cultivars and three herbicide concentrations (0, 0.06, and 0.12%), with four replicates of 50 seeds each.

The seedling growth test was conducted following the method described by Pereira et al. (2015), with the same modifications as in the germination test, in which the germinating paper was moistened. After seven days in the BOD incubator, the lengths of hypocotyls, radicles, and seedlings were measured using a millimeter ruler. The reduction in hypocotyl and root length was quantified based on the herbicide treatments, using the equation $Y(\%) = ((C - T)/C) \times 100$, where Y represents the reduction (percentage) in hypocotyl, radicle, or seedling length; C is the length (centimeters) in the control; and T is the length (centimeters) in the treatments with the herbicide at 0.06 and 0.12%. The experimental design for the length tests was completely randomized, in a 3x3 factorial arrangement with three cultivars and three herbicide concentrations (0.0, 0.06, and 0.12%). Each treatment had ten replicates of ten seeds. The data were subjected to the analysis of variance, and means were compared by the Tukey test, at 5% probability.

For cell-cycle analyses, samples of 20 seeds were used in a completely randomized design, in a 3x3 factorial arrangement with three cultivars and three glyphosate concentrations (0.0, 0.06, and 0.12%), with five replicates. The roots obtained from the germinated seeds, as described in the germination test, were collected after three, four, five, and seven days in the BOD incubator and immediately fixed in Carnoy's solution (3:1 ethanol: acetic acid) for 24 hours. After cell wall digestion with pectinase:cellulase (100:200U), the roots were hydrolyzed in 1N HCl for 10 min, at 60°C, and stained with Schiff's reagent for 90 min. The slides were prepared by the squash technique (Guerra & Souza, 2002) and evaluated under a light microscope with the AxioCam ERc 5s camera (Carl Zeiss Microscopy GmbH, Gena, Germany).

For each treatment, five slides were evaluated, each with approximately 500 analyzed cells, totaling 2,500 cells. The different stages of the cell cycle were quantified to determine the mitotic index and the rate of abnormalities of the studied genotypes (Leme & Marin-Morales, 2009). Percentage data were transformed using the arcsine square root transformation to perform the analysis of variance. Means were compared using the Scott-Knott test, at 5% probability.

Results and Discussion

Based on the parameters of Regras para Análises de Sementes (Brasil, 2009), an abnormal seedling growth was observed for the Capinópolis (UFV-16) cultivar, sensitive to glyphosate, at the herbicide concentrations of 0.06 and 0.12% (Table 1). However, for the 'P98Y11' and 'NS 6906 IPRO' glyphosate-tolerant genotypes, the percentage of normal seedlings was only affected at the concentration of 0.12%. Adverse effects of 0.12% glyphosate on the development of normal seedlings were also reported for other cultivars tolerant to this herbicide (Bervald et al., 2010; Pereira et al., 2018).

Table 1. Percentages of normal, abnormal, and ungerminated soybean (*Glycine max*) seedlings of three different cultivars under each glyphosate concentration treatment[(1)].

Soybean seedlings	Glyphosate (%)	Cultivars		
		'Capinópolis (UFV-16)'	'Capinópolis (UFV-16)'	'NS 6906 IPRO'
Normal seedlings	0.0	95Aa	79Ab	76Ab
	0.06	0Bb	77Aa	77Aa
	0.12	0Bb	40Ba	43Ba
Abnormal seedlings	0.0	4Bc	15Bb	20Ba
	0.06	99Aa	19Bb	18Bb
	0.12	99Aa	50Ab	50Ab
Ungerminated seeds	0.0	1Aa	6Aa	4Aa
	0.06	1Aa	4Aa	5Aa
	0.12	1Aa	10Aa	7Aa

[(1)]Means followed by equal letters, uppercase in the columns and lowercase in the rows, do not differ by the Tukey test, at 5% probability.

These findings show that the applied glyphosate concentrations affect the development of conventional soybean genotypes, but that higher concentrations can also affect genotypes that are tolerant to this herbicide. Therefore, it is relevant to study the potential impacts of the use of glyphosate even in tolerant cultivars due to the significant effects observed on seedling development depending on the applied herbicide concentration.

