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Response of gamma-irradiated banana plants to *in vitro* and *ex vitro* salinity stress

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ABSTRACT

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Stress caused by abiotic factors, such as salinity, decreases production of bananas, because it is very sensitive to salinity. This study aimed to investigate the effect of gamma (γ) ray-induced *in vitro* mutagenesis as well as *in vitro* and *ex vitro* reaction to salt stress (NaCl) in banana (*Musa* AAA cv. 'Dwarf Cavendish'). Shoot tips of banana were irradiated with gamma rays at doses of 25, 35 and 45 Gy, and subjected to MS medium containing additional NaCl (0, 100, 120, 140 and 160 mM) for two months (1st salinity stress) as factorial based on completely randomized design with five replications. The surviving shoots were transferred to a salt-free MS medium for one month, and then the salinity stress, as before, was re-applied (2nd salinity stress). Increasing NaCl concentrations resulted to a decrease in growth rate during 1st salinity stress. Also, irradiated explants had higher survival percentage, shoot number and shoot fresh weight than non-irradiated ones. In 2nd salinity stress, only the irradiated explants under 160 mM NaCl had decreased in shoot number compared to other salinity treatments. *In vitro*-regenerated plants were rooted and acclimatized in the greenhouse and evaluated under normal and saline conditions (3rd salinity stress). A sharp decrease in the survival percentage and leaf number observed with an increase of salinity, while irradiated plants had more survival rate and leaves number than non-irradiated plants. In addition, as the salt concentration increased, the leaf burn and yellowing rate increased and its intensity was higher in non-irradiated plants. Overall, banana shoot tips exposed to different doses of gamma irradiation had higher growth parameters under *in vitro* and greenhouse salt stress. However, further studies are required to evaluate agro-morphological characteristics of these mutants in the field conditions under salinity stress.

Keywords: banana, gamma-rays, mutagenesis, In vitro selection, salinity stress

INTRODUCTION

Banana (*Musa* spp.) is one of the world's most important fresh fruit commodity in terms of volume of trade. It has grown in its popularity for its adaptation to many agro-climatic conditions, resilience to climatic changes, non-specific seasonal fruit production, year-round availability of fruits, and high productivity per unit area. The popularity of the fruit led to its adoption and cultivation in more than 150 countries (Pillay and Tenkouano, 2011).

Although several banana varieties are under cultivation, the global market is dominated by the Cavendish types owing to their higher yields and early maturity (Kulkarni *et al*., 2007). 'Dwarf Cavendish' is very abundant and widespread, as well as the shortest commercially grown banana. It used to dominate the banana industries in subtropical countries where it was considered adapted, standing against subtropical winds, and high yielding (Robinson and Galán Saúco, 2010).

Banana production in Iran is restricted to certain areas along the southern regions, especially in Sistan and Baluchestan province (Miri *et al*., 2009). Generally, several bacterial, fungal, viral diseases and pests threaten the production of banana, and building-up of genetic resistance towards these biotic factors is urgently needed. There is also a greater need to develop tolerant genotypes for salinity, drought, cold and unfavorable soils conditions (Kulkarni *et al*., 2007).

One of the most serious problems limiting the extension of banana plantation in Iran is the salinity of soil and water (Miri *et al*., 2009). High salt levels in the soil can cause plasmolysis in plants as the osmotic pressure in the soil is higher than that in the plant cells. Also, high salinity due to low water potential in plants, toxic effects of Na^+ and Cl^- ions and unbalanced nutrients in the plant adversely affect plant growth (Dikayani *et al*., 2017). Therefore, to extend banana cultivation, it is important to develop salinity tolerant genotypes (Miri *et al*., 2009).

For most vegetatively propagated crops like banana that have a narrow genetic basis, it is essential to generate additional genetic variability to facilitate selection of desirable traits (Pillay and Tenkouano, 2011; Spencer-Lopes *et al*., 2018). Currently, it is believed that induced mutations, *via* the use of mutagenic agents like gamma-rays irradiation, have a high potential to enhance genetic variability for the development of new mutant varieties (Robinson and Galán Saúco, 2010; Çelik and Atak, 2017; Miri, 2018; Miri and Roughani, 2018).

In vitro mutagenesis makes the induction and selection of induced somatic mutations more effective (Pillay and Tenkouano, 2011). The most improved features in mutant banana by gamma radiation were earliness, bunch size, reduced height, large fruit size, tolerance to aluminum stress, resistance to *Fusarium oxysporum* f. sp. cubense, as well as putative mutant resistant to black sigatoka disease (Pillay and Tenkouano, 2011Çelik and Atak, 2017). Two banana cultivars 'Klue Hom Thong KUI' and 'Novaria' are products of *in vitro* mutation (Roux, 2004). In addition, *in vitro* mutation breeding by using gamma irradiation and chemical mutagens has resulted in creation of superior mutants of 'Robusta' with good bunch traits (Bakry *et al*., 2009). There are over 3220 officially released mutant cultivars in over 210 plant species (Bado *et al*., 2015).

