Original Research Article

Cytogenetic activity of root meristems of rice in response to conditioning in carrot extract and salinity

Sheila Bigolin Teixeira[1]*, Stefânia Nunes Pires[2], Gabriele Espinel Ávila[2], Diogo da Silva Moura[2], Rejane Peter[3], Vera Lucia Bobrowski[4], Sidnei Deuner[4]

[1] Postgraduate Program in Seed Science and Tecnology, Faculty of Agronomy 'Eliseu Maciel', Federal University of Pelotas (FAEM-UFPel), Pelotas, RS, Brazil.

[2] Postgraduate Program in Plant Physiology, Federal University of Pelotas (UFPel), Pelotas, RS, Brazil.

[3] Postgraduate Program in Animal Biology, Federal University of Pelotas (UFPel), Pelotas, RS, Brazil.

[4] Department of Botany, Federal University of Pelotas (UFPel), Pelotas, RS, Brazil.

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ABSTRACT

Aims: This study was conducted to evaluate the role of *Daucus carota* L. root extract in cytogenetic parameters of Oryza sativa L. seeds exposed to salinity.
Study design: The experiment installed was in a completely randomized design, in a 2x4 factorial scheme, with four replicates.
Place and Duration of Study: The experiment was conducted between March and June 2017, at the Laboratory of Seed Physiology and at the Laboratory of Genetics both belonging to the Universidade Federal de Pelotas (UFPel), in Capão do Leão, RS, Brazil.
Methodology: Seeds of cultivars BRS Querência (indica subgroup) and BRS 358 (japonic subgroup) were soaked at concentrations of 0% (water), 25%, 50% and 100% of aqueous extract of carrot for 48h. Then, were germinated on germitest paper moistened with solutions of 0, 25, 75 and 150 mM NaCl and placed in a germination chamber under a mean temperature of 25 ºC. The evaluation of mitotic index was performed in meristem cells of rice roots when they reached 0.5 to 1 cm in length.
Results: An increase in mitotic index was observed with the use of increasing doses of extract for cv. BRS 358, mainly by increasing the number of prophase cells. The salinity affected the two cultivars differently, with increase in the mitotic index for cv. BRS Querência and decrease for cv. BRS 358, but in this, the use of 50% of extract increased the index of cell division even with the increase of salinity. In addition, salinity above 75 mM caused an increase in the number of chromosomal alterations when associated to use of 50% of extract.
Conclusion: The results show that there is positive effect of the use of carrot extract on the mitotic index of root meristems of rice, cv. BRS 358, when germinated in the presence or absence of salt stress.

Keywords: Oryza sativa L., Daucus carota L., salinity, mitotic index.

1. INTRODUCTION

Plants during their growth and development are exposed to several abiotic factors that, depending on the climatic conditions, such factors can constitute stressful conditions, the main of them, extreme temperatures, water deficit or high salinity [1]. The average yields of the main cultivated plants are reduced by more than 50% by the occurrence of stress conditions [2]. Salt stress is one of the main agents that seriously affects crop production worldwide. More than 800 million hectares of arable land are affected by salinity [3]. The use of low-quality water for irrigation and poor drainage of soils

increases salt content in growing areas, directly affecting the productivity of important crops such as rice.

Rice is one of the most consumed foods in the world, and estimates show that, with the increasing world population, demand for this cereal tends to double by 2050, necessitating the adoption of new management strategies for maintaining crop productivity, since yield and quality of rice are influenced by a series of biotic and abiotic stress factors [4].

Thus, soil and water management practices can be used to reduce soil salinization, but are usually associated with very high costs. Therefore, sustainable production of crops in saline soils requires alternative approaches such as the creation of salinity-tolerant varieties or management techniques that can reduce the effects of stress. Research activity in terms of agricultural systems has been oriented for years to increase crop yields without regard to product quality and rational use of resources. In contrast, today the attention is mainly focused on product quality and the sustainability of cropping systems [5]. Due to the difficult task of preventing damage caused by stress, plant protection should be based on the stimulation of its growth and development, which can be achieved through the application of biostimulants. Plant biostimulators or phytostimulators are non-toxic substances of natural origin that enhance and stimulate plant life processes without altering their natural pathway [6].