The treatments did not affect the percentage of ungerminated seeds, in alignment with the findings of other authors (Bervald et al., 2010). Therefore, as previously noted, glyphosate does not compromise germination but rather the development of normal seedlings. Although the herbicide inhibits the action of the EPSPS enzyme, hindering the biosynthesis of aromatic amino acids and various other metabolites (Sammons & Gaines, 2014; Ruszkowski & Forlani, 2022), the seed's reserves of amino acids are sufficient for germination. However, it is possible to conclude that seedling development is impaired when the availability of aromatic amino acids becomes limited and the EPSPS enzyme is inhibited by the herbicide, as illustrated by the percentage of abnormal seedlings in the germination test.

The sensitivity of the Capinópolis (UFV-16) cultivar to glyphosate was evidenced by the morphology of its seedlings, particularly the radicle (Figure 1 A–D). During germination, the primary root emerges from the seed as a radicle, and, subsequently, secondary roots develop from the primary root (Torrion et al., 2012). Considering this sequence of events, glyphosate negatively affected the development of the root system, especially of the secondary roots, whose formation is the primary morphological characteristic that distinguishes glyphosate tolerance from sensitivity in cultivars (Pereira et al., 2018). This finding provides evidence of the inhibition of the shikimate pathway and, consequently, of the biosynthesis of tryptophan, an essential cofactor for root development. Tryptophan is converted into indoleacetic acid through various metabolic pathways and plays a crucial role in the

