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Morphophysiological and biochemical alterations in *Ricinus communis* L. seeds submitted to cobalt⁶⁰ gamma radiation

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ABSTRACT

This study aimed to evaluate the radiosensitivity of castor bean seeds after applications of different doses of Cobalt⁶⁰ gamma radiation. Seeds were pre-soaked for 24 hours in distilled water and then irradiated with 50, 100, 150, and 200 Gy, except the control. Sowing was performed in trays, which contained soil as substrate and were maintained in a greenhouse. The electrical conductivity, emergence, emergence speed index, growth parameters and activities of antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, and catalase) were evaluated in the leaves and roots of castor bean seedlings. Gamma radiation did not affect the electrical conductivity of the seeds; however, at a dose of 200 Gy, the emergence and emergence speed index of the seedlings was negatively affected. An analysis of the morphophysiological parameters revealed a reduction in seedling size as the radiation dose increased. There was a significant increase in superoxide dismutase and ascorbate peroxidase activities at higher radiation doses in the leaves, but not in roots. Thus, the analysis of all the variables suggests a response pattern as to the morphophysiological and biochemical changes of castor bean seedlings due to the increase of gamma radiation, which may serve as a tool for generating greater genetic variability.

Key words: Castor bean, radiosensitivity, germination, growth, antioxidant enzymes.

INTRODUCTION

Ricinus communis L., also known as castor bean, is regarded as one of the major oilseeds in the world, mainly due to two main features: the peculiarities of its oil that is not edible and its drought tolerance

(Foster et al. 2010). Biodiesel obtained from castor bean oil has a lower cost compared to the ones obtained from other oils due to its solvability in alcohol transesterification occurs without heating (Conceição et al. 2007). In addition, biodiesel produces fewer particulates, hydrocarbons, nitrogen oxides and sulphur dioxides than mineral diesel

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and therefore reduces vehicle exhaust pollutants that are harmful to human health (FAO 2010).

Its cultivation has gained great prominence in recent years, which justifies studies related to tolerance of this crop to biotic and abiotic stresses, as well as increased productivity. The genetic variability is essential for the improvement of any species and can be obtained through the use of ionizing radiation on seeds to mutation induction.

An important type of ionizing radiation is gamma radiation, which is high-frequency electromagnetic radiation consisting of high-energy photons. These photons have a high penetrating power and can interact indirectly with matter, causing ionization and excitation. Previous studies in plants have reported modifications of growth and development, a decrease in reproduction capacity, metabolic alterations and DNA damage under radiation stress (Vanhoudt et al. 2010, Silva et al. 2011). In addition, gamma radiation, for example, through the radiolysis of water can result in the formation of reactive oxygen species (ROS) which on their turn potentially lead to cellular damage (Esnault et al. 2010). However, plant cells have a well-equipped antioxidative defense system comprising enzymes and metabolites to regulate the amount of ROS (Karuppanapandian et al. 2011). Thus, the induction of the defense antioxidant system seems to be an important mechanism of response to stress after gamma irradiation. While the deleterious effects of radiation would intuitively be expected, various studies have reported hormesis effects, such as growth stimulation, following exposure to relatively low doses of ionizing radiation (Marcu et al. 2013).

Crops with improved characteristics have successfully been developed by mutagenic inductions (Majeed and Zahir 2010). Research on castor bean for the induction of mutations is still incipient. Thus, all studies aiming to understand the radiosensitivity of these plants and the physiological and enzymatic responses to Co^{60} gamma radiation

will contribute to genetic improvement programs solving problems that traditional breeding cannot overcome.

This study aimed to evaluate the radiosensitivity of castor bean seeds cv IAC 80, the morphological responses of these irradiated seeds, and the activity of antioxidant enzymes in seedlings after submitted to different doses of Co^{60} gamma radiation.