Some plants produce compounds of the secondary metabolism that act by inhibiting or favouring the germination process as well as the process of cell division, as in the case of carrots. Chemical analyzes of the extract of carrot roots have presented high content of vitamin A (β-carotene), hormones, amino acids, proline, sugar, protein, carbohydrates, macro and micronutrients, phenolic compounds, fats and fibers [7] which, associated with another plant can favor its development under stress due to its composition rich in antioxidants and nutrients. Phenolic compounds, as effective antioxidants at low concentrations, influence many biochemical and physiological processes in plants [8]. Thus, studies regarding the effect of allelochemicals, negative or positive, of a plant on the germination and/or development of another are secondary manifestations of processes occurred at molecular and cellular level initially [9,10].

Although numerous studies have addressed the effect of saline stress on the growth and development of the most diverse crops of agricultural importance, little is known about cell division and how the use of plant extracts acts in the salinity tolerance process. The objective of the present study was to evaluate the effect of the application of carrot extract on the conditioning of rice seeds on mitotic activity and chromosomal damage in root meristems of seedlings as well as to investigate their capacity to mitigate the effects of salinity (NaCl).

2. MATERIAL AND METHODS

The experiment was conducted at the Laboratory of Seed Physiology located at the Department of Botany and at the Laboratory of Genetics located at the Department of Ecology, Zoology and Genetics, both belonging to the Biology Institute of the Federal University of Pelotas (UFPel), in Capão do Leão, RS, Brazil. Seeds of two rice cultivars of local interest were used: BRS Querência and BRS 358, from EMBRAPA – Clima Temperado/Estação Experimental Terras Baixas.

For the experiment, seeds were subjected to disinfestation with sodium hypochlorite at 2% for 5 minutes, washed in distilled water and then placed in a beaker to soak in 0%, 25%, 50% and 100% aqueous extract of carrot, for 48 hours. To obtain the extract, freshly harvested carrots, and its roots, after cleaning, were crushed and centrifuged (model Mondial premium), on the day of its use. The resulting extract was diluted in distilled water to obtain the different established percentages, being characterized by pH, osmotic potential (Ψs) and electrical conductivity (EC) (Table 1).

Table 1. Characterization of the carrot extract as pH, osmotic potential (Ψs) and electrical conductivity (EC).

Carrot extract (%)	pH	Ψs (MPa)	EC (mS cm^{-1})
25	6.04	-0.13	1.19
50	6.04	-0.31	1.98

100	6.04	-0.61	3.63

After soaking, the seeds were arranged in rolls of germitest paper, moistened at a volume of 2.5 times its weight with water, to evaluate the effect of the carrot extract alone.

For the analysis of the protective effect of carrot extract, seeds soaked in 0% and 50% of extract were also sown on germitest paper moistened with in three different salt concentrations, 25, 75 and 150 mM sodium chloride (NaCl). The germination was conducted in B.O.D. type chamber, set at 25 °C.

The effects of salts and carrot extract were observed on cytogenetic activity in rice root meristematic cells, analyzing mitotic index (MI), mitotic phase index (prophase, metaphase, anaphase and telophase), presence of chromosomal alterations and the interface index. Were observed 250 cells per slide, with 8 replicates, totaling 2000 cells per treatment.

The analyzes were carried out by the crushing technique [11]. Therefore, the seedlings were collected after the protrusion of the radicle (between 0.5 and 1 cm in length), from the second to the fourth day of sowing, and fixed in Carnoy 3:1, remaining at room temperature for a period of 2 to 24 hours. Afterwards, they were stored in a freezer for later microscopic analysis. For the preparation of the slides, the radicles were immersed in distilled water for 5 minutes. Because the cell wall of the rice cells was fairly rigid, the rootlets were dipped in cellulase-pectinase solution (2:20% v/v) for 10 minutes prior to hydrolysis and then placed in 5.0 N hydrochloric acid (HCl) for a period of 15 minutes, returning to the distilled water for another 5 minutes. Subsequently, the radicles were transferred to slides and under a stereomicroscopic microscope the hood was removed to obtain the root meristem, being stained with 2% acetic orcein, placed on the crushed material and then heated. The slides of each treatment were analyzed by scanning method in an optical microscope at a magnitude of 400x.

The experimental was conduted completely randomized design with four replicates. The results of mitotic indexes and chromosomal changes were analyzed by polynomial regression using the statistical program SigmaPlot 12.0. The mean values of the phase indices as a function of the extract and the salt were compared by the Tuckey test at 5% of probability, using the software Rbio [12].