Although studies of salt tolerance selection are available for diverse plant species (Nikam *et al*., 2015), limited research has been conducted for banana. The objective of this study was to investigate the effect of gamma irradiation on response of banana to *in vitro* and *ex vitro* salinity stress.

MATERIALS AND METHODS

In vitro shoot tips of *Musa* AAA cv. 'Dwarf Cavendish' were irradiated in a gamma cell with a $cobalt^{60}$ source at Agricultural, Medical and Industrial Research School, Atomic Energy Organization of Iran (AEOI), Karaj, Iran, at 25, 35 and 45 Gy doses (M_1V_0) in 2009. The propagules were immediately transferred onto multiplication medium consisting MS (1962) medium supplemented with 1.5 µM BAP, 7% Agar and 30% sucrose (Miri, 2009).

Cultures were incubated at 25±2°C under 16 hour's illumination (2500 lux, day light fluorescent tubes). Further subculturing was performed three times at an interval of 30 days in order to separate chimeras before applying salinity stress (M_1V_3) (López *et al*., 2017). Subsequently, 3350 irradiated and non-irradiated shoots (1-1.5 cm in length) were isolated and cultured on MS medium containing 0.5 µM BAP, 7% Agar and 30% sucrose with different concentrations of NaCl (0, 100, 120, 140 and 160 mM) (1^{st} salinity stress, M_1V_4) to evaluate their reaction to salinity stress.

After two months of incubation, the survival rate, shoot number, shoot length and shoot fresh weight were determined. Then, the 1664 vigorous shoots were transferred to fresh salt-free medium for one month (M_1V_5). Similar to 1^{st} salinity stress, 1451 shoots were again subjected to the selection medium for two months (2^{nd} salinity stress, M_1V_6) and after measuring the growth parameters, the surviving shoots were cultured on the rooting medium (MS containing 0.2 µM BAP, 2.5 µM NAA, 6% Agar and 20% sucrose) (M_1V_7). The plantlets with well-developed root-system were carefully removed from the culture vessels and gently washed in running tap water to remove the entire gelled medium. They then were transferred to polythene bags containing a 1:1 mixture of peat moss and vermiculite, and hardened in the greenhouse under natural light with relative humidity of 90–100%, which gradually decreased to 70-75%, and an ambient temperature of 26-32°C.

To assess the *ex vitro* evaluation of saltinity stress, the 224 hardened irradiated and non-irradiated NaCl-selected plants with a height of 15-20 cm were irrigated once a week with saline water containing 0, 100, 120, 140 and 160 mM NaCl for two months (3rd salinity stress), and watered with salt-free solution between two irrigation intervals to avoid high salt accumulation. Thereafter, plant survival percentage and number of leaves were recorded.

All stages of this research were laid out as a factorial experiment with two factors, gamma irradiation and salinity, in a completely randomized design with five replications. Analysis of variance was carried out by SPSS software and the means were compared using the Duncan's Multiple Range Test ($p \leq 0.05$).

RESULTS

Gamma rays and salinity had significant effects on *in vitro* survival percentage only at 1st salinity stress (Table 1). The shoot tip survival (%) was found to be enhanced at 45 Gy (72.6%) compared to non-irradiated explants (Fig. 1). Lower survival (%) was observed with increase in salt concentration at 1st salinity stress, thus significant reduction up to 80.8%, 67.5%, 56.0% and 41.3% was observed at 100, 120, 140 and 160 mM NaCl, respectively (Fig. 1). The extent of leaves browning also increased with salt concentrations (Fig. 2).

Interaction of gamma rays and salinity had a significant effect on shoot number at 1st salinity stress (Table 1). In general, non-irradiated shoot tips showed higher reduction in shoot number with increase in NaCl concentration than in irradiated ones (Fig. 3).

Table 1. Analysis of variance of the effect of gamma-rays and salinity on growth parameters of banana in 1st and 2nd salinity stress

		Mean Square							
		1st salinity stress				2nd salinity stress			
S.O.V.	df	SP	SN	SL	SFW	SP	SN	SL	SFW
γ-ray	3	10.39*	18.68*	37.55*	2.09*	0.37	4.54*	10.52	1.38
Salinity	4	88.15*	62.22*	70.51*	1.91*	0.92	3.72*	37.11*	1.07
γ-ray × salinity	12	0.89	2.80*	1.31	0.70	0.18	0.49	0.90	0.94
Error	95	1.57	1.42	1.66	0.47	0.65	1.39	1.56	0.44

*: Significant at the 5% probability level.
SP: survival percentage, SN: shoot number, SL: shoot length, SFW: shoot fresh weigth.