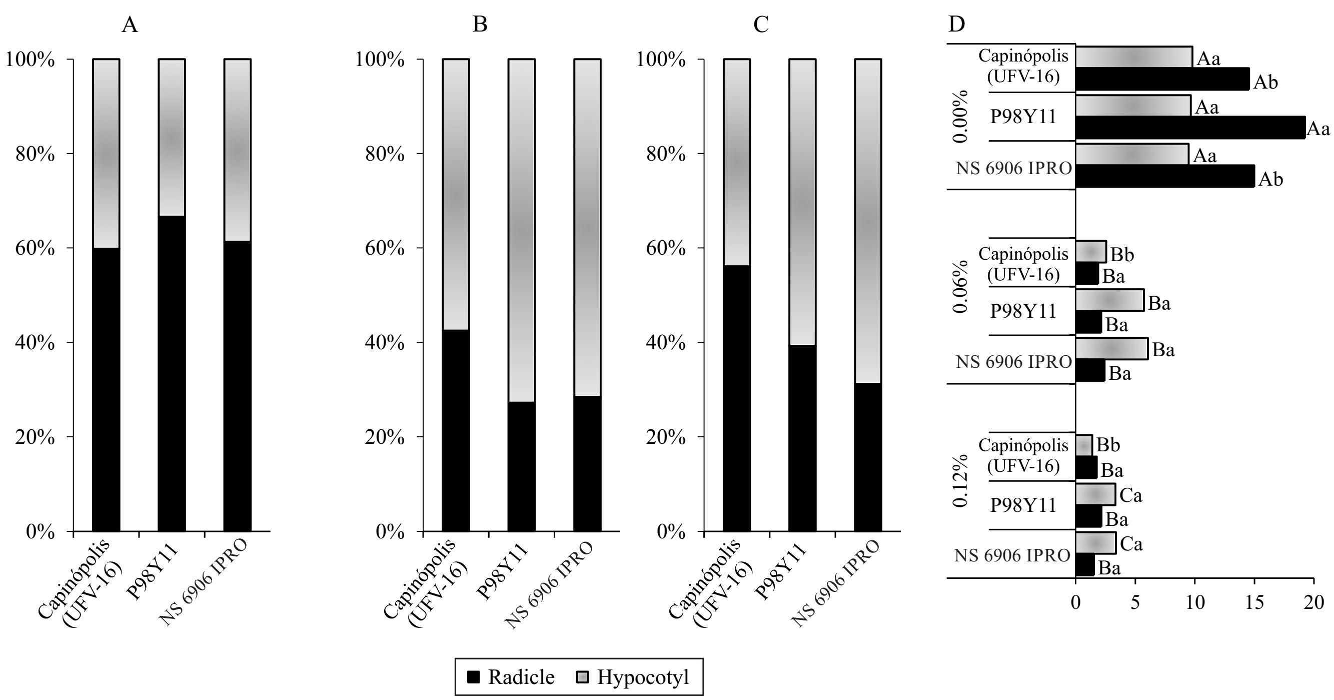

Figure 1. Soybean (*Glycine max*) seedling architecture regarding the proportion (%) between hypocotyl and radicle in the different treatments with the glyphosate herbicide at the concentrations of 0% (A), 0.06% (B), and 0.12% (C), as well as the average length (cm) of the hypocotyl and radicle in the different treatments (D). Means followed by equal letters do not differ from each other by the Tukey test, at 5% probability. Uppercase letters compare the effects of herbicide concentration within each soybean cultivar, while lowercase letters compare cultivars to each other at the same herbicide concentration.

initiation and establishment of the root-shoot axis in plants (Hu et al., 2021). Therefore, considering that transgenic cultivars have a version of the CP4-EPSPS enzyme that is tolerant to glyphosate (Pline-Srnic, 2006), it is likely that the tryptophan biosynthesis is maintained, leading to a normal rooting.

The average lengths of the hypocotyls, radicles, and seedlings decreased due to the concentration of the herbicide (Figure 1 D). Under the glyphosate treatments, both tolerant cultivars showed seedlings with larger hypocotyls, whereas the three evaluated genotypes presented a similarly reduced main-root length (Costa et al., 2023). Therefore, root length alone is an inadequate parameter for discriminating genotypes as to their tolerance or sensitivity to glyphosate.

In the control treatment (0% glyphosate), the average length of the radicles was greater than that of the hypocotyls, roughly in a ratio of 2/3 to 1/3 (Figure 1 A). However, in the glyphosate treatments, the radicles became shorter than the hypocotyls, indicating a change in the morphological structure of the seedlings under the experimental conditions (Figure 1 B and C), as also found by Costa et al. (2023).

When testing the rate of reduction in seedling length as a function of each herbicide concentration (0.06 and 0.12%), the radicles were generally more affected than the hypocotyls (Figure 2). At both concentrations, the rate of reduction in hypocotyl length was higher for cultivar Capinópolis (UFV-16) than for P98Y11 and NS 6906 IPRO. Other authors also reported a reduction in seedling length due to glyphosate application (Bertagnolli et al., 2006; Pereira et al., 2009; Zonetti et al., 2011; Pádua et al., 2012; Melo et al., 2013). The reduction in radicle length, however, was higher for cultivar P98Y11 at the concentration of 0.06%, but similar (above 80%) for all three cultivars at the concentration of 0.12%. The highest reduction in total length was observed for seedlings of the sensitive cultivar under both herbicide concentrations. These results are an indicative that the addition of glyphosate to germinating paper compromises the development of

