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MECHANISMS OF TOLERANCE TO SALINITY IN BANANA: PHYSIOLOGICAL, BIOCHEMICAL, AND MOLECULAR ASPECTS[1]

LILIA WILLADINO[2], TEREZINHA RANGEL CAMARA[3], MARTA BARBOSA RIBEIRO[4], DANIEL OLIVEIRA JORDÃO DO AMARAL[5], FLAVIA SUASSUNA[6], MÁRCIA VANUSA DA SILVA[7]

ABSTRACT - In the northeastern region of Brazil, saline soils are constraints to banana production, becoming necessary to understand the mechanisms of salt tolerance. Two bananas genotypes, Tap Maeo, tolerant, and Berlin, sensitive, were subjected to treatment with 50 mol m^{-3} NaCl or without salt. This study evaluated the effects of salt on the following physiological aspects: leaf area, content and distribution of Na^+, membrane integrity, proton ATPase activity. Besides, a search for differentially expressed genes was performed using the *Differential Display* technique. Tap Maeo genotype showed the smallest reduction in leaf area, smaller accumulation of Na^+ and malondialdehyde (MDA), and higher activity of proton ATPase activity. Two sequences differentially expressed in the tolerant genotype, (*Musa* 07, *Musa* 23), shared a high degree of identity with the amino acid sequences of the genes *SOS1* and *SOS2*, respectively. The clone *Musa* 10 was highly similar to amino acid sequence of the ascorbate peroxidase gene, and *Musa 26*, encodes the enzyme betaine aldehyde dehydrogenase. These significant biological markers indicate that salinity tolerance in banana involves at least two simultaneous mechanisms: the activation of the SOS pathway, increasing the extrusion of Na^+, and the activation of antioxidative system, increasing the synthesis of APX and betaine aldehyde dehydrogenase enzyme.

Index terms: DDRT-PCR, leaf area, *Musa*, Na^+, antioxidative system, SOS pathway.

MECANISMOS DE TOLERÂNCIA À SALINIDADE EM BANANEIRA : ASPECTOS FISIOLÓGICOS, BIOQUÍMICOS E MOLECULARES

RESUMO - Na região Nordeste do Brasil, solos salinos são restritivos à produção de banana, tornando-se necessário compreender os mecanismos de tolerância de sal. Dois genótipos de banana, Tap Maeo, tolerante, e Berlim, sensível, foram submetidos ao tratamento com 50 mol m-3 NaCl ou sem sal. Este estudo avaliou os efeitos do sal sobre os seguintes aspectos fisiológicos: área foliar, conteúdo e distribuição de Na^+, integridade da membrana, atividade da ATPase. Além disso, uma busca por genes diferencialmente expressos foi realizada usando a técnica *Differential Display*. O genótipo Tap Maeo apresentou a menor redução na área foliar, menor acúmulo de Na^+ e malondialdeído (MDA) bem como maior atividade da H^+ATPase. Duas sequências diferencialmente expressas no genótipo tolerante (*Musa 07, Musa* 23) compartilham alto grau de identidade com as sequências de aminoácidos dos genes *SOS1* e *SOS2*, respectivamente. O clone *Musa* 10 é muito semelhante à sequência de aminoácidos do gene da peroxidase do ascorbato, e o *Musa* 26 codifica a enzima aldeído betaína desidrogenase. Estes marcadores biológicos significativos indicam que a tolerância à salinidade em banana envolve pelo menos dois mecanismos simultâneos: a ativação da via SOS, aumentando a extrusão do Na^+, e a ativação do sistema antioxidante, aumentando a síntese de APX e da enzima aldeído betaína desidrogenase.

Termos para indexação: área foliar, DDRT PCR, *Musa*, Na^+, rota SOS, sistema antioxidativo.