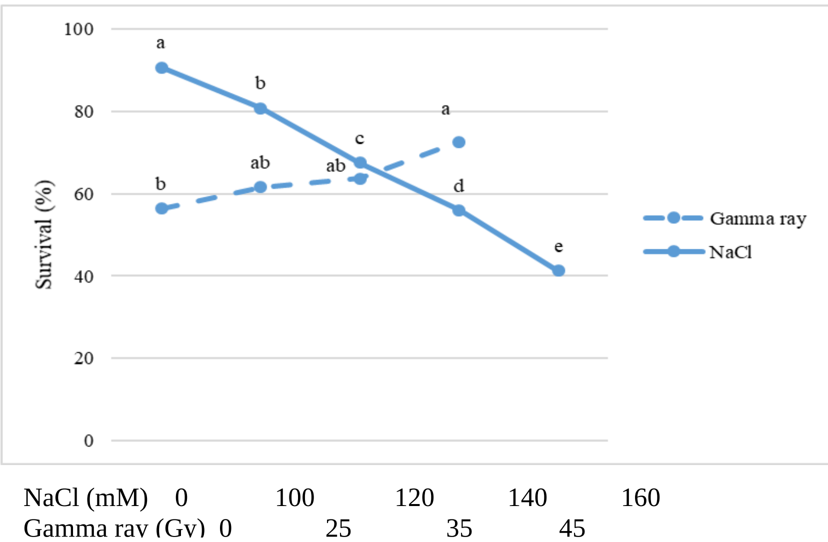

Fig. 1. Effect of gamma rays and salinity on survival (%) of *in vitro* banana cv. ‘Dwarf Cavendish’ at 1st salinity stress

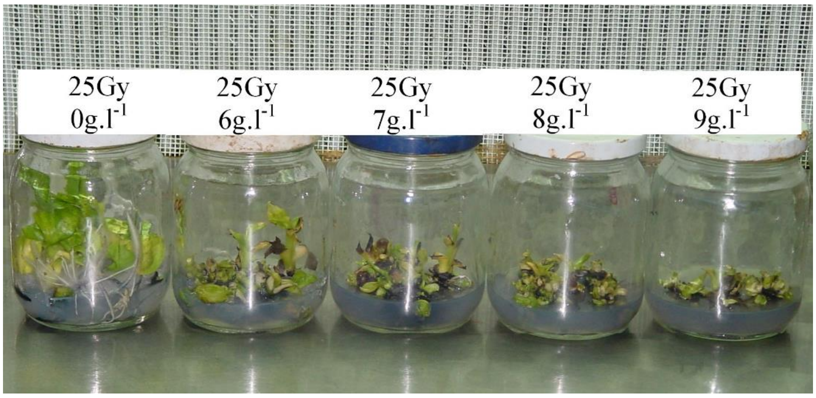

Fig. 2. Shoot growth and leaves browning of *in vitro* banana cv. ‘Dwarf Cavendish’ at 1st salinity stress

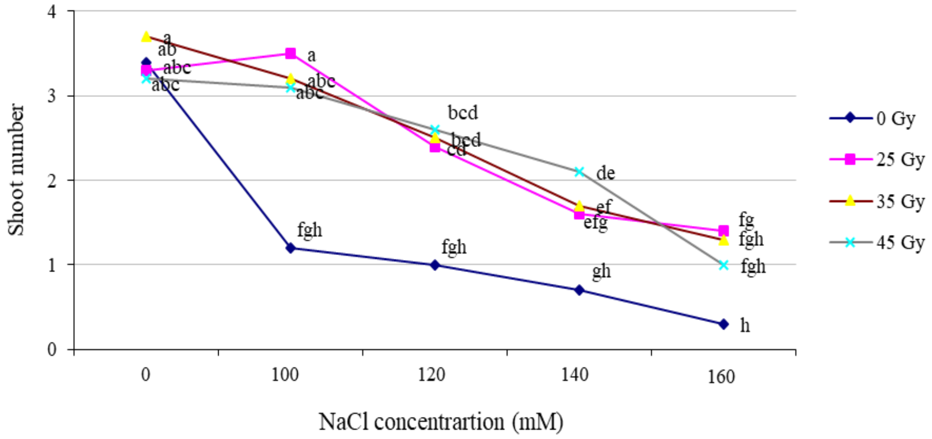

Fig. 3. Gamma rays and salinity interaction effect on shoot number of *in vitro* banana cv. ‘Dwarf Cavendish’ at 1st salinity stress

At 2nd salinity stress, irradiated shoot tips showed significantly higher shoot number over control (Fig. 4). A significant reduction in shoot number was observed only in treatment with 160 mM NaCl (Fig. 5).