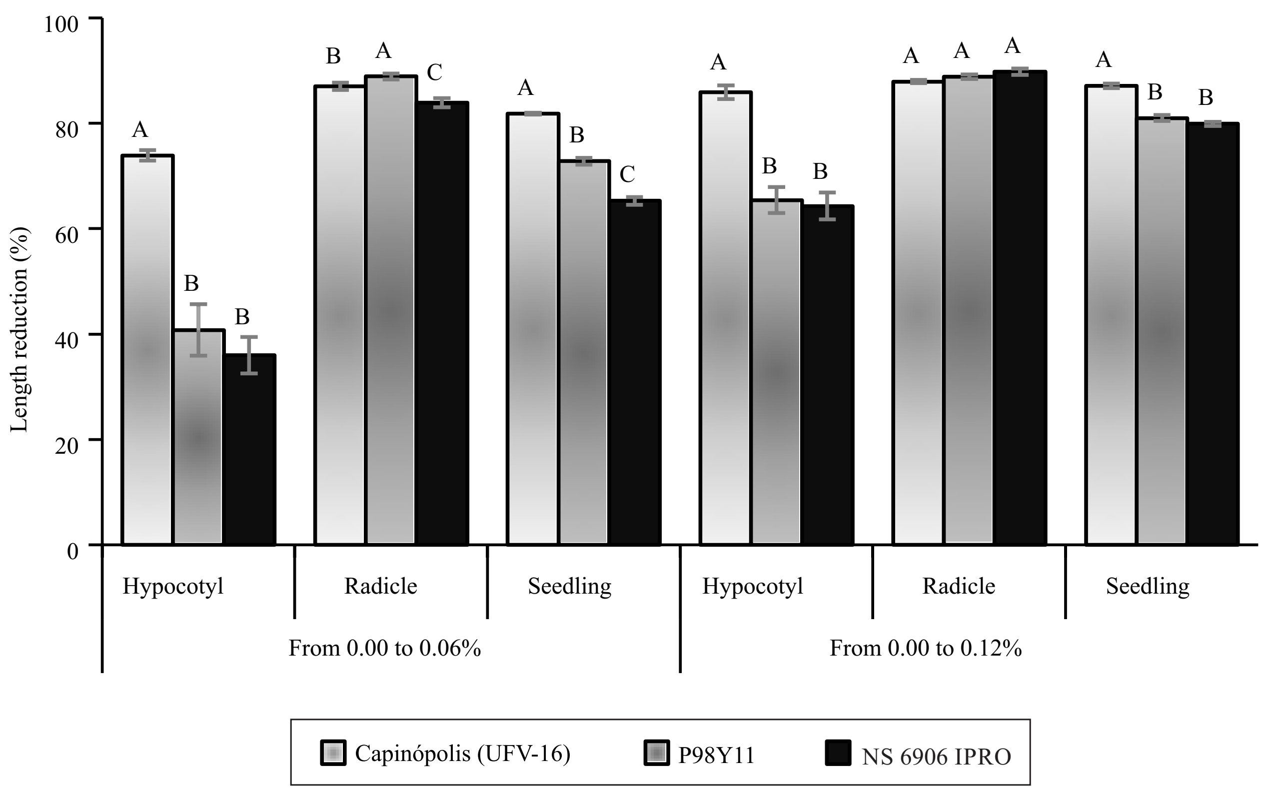

Figure 2. Percentages of reduction in hypocotyl, radicle, and seedling length as a function of the applied herbicide concentration (0.06 and 0.12% glyphosate) in relation to the control (0.0% glyphosate). Means followed by the equal letters do not differ from each other by the Tukey test, at 5% probability.

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the seedlings in the germination test. It should be noted that only tolerant seedlings developed secondary roots (Bertagnolli et al., 2006; Pereira et al., 2009; Pádua et al., 2012; Melo et al., 2013).

The analysis of the mitotic index showed three distinct patterns (Figure 3). The first occurred in the control treatment on the third, fourth, and fifth days after sowing (Figure 3 A–C), with no significant differences among the three studied genotypes, except on the seventh day when the index of the sensitive genotype was higher than that of both tolerant ones (Figure 3 D). The second pattern was observed in the treatments with the herbicide, in which the tolerant genotypes showed a higher mitotic index than the sensitive one, regardless of the applied concentration. The third pattern represented the individual effects of glyphosate on the mitotic index of each genotype. Therefore, the herbicide had a detrimental effect on this index, with significant effects on all evaluation days, except on the seventh day for the 'NS 6906 IPRO' genotype. These results show that, although glyphosate affected the cell division rates in all studied soybean cultivars, the sensitive one is the most impacted. Moreover, the tolerant cultivars showed a higher index in the glyphosate treatments, whereas all evaluated cultivars did not differ significantly in the control treatment.

In the literature, the suppressive effects of glyphosate on the development of sensitive soybean seedlings have also been reported (Bervald et al., 2010;