MATERIALS AND METHODS

Castor bean seeds cv IAC 80, after manual removal of the caruncle, were soaked for 24 hours in distilled water and then irradiated with gamma rays using the Cobalt⁶⁰ source “Eldorado 78” (Atomic Energy of Canada, Ltd). The different doses of radiation (0, 50, 100, 150, and 200 Gy) were obtained by varying the exposure time of the seeds. After irradiation, seedling emergence and growth tests were performed in a greenhouse at the Centro de Pesquisa Agropecuária de Clima Temperado (EMBRAPA), located in Pelotas, Rio Grande do Sul State, latitude 31°41’S, longitude 52°21’W and altitude 60 m asl, and biochemical analysis were conducted at the Laboratory of Genetics of the Zoology and Genetics Department at the Federal University of Pelotas (UFPeL).

SEEDLING VIGOR AND BIOMASS

Electrical conductivity - Tests were conducted in the Laboratory of Genetics of the Zoology and Genetics Department at the Federal University of Pelotas (UFPeL), and evaluated after 3, 6, and 24 hours of seed induction. Four subsamples of 25 seeds were used, which were placed in a beaker with 80 mL of deionized water and maintained in a germination chamber under constant temperature ($25 \pm 2^\circ\text{C}$) and 12 hour (light/dark) photoperiod. The electrical conductivity reading was performed with a bench-top conductivimeter (Digimed CD-21), and the data was expressed in $\mu\text{S cm}^{-1} \text{ g}^{-1}$ of

seeds as a function of the initial weight (Aosa 1983).

Seedling emergence - To determine the percentage of seedling emergence (E%), we utilized four replications of 50 seeds. The seeds were placed inside multicellular expanded polystyrene trays containing soil as a substrate for 21 days, arranged in a greenhouse with an average temperature of $25 \pm 2^\circ\text{C}$ and irrigated daily. The substrate was irrigated with distilled water when necessary. After 21 days, the number of normal and emerged plants was counted, and the data were expressed as percentages (Popinigis 1985).

Emergence speed index (ESI) - The ESI was established together with the emergence test, which was performed by counting the number of emerged seedlings daily until stabilization of the stand. The ESI was calculated according to the formula proposed by Maguire (1962): $ESI = E_1/N_1 + E_2/N_2 + \dots + E_i/T_i$, where E_1 , E_2 , and E_i refer to the number of emerged seedlings in each day, and, T_1 , T_2 , T_i refer to the days after sowing.

Shoot and root length of the seedlings - The length of the shoots and the roots was measured in 10 seedlings of each treatment. The data are expressed in mm per seedling.

Fresh weight of the seedlings - This parameter was evaluated by weighing the shoots and roots separately and the data are expressed as mg per seedling.

Dry weight of the seedlings - Shoots and roots were placed in paper envelopes and transferred to an incubator at $70 \pm 1^\circ\text{C}$ until a constant weight was obtained. The data are expressed as mg per seedling.

ANTIOXIDANT ENZYME ACTIVITY

On day 21 after sowing, the roots and the first completely expanded leaves were collected. The tissues were placed in aluminum foil envelopes and stored in an ultra-freezer at -80°C for subsequent

analysis. For enzyme extraction, approximately 200 mg of leaf and root tissue was macerated in liquid nitrogen plus 20% insoluble polyvinylpyrrolidone (PVPP). After maceration, the tissues were homogenized in 1.8 mL of extraction buffer (100 mM potassium phosphate (pH 7.8), 0.1 mM EDTA and 10 mM ascorbic acid). The homogenate was centrifuged at 13,000 g for 15 minutes at 4°C , and the supernatant was collected for protein quantification by the Bradford (1976) method. Absorbance values were measured using a spectrophotometer at 595 nm. These samples were employed to analyze the activity of SOD, CAT, and APX enzymes.

The SOD activity was estimated based on the capacity of the enzyme to inhibit the photoreduction of nitrotetrazolium blue (NBT) (Giannopolitis and Ries 1977). The reaction buffer was composed of 100 mM potassium phosphate (pH 7.8), 14 mM methionine, 0.1 μM EDTA, 75 μM NBT, and 2 μM riboflavin. Readings were obtained at 560 nm, and one unit of SOD was considered as the amount of enzyme necessary to inhibit NBT photoreduction by 50% under the assay conditions.

Catalase activity was determined according to Azevedo et al. (1998), with minor modifications. The activity was monitored based on the decrease in absorbance at 240 nm that was observed during two minutes of incubation at 28°C in a reaction buffer containing 100 mM potassium phosphate buffer (pH 7.0), 12.5 mM H_2O_2 , and enzyme extract.