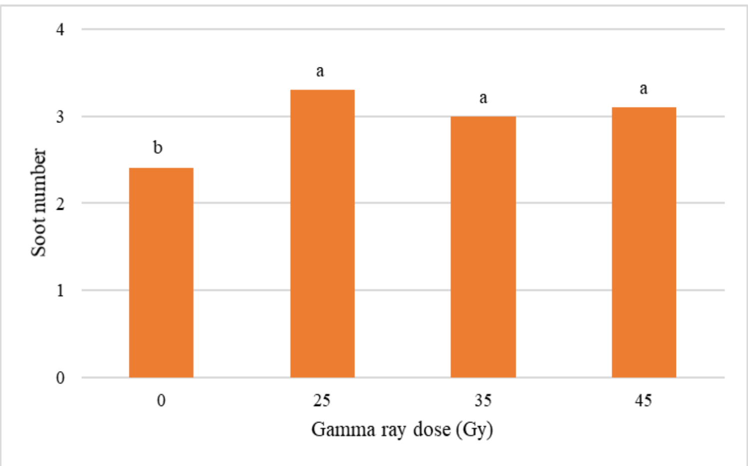

Fig. 4. Effect of gamma rays on shoot number of *in vitro* banana cv. ‘Dwarf Cavendish’ at 2nd salinity stress

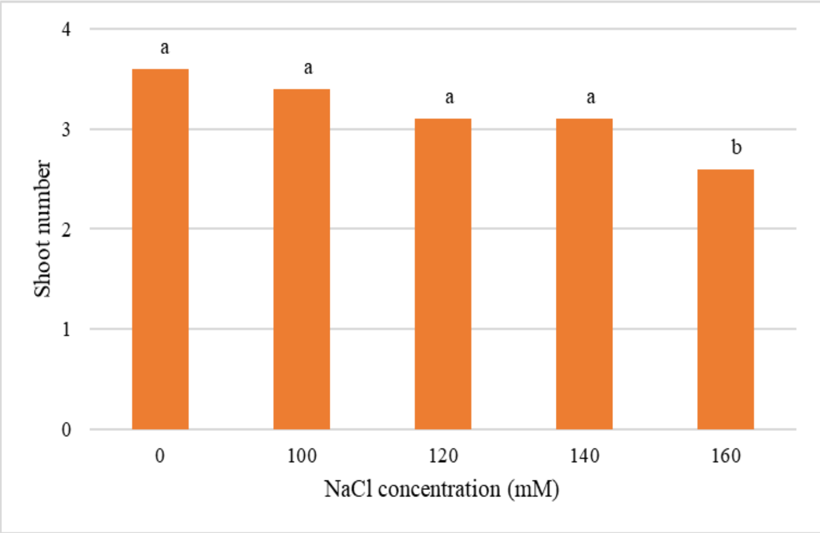

Fig. 5. Effect of salinity on shoot number of *in vitro* banana cv. ‘Dwarf Cavendish’ at 2nd salinity stress

At 1st salinity stress, shoot tips exposed to gamma rays showed significant shoot length reduction over control (Fig. 6), whereas at 2nd salinity stress it had no effect (Table 1). However, shoot length was affected by salinity stress in both stages (Table 1). Irradiated and non-irradiated shoot tips cultures on NaCl selection media showed significant shoot length reduction with increasing salt concentration compared to control at 1st salinity stress. Shoot tips exposed to 100-160 mM NaCl showed 20.7-27.6% reduction over control at 2nd salinity stress, whereas >50% reduction in shoot length was observed in treatment with 160 mM NaCl (Fig. 7).

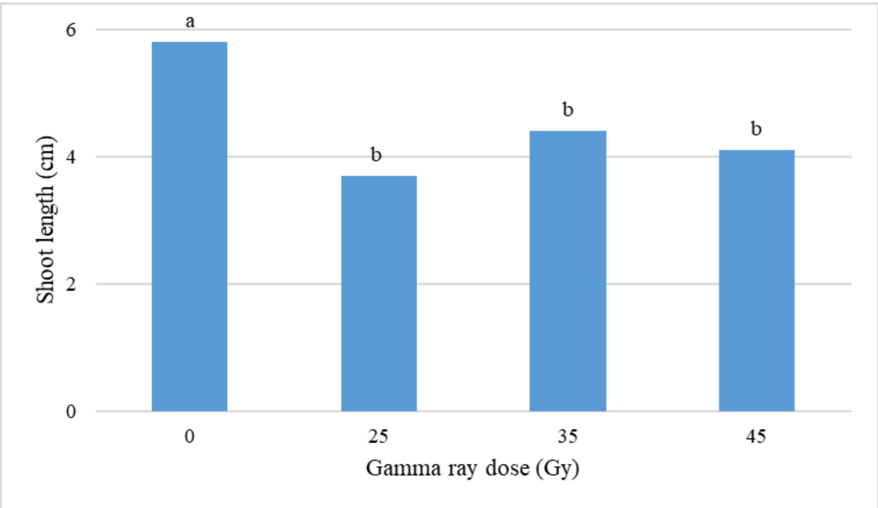

Fig. 6. Effect of gamma rays on shoot length of *in vitro* banana cv. ‘Dwarf Cavendish’ at 1st salinity stress