3. RESULTS

The cytogenetic analysis of the root meristematic rice cells subjected to the carrot extract in the different percentages showed that there was a significant effect for the mitotic index variable (IM) only in cv. BRS 358, for which a 32% increase was observed for 56% cells dividing from 0% to 100% extract. For cv. BRS Querência results remained between 47 and 49% (Figure 1).

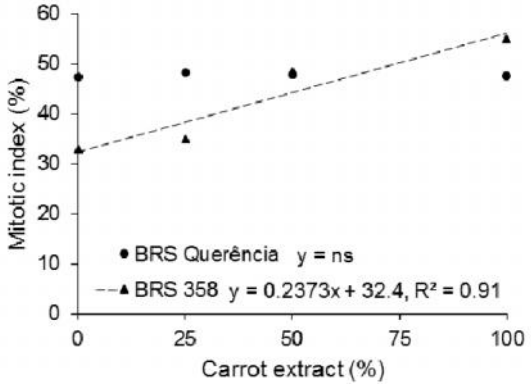

Figure 1. Mitotic index in root meristems of two rice cultivars, BRS Querência and BRS 358, subjected to the seeds conditioning in carrot extract.
ns: not significant

When the phase index of the cell cycle was considered, no significant difference was observed in the prophase (P) and metaphase (M) index in BRS Querência, but with the use of 25% of extract the percentage of cells in anaphase and telophase was significantly higher in relation to 50% of carrot extract (Figure 2A). In cv. BRS 358 there was a significant increase of prophase cells at 50 and 100% of extract and of cells in anaphase at 25% and 100% of extract (Figure 2B).

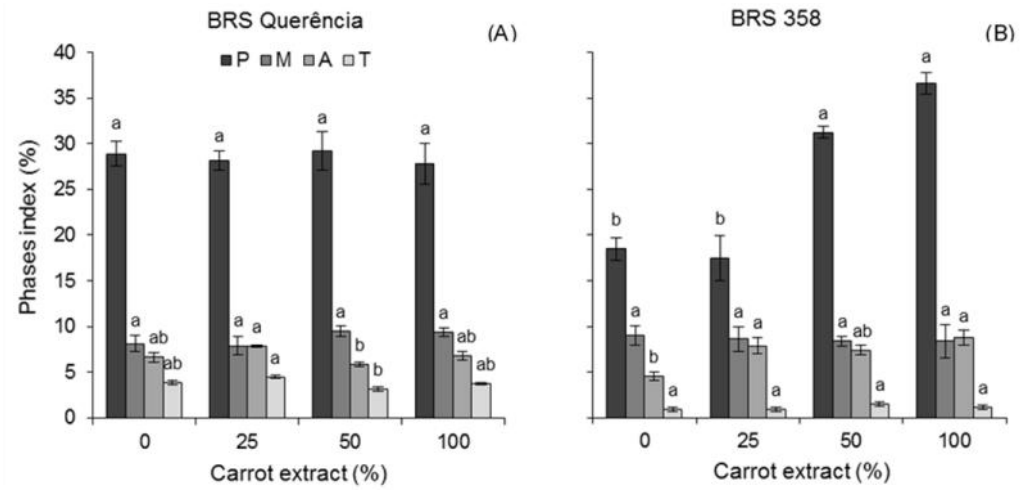

Figure 2. Phases index of cell division (P-Prophase, M-Metaphase, A-Anaphase and T-Telophase) in root meristems of two rice cultivars, (A) BRS Querência and (B) BRS 358 subjected to the seeds conditioning in carrot extract.

Means followed by the same letters, comparing each index alone in the different percentages of extract, didn't differ by Tukey's test (P <.05). Bars represent the standard error of the mean of four replicates.

The cytogenetic analysis of root meristematic cells of rice after soaking of seeds in 0% and 50% of carrot extract, followed by germination at increasing salt concentrations, showed different behavior between the two cultivars. For cv. BRS Querência, there was a significant effect of salt only when the seeds were soaked in water without carrot extract, with higher values in the 150 mM NaCl concentration (Figure 3A). Conversely, the cv. BRS 358 when imbibed in water, expressed reduction in mitotic index in response to salinity, but when associated with 50% of carrot extract, there was quadratic behavior with a minimum point in 58 mM NaCl (Figure 3B).