The activity of APX was determined according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. The reaction buffer, which consisted of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and enzyme extract, was incubated at 28°C . Decreases in the absorbance were monitored for two minutes after the start of the reaction.

EXPERIMENTAL DESIGN

The experimental design was completely randomized with five treatments and four replications. The results were subjected to one-way analysis of variance (ANOVA), and the medians were compared using Tukey's post hoc test. The significance was set to 5%. The statistical analysis was performed using the STATISTICA 7.0® software.

RESULTS AND DISCUSSION

SEEDLING VIGOR AND BIOMASS

Electric conductivity test performed on the seeds of castor bean cv IAC 80 after 3, 6, and 24 hours of incubation in water revealed that there were no significant effect of on the membrane integrity of seedlings (Figure 1).

Thus, different doses of radiation did not alter the reorganization speed of the cellular membrane system, and for this reason, cellular solutes did not leak out into the incubation buffer. These results are different from those presented by Amjad and Anjum (2003), who examined onion seeds irradiated with doses of 0, 10, 20, 40, 80 and 100 Gy. These authors verified an increase in conductivity in irradiated seeds relative to control seeds. The same authors also indicated that the conductivity test is commonly used to evaluate loss of seed quality. Nevertheless, there are few reports in the literature describing the measurement of damages caused by radiation on membrane integrity.

A significant decrease was observed in seedling emergence between the control plants (0 Gy) and the 200 Gy treatment plants. Although there was no significant difference in emergence in the intermediate doses (50, 100, and 150 Gy) when compared to the control group, but a decreasing trend was observed (Figure 2a). Previously, gamma radiation was found to reduce seedling emergence in wheat (Grover and Khan 2014), rice (Pons et

al. 2001, Silva et al. 2011), and sesame (*Sesamum indicum* L.) (Boureima et al. 2009). Seeds that were not irradiated showed better germination and there was a reduction in emergence as the Co⁶⁰ gamma radiation dose increased.

The ESI presented showed a response similar to that of emergence at 200 Gy; at this dose, a significant decrease in ESI was noted in relation to the control group and the 50 and 100 Gy treatments, illustrating the negative effect of this radiation dose on seedlings (Figure 2b). Similar results were observed in two rice cultivars (Silva et al. 2011). Thus, the decrease in germination may be related to the radiosensitivity of the embryonic axis, but the damage can occur in any part of the seed.

Radiation causes not only a reduction in the emergence percentage, but it also reduces the ESI proportionally to the radiation dose. Similar results were observed by Hameed et al. (2008). However, the morphological, structural, and functional changes depend on the strength and duration of gamma doses exposure.

Differences in seedling growth as a function of the radiation dose are illustrated in Figure 3. Negative effects were observed only at higher doses. These results were also confirmed through the analysis of biometric parameters. The shoot was longer in control plants than in the exposed to radiation; significantly smaller increases were noted at doses of 150 and 200 Gy (Figure 2c). There was a trend of reduced root length as the radiation dose increased, but significant differences were only noted between the control group and the 150 and 200 Gy treatments and between the 150 and 200 Gy treatments themselves (Figure 2d).

Vanhoult et al. (2014) exposed *Arabidopsis thaliana* seedlings to different gamma radiation doses and observed changes in root and leaf fresh weights but did not observe dose-dependent growth inhibition. However, these authors used lower dose rates compared to those utilized in the current study. Barros and Arthur (2005) observed