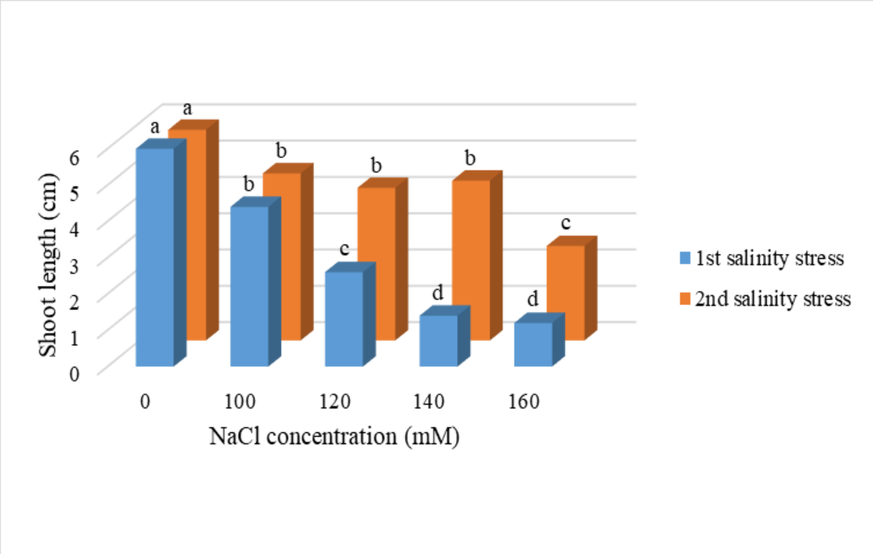

Fig. 7. Effect of salinity on shoot length of *in vitro* banana cv. 'Dwarf Cavendish' at 1st and 2nd salinity stress

Gamma rays and salinity had significant effect only on shoot fresh weight at 1st salinity stress (Table 1). The 35 and 45 Gy-irradiated shoot tips showed significantly higher shoot fresh weight than control and 25 Gy-irradiated ones (Fig. 8). Lower levels of shoot fresh weight were observed in shoot tips grown under NaCl stress, thus the lowest shoot fresh weight was observed in the 140 and 160 mM NaCl treated shoot tips (Fig. 9).

The survival rate of banana greenhouse plants in the 3rd salinity stress was affected by the interaction of gamma rays and NaCl concentrations (Table 2). As the salt concentration increased, the survival rate decreased, but the intensity of this decrease was higher in non-irradiated plants, thus by increasing the salt concentration from 100 to 160 mM, the survival rate in non-irradiated and irradiated plants decreased from 71.4 to 25.0% and 93.2 to 77.7%, respectively (Fig. 10).

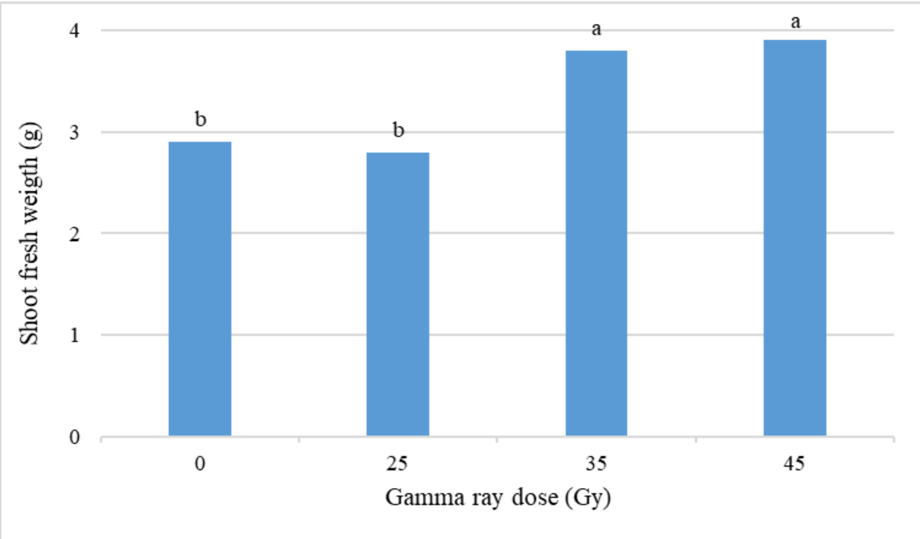

Fig. 8. Effect of gamma rays on shoot fresh weight of *in vitro* banana cv. 'Dwarf Cavendish' at 1st salinity stress

Table 2. Analysis of variance of the effect of gamma-rays and salinity on growth parameters of banana in 3rd salinity stress