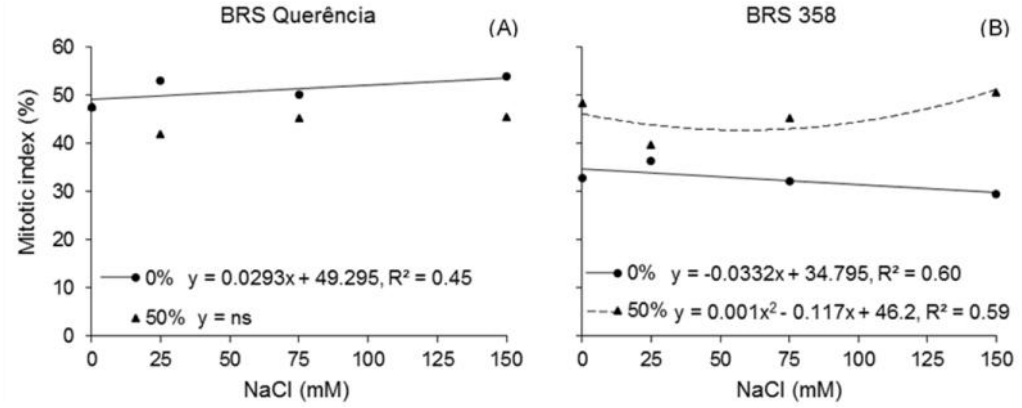

Figure 3. Mitotic index in root meristems of two rice cultivars, BRS Querência (A) and BRS 358 (B), subjected to the seeds conditioning in carrot extract and germinated under different salt concentrations.

ns: not significant.

For the phases index of mitosis in response to saline treatments, analyzing cv. BRS Querência, when the seeds were only soaked in water, there was no significant difference in the percentages of cells in prophase and telophase, however, there was a significant increase in metaphase and anaphase cells in the treatment with 25 mM NaCl (Figure 4A). Similarly, when soaked in 50% of extract, there was also significant difference only on the percentage of cells in metaphase and anaphase, but in a different way (Figure 4B). For metaphase, the concentration of 25 mM NaCl had lower percentage of cells in this phase and in anaphase the highest percentage was observed with 75 mM of NaCl.

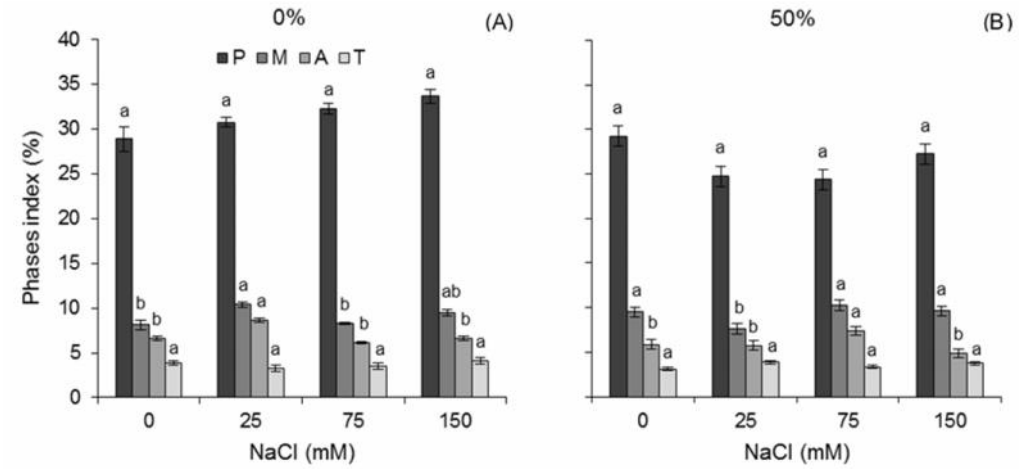

Figure 4. Phases index of cell division (P-Prophase, M-Metaphase, A-Anaphase and T-Telophase) in root meristems of the cultivar BRS Querência subjected to the seeds conditioning in 0% (A) and 50% (B) of carrot extract and germinated under different salt concentrations.

Means followed by the same letters, comparing each index alone in the different percentages of extract, didn't differ by Tukey's test (P <.05). Bars represent the standard error of the mean of four replicates.

Comparing the seeds soaked in water and those soaked in 50% of carrot extract for each germination condition (saline treatment) of cv. BRS Querência (Table 2). It was observed that in the presence of carrot extract the prophase cell index was significantly lower in the saline treatments, and there was a significant increase in response to the extract only in the percentage of cells in metaphase (zero and 75 mM treatments NaCl) and anaphase (75 mM NaCl).