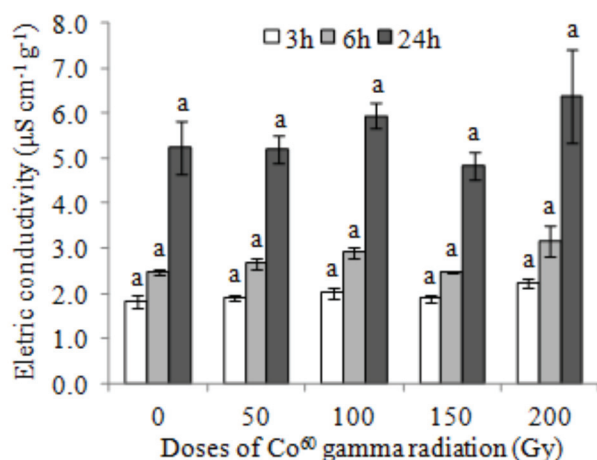


Figure 1 - Electrical conductivity ($\mu\text{S cm}^{-1} \text{g}^{-1}$) in castor bean seeds (cv IAC 80) exposed to different doses of Co^{60} gamma radiation (Gy) after 3 (□), 6 (▒) and 24 hours (■) of incubation. Means followed by different letters are significantly different based on Tukey's test ($P < 0.05$). Bars represent the standard error of the mean of four replications.

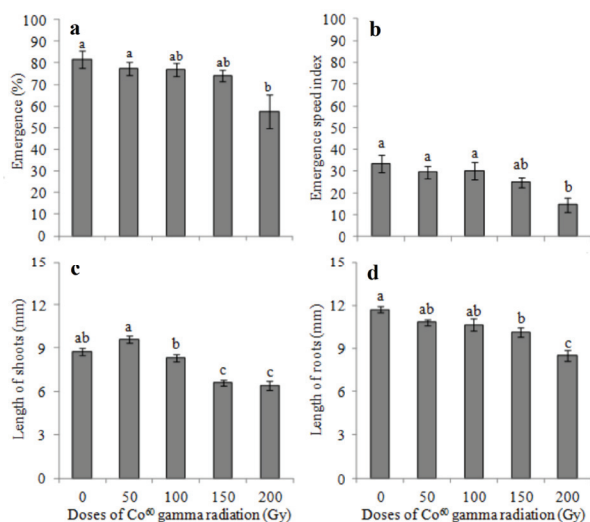


Figure 2 - Emergence (a), Emergence speed index (b), length of shoots (c) and length of roots (d) of castor bean seedlings exposed to different doses of Co^{60} gamma radiation. Means followed by different letters differ according to Tukey's test ($P < 0.05$). Bars represent the standard error of the mean of four replications.

that radiation also caused a drastic decrease in the total length of soybean seedlings as the radiation dose increased, which agrees with the data in the present study (Figures 2c, 2d and 3). Decreases in plant biomass were also described in cultivated species such as corn (Al-Salhi et al. 2004), whip



Figure 3 - Analysis of castor bean seedlings showing the total length of seedlings exposed to different doses of Co^{60} gamma radiation after 21 days.

beans (Kon et al. 2007), rice (Silva et al. 2011), and wheat (Grover and Khan 2014). This decrease in growth may be attributed to a reduction in mitotic activity in meristematic tissues and a decrease in seed humidity (El-Sherif et al. 2011).

These results are partially concordant with the As Low As Reasonably Achievable (ALARA) principle of radiologic protection (International Commission on Radiological Protection-ICRP, 1990), which affirms that radiation at any dose is harmful to organisms. However, the hormesis theory described by Luckey (1980) states that low radiation doses have the capacity to stimulate organisms, and this effect is inverted with increasing radiation doses. In seeds, this effect is also called the priming effect, which results in a stronger and faster defense response when the plant is stressed (Guan et al. 2009). This theory is in agreement with the results found in the present study with respect to the shoot length of castor bean seedlings at 50 Gy (Figures 2c and 3).

We observed an increase in the fresh weight of shoots in the 50 Gy group in relation to the control group; however, there was no significant difference between the control (0 Gy) and the 100 Gy treatments (Figure 4a). Decreased biomass

production was noted in the 150 and 200 Gy treatments, which is reflected in the differences observed in shoot length as a function of radiation dose (Figure 2c). A significant decrease in root fresh weight was observed only at a dose of 200 Gy (Figure 4b). Tabasum et al. (2011) also observed a significant reduction in the shoot and root fresh weight of three rice cultivars after irradiation at a dose of 400 Gy.

The dry weight of seedlings showed the same tendency as the fresh weight in both shoots (Figure 4c) and roots (Figure 4d). Similar results were described by Majeed et al. (2010) in *Lepidium sativum*: the fresh and dry weights increased at the lowest dose and decreased as the radiation dose increased. Kon et al. (2007) also revealed that the shoot dry weight of *Vigna unguiculata* tended to decrease after exposure to high doses of gamma radiation.