		Mean Sqaure	
S.O.V.	df	SP	LN
γ-ray	1	7.86	1.19
Salinity	4	66.34*	3.38**
γ-ray × salinity	4	2.14*	0.36
Error	45	1.57	0.21

*and **: Significant at the 5% and 1% probability levels, respectively.
SP: survival percentage, LN: Leaf number

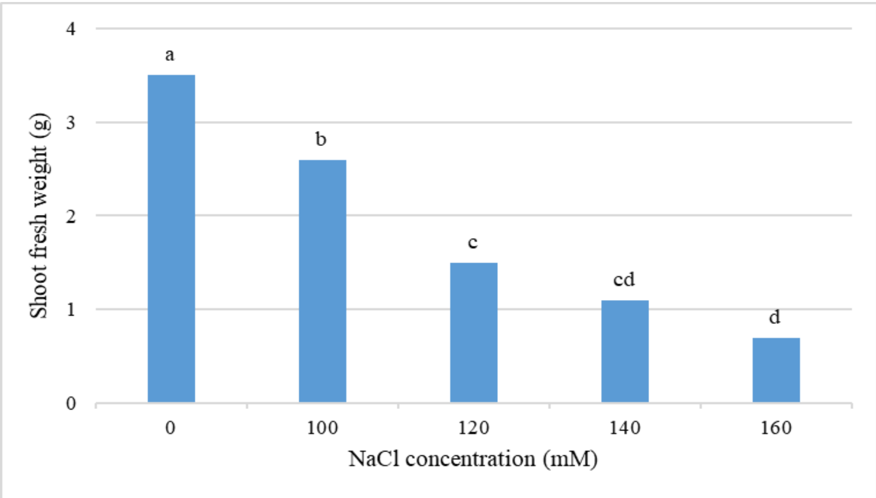

Fig. 9. Effect of salinity on shoot fresh weight of *in vitro* banana cv. ‘Dwarf Cavendish’ at 1st salinity stress

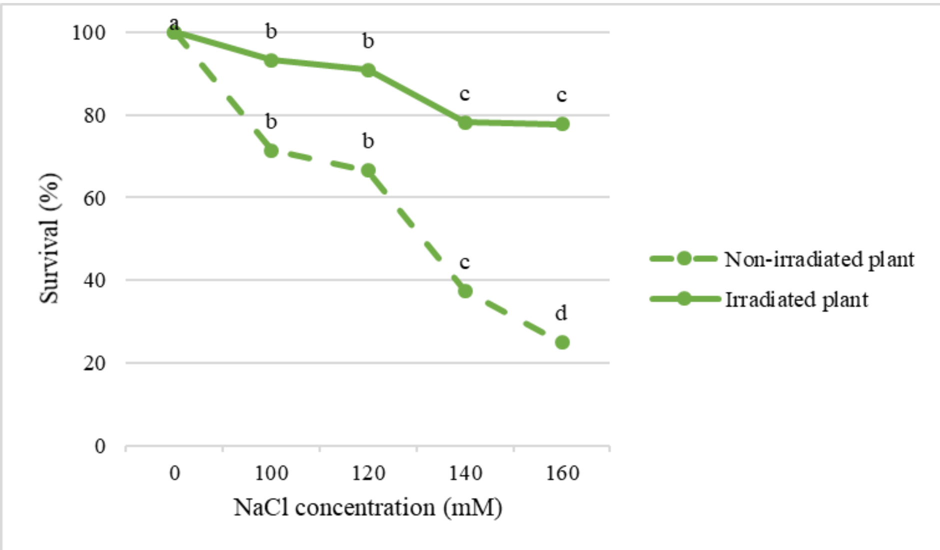

Fig. 10. Effect of gamma rays and salinity on survival (%) of greenhouse cv. banana ‘Dwarf Cavendish’ at 3rd salinity stress

Irradiated plants (184 plants) had more mean leaves than 35 non-irradiated ones (2.4 leaves in irradiated plants compared to 0.5 leaves in non-irradiated plants). As the salt concentration increased, the number of new leaves decreased (Fig. 11), whereas burnnin and yellowing of the leaf margins and tip increased (especially the older leaves) and its intensity was higher in non-irradiated plants (Fig. 12). When salt concentration increased to 140-160 mM, all non-irradiated plants appeared to show very severe injury symptoms.