Table 2. Interphase index and phases index of the cell division of root meristems of cultivar BRS Querência, subjected to the seeds conditioning in 0% and 50% of carrot extract and germinated under different salt concentrations.

	Carrot extract (%)	NaCl concentration (mM)			
		0	**25**	**75**	**150**
Interphase (%)	0	52.2 a	46.6 b	49.5 b	45.7 b
	50	52.0 a	57.6 a	54.6 a	53.4 a
	Mean	52.1	52.1	52.1	49.6
	CV (%)	4.8			
Prophase (%)	0	28.9 a	30.7 a	32.2 a	33.6 a
	50	29.2 a	24.7 b	24.4 b	27.2 b
	Mean	29.1	27.7	28.3	40.4
	CV (%)	10.1			
Metaphase (%)	0	8.1 b	10.4 a	8.2 b	9.5 a
	50	9.5 a	7.6 b	10.2 a	9.6 a
	Mean	8.8	9.0	9.2	9.6
	CV (%)	9.6			
Anaphase (%)	0	6.6 a	8.6 a	6.1 b	6.6 a
	50	5.9 a	5.7 b	7.4 a	4.9 b
	Mean	6.2	7.2	6.7	5.7
	CV (%)	10.1			
Telophase (%)	0	3.87 a	3.25 a	3.50 a	4.12 a
	50	3.12 a	3.87 a	3.37 a	3.75 a
	Mean	3.50	3.56	3.44	3.94
	CV (%)	17.5			

** Means followed by the same letter in the column, for each variable, didn't differ by Tukey's test (P <.05).*

For cv. BRS 358 when the seeds were soaked in water, the prophase cell index was significantly higher in the treatment with 150 mM NaCl, however, high salt concentration prevented the increase in

the percentage of cells in the other phases of mitosis (metaphase, anaphase and telophase) (Figure 5A). In response to carrot extract (50%), even at high salt concentrations (75 and 150 mM NaCl), there was a significant increase in the percentage of cells in telophase, the final phase of mitosis (Figure 5B).

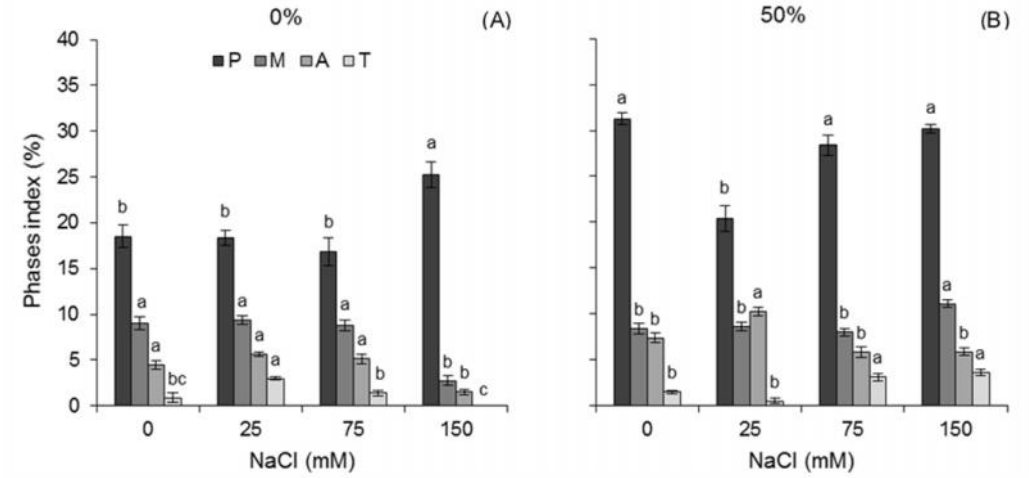

Figure 5. Phases index of cell division (P-Prophase, M-Metaphase, A-Anaphase and T-Telophase) in root meristems of the cultivar BRS 358 subjected to the seeds conditioning in 0% (A) and 50% (B) of carrot extract and germinated under different salt concentrations.

Means followed by the same letters, comparing each index alone in the different percentages of extract, didn't differ by Tukey's test (P <.05). Bars represent the standard error of the mean of four replicates.