According to El-Sherif et al. (2011) and Majeed et al. (2010), a decrease in biomass production may be due to stature reduction or a reduction in water content as a result of stress caused by radiation. Barros and Arthur (2005) reported that the growth reduction of plants depends on their radiosensitivity, which can change depending on the species, the humidity, the developmental stage, the radiation dose employed and, importantly, the criteria used to measure physiological effects.

ANTIOXIDANT ENZYME ACTIVITY

Stress due to radiation occasionally increases ROS production, which can activate mechanisms of damage repair, such as those involving antioxidant enzymes, to prevent oxidative stress. It is known that gamma radiation can cause alterations in enzyme capacities; however, the results reported by previous studies are dependent on the radiation dose and the plant species used (Silva et al. 2011, Vanhoudt et al. 2014). In the present study, we evaluated the SOD activity in castor bean seedling

leaves. There was an increase in this activity proportional to the radiation dose; however, this increase was only significant in relation to the control group at the highest concentration tested (200 Gy) (Figure 5a). Moreover, we did not observe a significant difference between treatments in the roots (Figure 5b). This response suggests that leaves are more sensitive to the effects of radiation, and the increase in antioxidant activity observed at elevated doses likely occurred to prevent oxidative stress due to ROS generation.

Increased SOD activity due to different radiation doses was also described by Al-Rumaih and Al-Rumaih (2008), who studied different species of *Trigonella* and found significant effects at doses greater than 40 Gy, and by Silva et al. (2011), who studied seedlings of the rice cultivars BRS Querência and BRS Fronteira.

APX also showed the highest activity in leaves as the radiation dose increased. In this case, there was a significant increase at doses of 150 and 200 Gy compared to the control and 50 Gy treatments

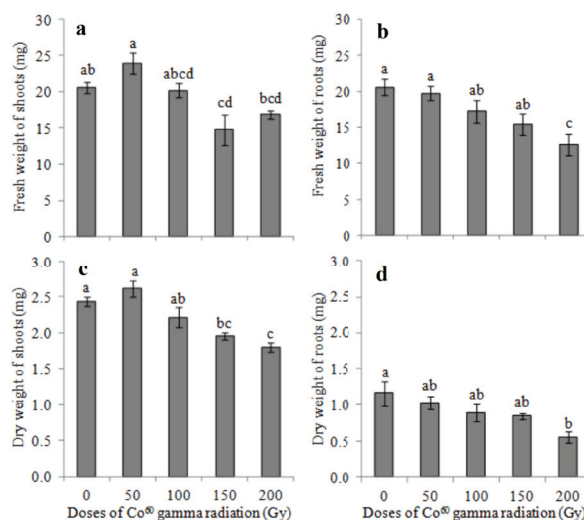


Figure 4 - The fresh weight of shoots (a) and roots (b) and the dry weight of shoots (c) and roots (d) of castor bean seedlings exposed to different doses of Co^{60} gamma radiation and evaluated after 21 days. Means followed by different letters differ by Tukey's test ($P < 0.05$). Bars represent the standard error of the mean of four replications.