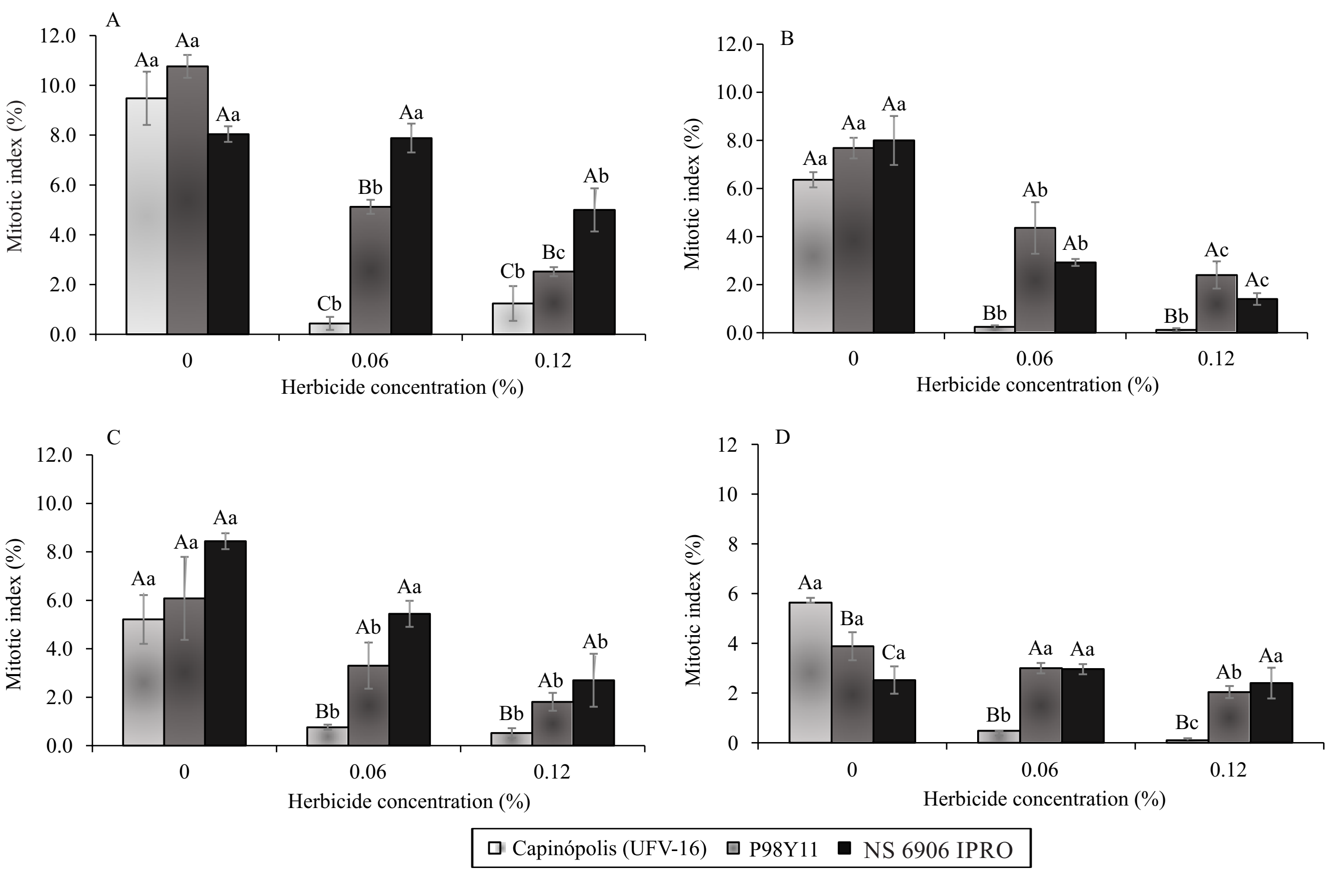

Figure 3. Effect of glyphosate on the mitotic index in seedlings of soybean (*Glycine max*) cultivars Capinópolis (UFV-16), P98Y11, and NS 6906 IPRO subjected to the germination test with 0.0, 0.06, and 0.12% glyphosate on the third (A), fourth (B), fifth (C), and seventh (D) days after sowing. Standard error bars of the means are shown. Uppercase letters compare the genotypes to each other within each treatment, while lowercase letters compare the same genotype under different treatments by the Scott-Knott test, at 5% probability.

Melo et al., 2013), but without cell-cycle analyses. In addition, similar mitodepressive effects of this herbicide have been observed in other species such as onion (Çavuşoğlu et al., 2011), buckwheat (*Fagopyrum esculentum* Moench) (Kumar & Srivastava, 2015), barley (Truta et al., 2011), and black gram (Khan et al., 2021). As mentioned previously, glyphosate inhibits the action of EPSPS, negatively impacting the biosynthesis of aromatic amino acids and other secondary metabolites in plants (Ruszkowski & Forlani, 2022). Khan et al. (2021) pointed out that the scarcity of these amino acids due to glyphosate exposure may disrupt protein synthesis, enzyme activities involved in the cell cycle, DNA replication, and microtubule dynamics.