Still, in the comparison of the soaking treatments (zero and 50%), it can be confirmed that for cv. BRS 358 the carrot extract has been more significantly in the cell division process. In general, mainly for the highest saline concentrations, seeds that were soaked in 50% of carrot extract increased the percentage of cells in prophase, metaphase, anaphase and telophase (Table 3).

Table 3. Interphase index and phases index of the cell division of root meristems of cultivar BRS Querência, subjected to the seeds conditioning in 0% and 50% of carrot extract and germinated under different salt concentrations.

	Carrot extract (%)	NaCl concentration (mM)			
		0	25	75	150
Interphase (%)	0	67.1 a	63.6 a	67.9 a	70.5 a
	50	51.4 b	60.2 a	54.0 b	49.1 b
	Mean	59.2	61.9	60.9	59.8
	CV (%)	5.9			
Prophase (%)	0	18.5 b	18.4 a	16.9 b	25.2 b
	50	31.2 a	20.4 a	28.4 a	30.1 a
	Mean	24.9	19.4	22.6	27.7
	CV (%)	6.3			
Metaphase (%)	0	9.0 a	9.4 a	8.7 a	2.7 b
	50	8.4 a	8.6 a	8.0 a	11.1 a
	Mean	8.7	9.0	8.4	6.9
	CV (%)	13.5			
Anaphase (%)	0	4.5 b	5.6 b	5.1 a	1.5 b
	50	7.4 a	10.2 a	5.9 a	5.9 a
	Mean	5.9	7.9	5.5	3.7
	CV (%)	13.7			
Telophase (%)	0	0.87 a	3.00 a	1.37 b	0.00 b
	50	1.50 a	0.50 b	3.12 a	3.62 a
	Mean	1.19	1.75	2.25	1.81
	CV (%)	33.8			

**Means followed by the same letter in the column, for each variable, didn't differ by Tukey's test (P <.05).*

With regard to the chromosomal alterations index found in dividing cells, it had no significant effect on the use of aqueous extract of carrot roots for any of the cultivars (Figure 7A). However, when the seeds were soaked in water (0%) and 50% of carrot extract, germinated at increasing salt concentrations, there was a quadratic effect in the chromosomal alterations index for cv. BRS Querência, with the use of 50% of extract, presenting a minimum point at 63.5 mM NaCl, which was not observed when the seeds were soaked only in water (Figure 7B). For cv. BRS 358 showed a similar response, and the seeds soaked in 50% of the extract presented quadratic behavior with a maximum point at 75 mM NaCl (Figure 7C).