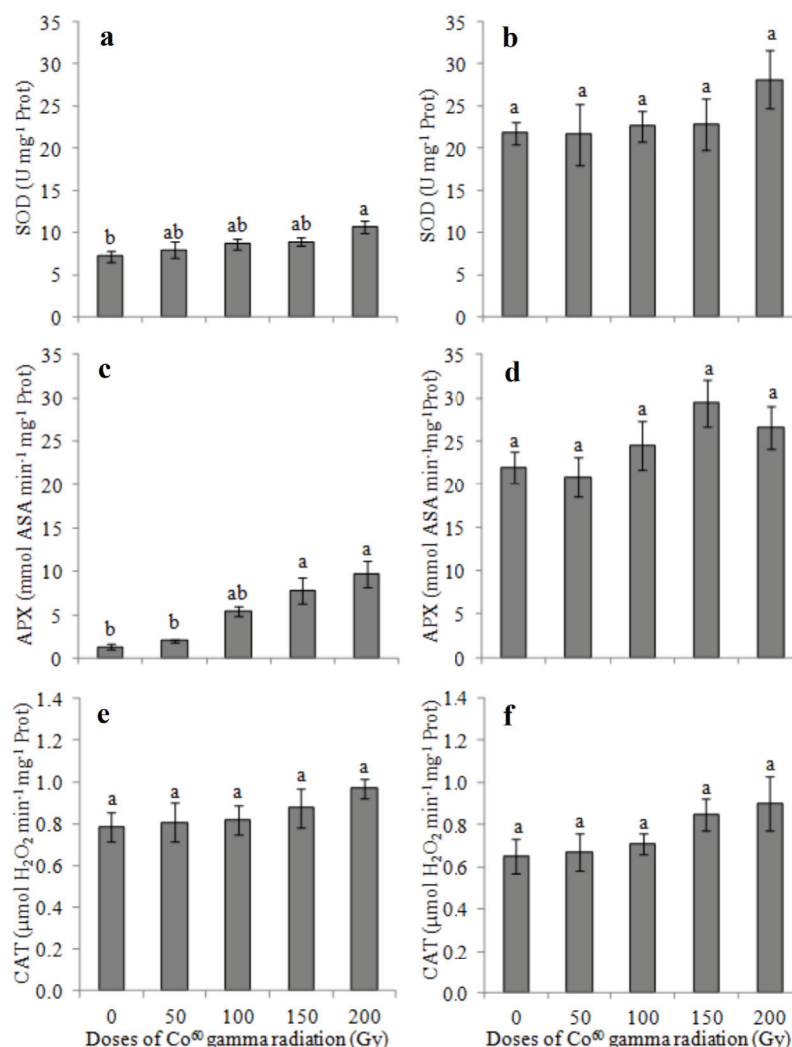


Figure 5 - Activities of the antioxidant enzymes SOD, APX and CAT in the leaves (first column) and roots (second column) of castor bean seedlings exposed to different doses of Co⁶⁰ gamma radiation. Means followed by different letters differ by Tukey's test ($P < 0.05$). Bars represent the standard error of the mean of four replications.

(Figure 5c). This response indicates that low doses of radiation do not have negative effects on castor bean cv IAC 80 seedlings, as observed in the 50 Gy treatment. In fact, the 50 Gy treatment caused an increase in shoot growth (Figure 3). Moreover, higher doses of radiation (approximately 200 Gy) enhanced SOD and APX activities and reduced the emergence percentage (Figure 2a). In roots, no significant differences were noted (Figure 5d). Silva et al. (2011) reported an increase in APX activity in rice at higher doses of radiation (150 and

200 Gy), which was similar to the results obtained in the present study.

CAT activity was not affected by radiation in either the leaves (Figure 5e) or the roots (Figure 5f). Similar results were described by Vanhoudt et al. (2010, 2014) in studies with *A. thaliana* and by Silva et al. (2011) in rice, in which different doses of gamma radiation also did not alter the activity of CAT.

SOD is the first enzyme to act within the antioxidant defense system by catalyzing

the dismutation of the superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2). However, the H_2O_2 produced by SOD is toxic to plant cells and may be broken down into H_2O and O_2 by the action of CAT or APX. The differences observed in the activities of the CAT and APX enzymes in the present study can be justified by the affinity of both enzymes for the substrate (H_2O_2): CAT is activated at higher concentrations of H_2O_2 , while APX is activated at low levels of H_2O_2 (Gechev 2006).

ROS are usually found at basal levels in cells, and in many cases act as signal for various metabolic pathways; however, they tend to increase under different stress conditions. In plants, the antioxidant system regulates and adjusts ROS levels according to the needs of the cell. Gamma radiation causes ROS generation because water molecules present in the organism absorb the energy released by the radiation, causing its dissociation (Vanhoudt et al. 2014). This results in the release of H^+ ions as well as H^{\cdot} and OH^{\cdot} free radicals, which have high chemical reactivity and tend to recombine to form ROS. The increase of enzymatic activity observed in the present study demonstrates its important role in plant metabolism, and in response to gamma radiation, these results can serve as a tool to generate greater genetic variability in plants.

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