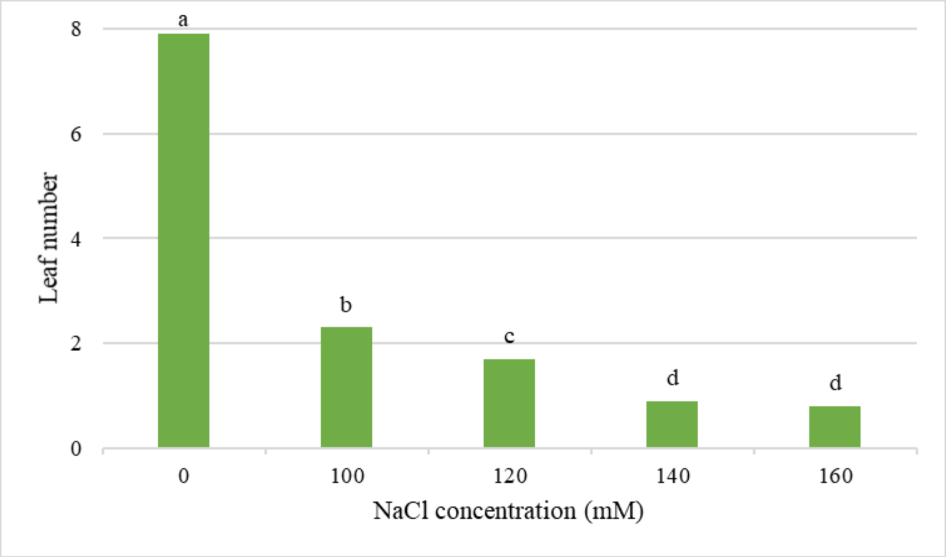

Fig. 11. Effect of salinity on leaf number of greenhouse banana cv. ‘Dwarf Cavendish’ at 3rd salinity stress

Fig. 12. Response of non-irradiated (top) and irradiated (bottom) greenhouse banana cv. ‘Dwarf Cavendish’ to 160 mM NaCl at 3rd salinity stress

DISCUSSION

The radiation-induced mutagenesis with *in vitro* culture can be employed as a promising technique that allows induction of genetic variation, selection, and multiplication of mutant clones (El-Sabagh *et al*., 2011; Nikam *et al*., 2015). Induced mutations change only one or a few specific traits of an elite cultivar without undesirable additional variations. One of the first steps in mutagenic treatment is the estimation of the most appropriate dose to apply (El-Sabagh *et al*., 2011).

In the present study, banana cv. 'Dwarf Cavendish' shoot tips were exposed to different doses of gamma radiation, and post-irradiation survival showed an increase with increasing doses of gamma radiation, however, shoot length decreased. Gamma irradiated shoot cultures of DENDROBIUM SONIA orchid at 15-45 Gy showed reduced shoot length (Billore *et al*., 2019). Nevertheless, the exposure to low-doses of gamma-irradiations can have stimulatory effects on specific morphological parameters and can improves seedling growth and ability to withstand water shortage (Jan *et al*., 2012; Geng *et al*., 2019).

Low doses of gamma-rays, generally, stimulated plant cell division, growth, and development. However, the way radiation influences plant growth and development is still unknown and the available data remains controversial. Although no conclusive explanations for the stimulation effects of low doseg-irradiation have been available until now, papers support the hypothesis that low dose irradiation will induce growth stimulation by changing the hormonal signalling network in plant cells or by increasing the antioxidative capacity of cells to easily overcome stress factors (Jan *et al*., 2012). Induced mutations also may alter the DNA and when such alterations affect gene function, changes to the phenotype are observed on account of the altered expression of the gene in question (Mba *et al*., 2007). It has been well documented that there are great differences of sensitivity to irradiation between species and varieties (Geng *et al*., 2019).

Our results suggest that doses of 25 to 45 Gy seem to be suitable for inducing mutation for banana cv. 'Dwarf Cavendish' improvement. Roux (2004) standardized the methodology to provide guidelines to mutation induction programs in *Musa* spp., and recommended doses of 30–40 Gy for triploid cultivars. The proliferation of banana genotypes was arrested beyond 50-60 Gy and a dose of more than 70 Gy was lethal (Rao *et al*. (1998).

In the present study, *in vitro* and *ex vitro* salinity stress were applied by inclusion of growth-inhibitory levels of NaCl in the selection medium. The growth rate and injury degree of banana cv. 'Dwarf Cavendish' shoot tips and greenhouse plants was aggravated when exposed to successively increasing NaCl concentration of 100-160 mM, and the injury degree in non-irradiated plants was higher than the irradiated ones. In banana, Na^+ and Cl^- concentrations increase was associated with the increase of NaCl levels (Gomes *et al*., 2011), and an inhibition *in vitro* plantlets growth occurred with 200 mM NaCl (Dikayani *et al*., 2017).

The leaf number reduction that occurs due to the increase of the levels of salt is a common response in banana and has been previously described by different researchers (Shapira *et al*., 2009; Gomes *et al*., 2011; Willadino *et al*., 2017). This reduction may have resulted from reduced water availability and membrane damage to explants due to increased NaCl stress in the medium or nutritional imbalance because of interference of salt ions with essential nutrients (Nikam *et al.*, 2015).