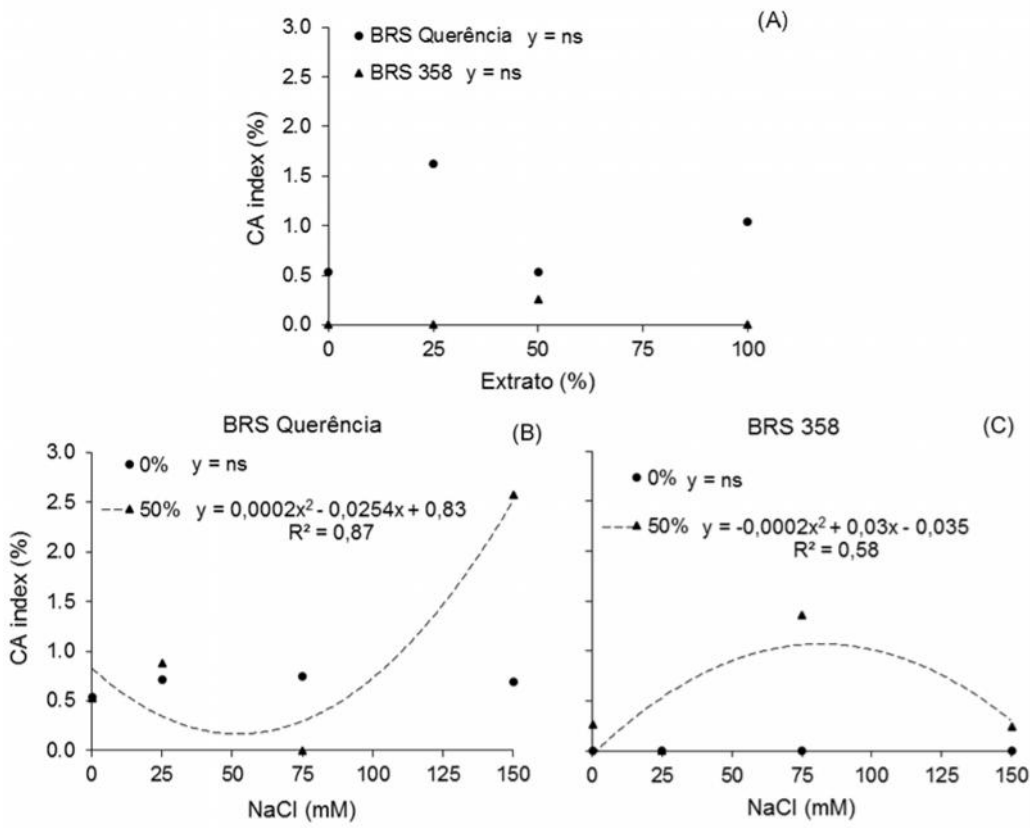

Figura 7. Chromosomal alterations index (CA) in root meristems of two rice cultivars. (A) Effect of soaking seeds of BRS Querência and BRS 358 in carrot extract at different percentages; (B) Effect of soaking seeds of cv. BRS Querência in water (0%) and 50% of carrot extract and germinated in different salt concentrations; C) Effect of soaking seeds of cv. BRS 358 in water (0%) and 50% of carrot extract and germinated at different salt concentrations.

4. DISCUSSION

Cells responses to stress include changes in the cell cycle and cell divisions, changes in the endomembrane system and vacuolization, as well as changes in cell wall architecture, which contributes to enhance the stress tolerance [13]. The frequency of cell division is defined by the mitotic index (IM), which also demonstrates cellular proliferation rate and root growth rate [14,15]. Changes and/or inhibition in mitotic activities are generally used to measure cytotoxicity effects on plants under stress. IM is a reliable parameter to estimate the frequency of cell division [16].

In this study, the soaking of rice seeds in carrot extract induced an increase in the mitotic index of the root cells of cv. BRS 358 (Figure 1). This was mainly due to the passage of the cells that were interphase, to the first phase of cell division, evidenced by the increase in the prophase index (Figure 2B). It was also observed that a greater number of cells reached anaphase in the presence of the extract. Similar results were observed in cv. BRS Querência, in which the use of 25% of extract

228 increased the anaphase and telophase index, demonstrating that the use of the extract made more
229 cells to reach the final stages of cell division (Figure 2A).

230 The cell cycle comprises the period of interphase and mitosis, consisting of a series of coordinated
231 periodic events, whereby the cellular material is duplicated and divided between the daughter cells
232 [17]. The interphase is characterized by a marked cellular activity, being divided into three phases,
233 G1, S and G2. The G1 phase is the period of intense biochemical activity, is characterized by the
234 growth and replication of the cytoplasmic organelles. The S phase is the period of DNA synthesis and
235 several proteins associated with this molecule. The G2 phase is the period in which the structures
236 directly involved with mitosis are formed, such as spindle fibers [18]. DNA replication is initiated after
237 the transition of the G1/S phases, and after duplication of the genetic material the mechanical
238 structures allow the separation of the chromosomes, starting with the cytoskeleton reorganization in
239 the G2 phase. Cell cycle initiation involves not only DNA synthesis but also the regeneration of the
240 microtubular cytoskeleton [19].

241 The germination of the seeds is an excellent model for the study of the cell cycle in plants because
242 the imbibition of the seeds consists in the resumption of the cell cycle progression in the meristematic
243 cells representing an important control in the initial development of the seedlings [17,20].

244 Salt, in this study, affected the mitotic index of the rice roots differently for the two cultivars tested,
245 with a reduction in the mitotic index for cv. BRS 358 and increase for BRS Querência (Figure 3A and
246 3B), but there was an increase in the prophase at 150 mM NaCl in both cultivars, with a decrease in
247 the number of cells in the other phases, mainly for cv. BRS 358 (Figure 4A and 5A). This is due to the
248 blocking of cell division in prophase or prolonged prophase, strongly delaying the new phases, which
249 decreases the indices of the other phases [21]. These data corroborate with authors [22], who noticed
250 an increase in prophase frequency in the root cells of three wheat cultivars exposed to salt stress.

251 The salt stress causes overproduction of ROS, and this imbalance in ROS causes a significant delay
252 in the transition from prophase to prometaphase, which affects chromosome separation in anaphase,
253 chromosomal movement, and nuclear involvement dynamics [23]. Oxidative stress accelerates cell
254 division displacement zone for elongation zone and differentiation resulting in shortened meristem
255 [24,25].