On all evaluation days, the expressive reduction in the mitotic index in the sensitive seedlings was accompanied by a significant increase in their rate of abnormalities. In the control treatment, no significant differences were observed among the evaluated genotypes on the third, fourth, and fifth days after sowing (Figure 4 A–C), but, on the seventh day, 'P98Y11' showed a lower rate of abnormalities (Figure 4 D). Overall, this rate increased with the application of the herbicide in the seedlings sensitive to it, but was similar in the tolerant genotypes under the different treatments, with some variations. Notably, on the fifth day, the 'P98Y11' genotype showed more abnormalities in the control and 0.06% herbicide treatments than at the highest herbicide concentration of 0.12% (Figure 4 C).

The reduction in the mitotic index and the increase in the abnormality rates in glyphosate-sensitive seedlings suggest a slowdown or halt in their development, which was not observed in tolerant genotypes. Part of the responses of the sensitive seedlings can be explained by the aforementioned effects of glyphosate at the cellular level, interfering in organogenesis, especially in the roots. Contrastingly, tolerant cultivars have mechanisms that enable them to maintain a certain stability in mitotic divisions even under the pressure

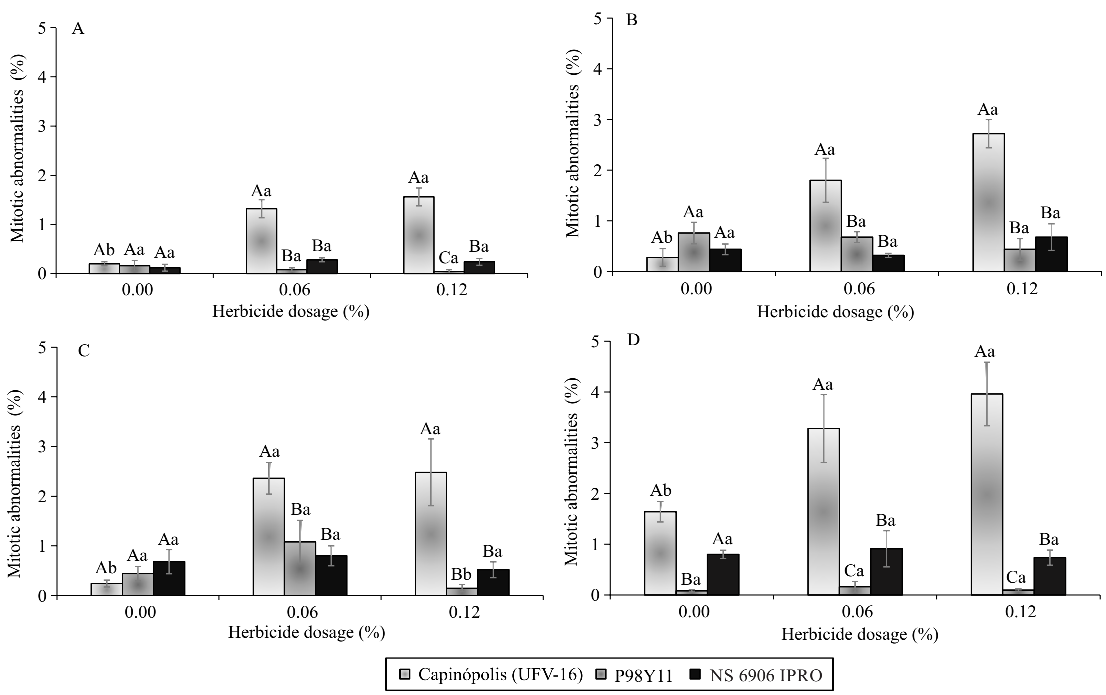

Figure 4. Effect of glyphosate on the mitotic abnormalities in seedlings of soybean (*Glycine max*) cultivars Capinópolis (UFV-16), P98Y11, and NS 6906 IPRO subjected to the germination test with 0, 0.06, and 0.12% glyphosate on the third (A), fourth (B), fifth (C), and seventh (D) days after sowing. Standard error bars of the means are shown. Uppercase letters compare genotypes to each other within each treatment, while lowercase letters compare the same genotype under different treatments by the Scott-Knott test, at 5% probability.

of the herbicide, i.e., the influence of glyphosate on the seedling cell cycle.