In addition, concentration of Na^+ ions has inhibited high intake of K^+ ions into the plant. The functions of the potassium ions are to maintain osmotic pressures in the cells, synthesize proteins and serve such as pyruvate kinase thus, the low concentration of K^+ in the cells causes chlorosis and necrosis (Dikayani *et al.*, 2017). Accumulation of toxic concentrations of sodium (Na^+) and/or chloride (Cl^-) ions, especially in older leaves, inducing tissue necrosis and early leaf senescence (Diego *et al*., 2017). Typical salt stress symptoms appear in banana only along the leaf margins. Xylem sap containing high concentration of Na^+ is pulled by water tension from the marginal vein back into the adjacent mesophyll without having to cross a layer of parenchyma tissue. The distinct anatomy of the marginal vein plays a major role in the accumulation of Na^+ in the margins, with the latter serving as a dumping site for toxic molecules (Shapira *et al*., 2009). We also observed that the toxicity caused by salinity increased the yellowing and senescence of older leaves margins and tip.

Gomes *et al*. (2001) compared the effect of saline stress in five genotypes of banana and stated that the genotypes Pacovan, Nanicão and FHIA18 were able to accumulate the Na^+ in the root and rhizoma when they were treated with the highest NaCl level. On the other hand, Calcuttá genotype presented the highest Na^+ concentration in the leaf that associated with a decrease in K^+ concentration. This behavior was reflected in a high reduction of leaf dry weight and leaf area. The Pacovan genotype, however, showed the lowest decrease of leaf dry weight and the lowest reduction in leaf area.

Willadino *et al*. (2011), similarly, evaluated 12 banana genotypes with for salt tolerance during initial growth stage. They found that the PA 42-44 genotype was the most sensitive one, because it showed 18.5% reduction of dry matter production as well as the highest Na^+ contents in both leaf blade and roots and rizome, and demonstarted low efficiency to extrude and to prevent the Na^+ translocation to leaf blade. On the other hand, the Preciosa genotype showed both the lowest Na^+ contents and the smallest reduction in dry matter production (0.2%) as well as a low Na^+/K^+ ratio indicating an efficient salt tolerance strategy by Na^+ extrusion. In addition, Willadino *et al*. (2017) subjected two bananas genotypes, Tap Maeo (tolerant) and Berlin (sensitive), to treatment with 50 mM NaCl, and observed that while the Tap Maeo genotype demonstrated a small increase in Na^+ concentration in the shoots, the Berlin exhibited an increase of more than 300%.

Salinity tolerance in *Musa* involves at least two simultaneous mechanisms including; the activation of the SOS system such as the extrusion of Na^+ from the cytoplasm, and antioxidative system like the increase in the synthesis of the enzyme ascorbate peroxidase and glycine betaine (Willadino *et al*., 2017). A trial was initiated for testing the concepts of salt tolerance like, Na exclusion, compartmentation of Na ions, dilution of Na due to tissue expansion and K/Na selectivity in banana. Among the concepts of salt tolerance studied, K/Na selectivity was found to be the most relevant salt tolerance mechanism in banana crop (Jeyabaskaran and Sundararaju, 2000).

Although the expression of the various mechanisms of salt stress depends on the effects of several genes, a mutation in one of the key genes that control these mechanisms, such as ability to exclude Na^+ and to absorb K^+, may be enough to transfer a sensitive plant into a relatively stress-tolerant one (Tal, 1994; Miri, 2009). Further studies on these non-irradiated sensitive and selected irradiated plants using morphological and molecular markers indicated the existence of considerable DNA variation (Miri *et al*., 2009; Miri *et al*., 2014). Analyzing by RAPD markers, a 240 bp band was presented in only irradiated selected plants (Miri *et al*., 2009). In addition, two specific microsatellite alleles appeared consistently in non-irradiated susceptible clones, but were absent in all selected irradiated clones for salinity stress (Miri *et al*., 2014).

In vitro mutagenesis of cultured explants represents a feasible method for induction of genetic variation, which can be subjected at the cellular level to selection for desirable traits. However, the response to salt stress in the *in vitro* culture does not always correlate with that at *ex vitro* cultures and success of *in vitro* mutagenesis programs will depend on evaluation of mutant clones under greenhouse and field conditions to confirm their performance for the selected traits of interest (Lee *et al*., 2003; Nikam *et al*., 2015). Therefore, in this study, clones were screened for responses to salinity at greenhouse. It seems that genetic changes resulted in higher growth characteriistics in irradiated plants was sustained, and response of surviving explants in 1st stress were also stable in second and third salinity stress due to:

1) no-significant difference of salt effect on survival and shoot number (with the exception of 160 mM NaCl treatment) in 2nd salinity stress,

2) higher survival and 4.6 fold of leaf number in irradiated plants contrasted to non-irradiated in 3rd salinity stress.

However, further study is required to evaluate the selected mutants under field conditions as well as to characterize the physiological, biochemical and molecular aspects of their response mechanisms to salinity stress.

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