256 In addition, salt may be lowering MI by inhibition of protein synthesis, DNA synthesis or effect on the S
257 or G2/M phase in the cell cycle and reduction of cyclin-dependent kinase (CDK) activity [26].

258 The studies currently seem to be consistent that the MI decreases with the application of different
259 concentrations of NaCl in maize [27], barley [28,29], sorghum [30] and onion [31]. In addition to saline
260 stress, irrigation with wastewater causes a decrease in MI in different plant species, such as wheat
261 [32]. However, in studies [33], the salt concentration of 150 mM NaCl markedly increased the mitotic
262 activity of root tip cells of Centaurea ragusina, however, higher salt concentrations showed total
263 absence of mitotically active root cells and induced massive damage of membrane and cytoplasm,
264 indicating high cytotoxicity.

265 It has been reported that the salt has chromotoxic effects and increases the total chromosomal
266 alterations [28,34]. The highest concentrations of salt (300, 450 and 600 mM NaCl) completely
267 inhibited mitotic activity and increased chromosomal abnormalities in an endemic plant, Centaurea
268 ragusina [33] and barley [35]. They lead to chromosomal abnormalities such as the formation of
269 micronuclei, anaphase bridges, chromosomal adhesivity, C-mitosis and multipolarity [21]. However,
270 there are few studies that indicate the effects of salt stress on chromosomal alterations in cereals,
271 such as barley [28,29], maize [27] and sorghum [30].

272 As an alternative, to reduce or eradicate the negative effects of salinity, the researchers studied the
273 use of various plant growth regulators and vitamins, on seed germination [36], seedling growth [37]
274 and mitotic activity [28,38]. As in this work, where carrot extract was used, which in addition to
275 causing increase in IM for cv. BRS 358, it was observed that in the association of extract and 150 mM
276 NaCl, a larger number of cells were observed reaching the final stages of cell division, anaphase and
277 telophase, showing that even under stress the cells of the roots were able to perform cytokinesis,
278 which was not observed in the absence of extract, for the same cultivar (Figure 5B).

In the cv. BRS Querência no change in the mitotic index with the use of extract, only an increase in the number of chromosomal alterations in the presence of 50% of carrot extract and salt concentrations above 63.5 mM, while in BRS 358 a decrease in CA was observed at concentrations above 75 mM NaCl (Figure 7B and 7C). The interference in the cell division, caused by the action of the extract, with effect on the development of the root system, probably represents one of the mechanisms of action of the extract on the development of plants [39].

The exogenous application of growth regulator may cause a positive or negative effect on seed germination and seedling growth under non-stress conditions [37]. Because, supplementation of plants with beneficial elements or organic compounds ready for use, for example, amino acids [40] including proline [41] and polyphenols [42] enable plants to save energy for other needs such as post-stress recovery processes. As reported, with the use of pre-treatments of exogenous gibberellin [43] and cytokinin [44], which stimulated cell division under normal conditions.

However, some growth regulators may cause mitotic irregularities, cellular distortions and chromosomal alterations even without stress conditions [28,38]. As well as some commonly used plant extracts [45,46]. Data that are consistent with those found in this study.

The reduced rates of cell division in this work and the low influence of salinity can be justified by the fact that the seeds, both the control and the extract, undergo a pre-soak for 48 hours, in the absence of salinity, only after already being hydrated is that they were placed in a saline medium. For although there are studies that demonstrate that during seed imbibition, the initial growth occurs by cellular expansion, and mitosis occurs only after the protrusion of the root, in other words, at the beginning of seedling growth [47,48].

There are controversial studies, where castor bean seeds were observed in mitotic configurations before radicle protrusion with 24 hours soaking in water [49]. In seeds of Jatropha curcas L., belonging to the same castor bean family, Euphorbiaceae, the cell divisions only occurred after root protrusion [48].

5. CONCLUSION

The aqueous extract of carrot increased the mitotic index in cultivar BRS 358, mainly due to the increase of the prophase cell index. Salt stress reduces the mitotic index of rice roots of cultivar BRS 358, as it impairs the process of cell division in the post-prophase stages. Carrot extract associated with salinity increases the telophase index in cultivar BRS 358. There is an increase in the chromosomal alterations with the increase of the salinity in the presence of the carrot extract, in the cultivar BRS Querência.

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