The occurrence of non-oriented chromosomes, c-metaphases, abnormal mitotic spindle formation, and micronuclei shows the cytotoxic potential of glyphosate (Figure 5), as reported in other studies (Alvarez-Moya et al., 2011; Çavuşoğlu et al., 2011; Truta et al., 2011; Kumar & Srivastava, 2015; Khan et al., 2021). These abnormalities can cause problems in chromosome orientation, segregation, and mitotic spindle arrangement, leading to aneuploidy and showing the aneugenic effect of glyphosate.

C-metaphases and spindle formation issues were also observed in buckwheat (Kumar & Srivastava, 2015) and black gram (Khan et al., 2021) after exposure to glyphosate, whereas the aneugenic effects of the herbicide were also reported for barley (Truta et al., 2011). Regarding micronuclei, their presence may be associated with the loss of one or more chromosomes, confirming the aneugenic action of glyphosate, or with the formation of fragments resulting from breaks, indicating a clastogenic effect (Leme & Marin-Morales, 2009) (Figure 5 D and G). Another indicator of herbicide toxicity that directly affects root growth is the presence of meristematic cells with an excessively condensed nuclei (Figure 5 I), suggesting apoptosis.

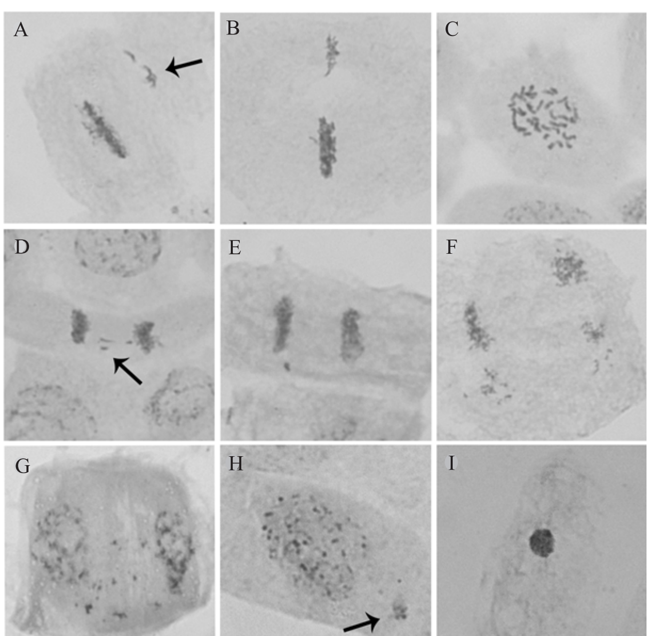

Figure 5. Cell cycle changes observed in cells of the root tip of soybean (*Glycine max*) cultivars exposed to glyphosate, showing: A, metaphase with non-oriented chromosome (arrow); B, two parallel and asymmetric metaphase plates; C, c-metaphase; D, anaphase with chromosome and delayed fragments (arrow); E, telophase with micronucleus; F, telophase with abnormal spindle; G, cytokinesis with micronuclei; H, interphase with micronucleus (arrow); and I, interphase nucleus with a high degree of condensation. Bar: 10 μm.

In summary, the data on the mitotic index and abnormality rates highlighted the cytological and genetic damage caused by glyphosate to sensitive genotypes. In contrast, less severe effects were observed on the cell division and abnormality rates of tolerant genotypes.

Conclusions

1. The glyphosate treatments affect the morphology of soybean (*Glycine max*) seedlings in terms of the proportion between hypocotyl and radicle, especially by severely impacting root development.
2. Both the length of the hypocotyl and the presence of secondary roots differentiate seedlings of glyphosate-tolerant genotypes from those of sensitive ones.
3. Glyphosate significantly influences the cell cycle of soybean seedlings, with specific implications for the sensitive cultivar, which has lower mitotic indices and higher abnormality rates.
4. Tolerant seedlings present unchanged abnormality rates despite the mitodepressive effect of the glyphosate treatments.

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