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[2]Biologist, PhD. Department of Biology, Federal Rural University of Pernambuco, Avenida Dom Manoel de Medeiros s/n, 52021060, Recife, Pernambuco, Brasil. E-mail: willadino.lilia@gmail.com (autor para correspondência);
[3]Agronomist, PhD. Department of Chemistry, Federal Rural University of Pernambuco, Avenida Dom Manoel de Medeiros s/n, 52021060, Recife, Pernambuco, Brasil. E-mail: teca.camara@gmail.com
[4]Forestry Engineer, Master in Chemistry, Department of Chemistry, Federal Rural University of Pernambuco, Avenida Dom Manoel de Medeiros s/n, 52021060, Recife, Pernambuco, Brasil. E-mail: martaribeiro21@hotmail.com
[5]Biologist, PhD. National Institute of the Semi-Arid, Avenida Francisco Lopes de Almeida, s/n, 58429970, Campina Grande, Paraiba, Brasil. E-mail: danieljornal@yahoo.com.br
[6]Biologist, Master in Botany, Department of Biology, Federal Rural University of Pernambuco, Avenida Dom Manoel de Medeiros s/n, 52021060, Recife, Pernambuco, Brasil. E-mail: flaviasuassuna@yahoo.com.br
[7]Agronomist, PhD. Department of Biochemistry, Federal University of Pernambuco, Avenida Prof. Moraes Rego s/n, 50670420, Recife, Pernambuco, Brasil. E-mail: marciavanusa@yahoo.com.br

INTRODUCTION

Salinity is one of the most significant abiotic stresses in the world, constituting a limiting factor that adversely affects the growth of plants in general and the productivity of agricultural crops. In the last decade, more than 900 million hectares worldwide were recorded as having been affected by salinity (FAO 2008).

Plant species, glycophytes or halophytes, that survive in saline environments exhibit adaptation strategies that involve physiological, biochemical and molecular mechanisms. Studies have shown that plants develop a tolerance to osmotic stress, ionic toxicity stress (FLOWERS et al., 2015), and oxidative stress (FOYER; SHIGEOKA, 2011). To alleviate Na^+ toxicity and re-establish cellular homeostasis is necessary the extrusion of Na^+ by the salt overly sensitive pathway (SOS) (PARDO, 2010), and this transport across the membrane is dependent of proton ATPase activity (VITART et al., 2004). The salt SOS pathway functionally consists of the Na^+ transporter, SOS1, the protein kinase SOS2, and the $Ca2^+$ sensor SOS3, which constitute a functional model that ensures ionic homeostasis in plants that are adapted or tolerant to saline stress (FEKI et al., 2014). Another important mechanism for tolerance is the increase in the activity of antioxidant enzymes, to avoid the effects of oxidative stress (FOYER; SHIGEOKA 2011). This is a mechanism that avoid the accumulation of reactive oxygen species (ROS) that causes damage to nucleic acids, proteins, membrane lipid peroxidation and disrupt the redox homeostasis (AZOOZ et al., 2009).

Tolerance is affected by many different genes involved in these various metabolic pathways, such as ion extrusion, ion compartmentalization, synthesis of compatible solutes and scavenging ROS (SHI et al., 2000; SUN et al., 2010; KUMAR et al., 2009). Genotypes that differ with regard to their tolerance for salinity therefore exhibit qualitative and/or quantitative differences in gene expression. The identification and understanding of the molecular control mechanisms involved in the tolerance to abiotic stresses may result in the use of molecular tools to produce new more tolerant commercial cultivars (GUPTA; HUANG, 2014) In many circumstances, it is the sensitivity of the roots to stress that limits the plant productivity. Despite this direct action of stress on the roots, the majority of studies focus primarily on the leaf tissue in experiments involving the exposure of plants to saline stress. However, the combination of the physiological relevance and structural simplicity of the root tissues makes them an obvious target for functional genomic analysis (KUMAR et al., 2015) Several techniques have been developed to identify genes that are differentially expressed and that are involved in the mechanism of tolerance to abiotic stress in plants (GUPTA; HUANG, 2014). Among these techniques, DDRT-PCR, still managing a lower volume of information than the other techniques, has a relatively low cost and is easy to perform (LIANG; PARDEE, 1995). Several plant-related studies have shown that DDRT-PCR can be effectively used to clone genes that are preferentially expressed in plants by abiotic stress (UEDA et al., 2002).

Considering the importance of saline stress as a limiting factor for banana production and given that the physiological and biochemical specific responses to salinity represent a combination of prior molecular events activated by the perception of salt, it is necessary to identify and characterize the genes involved in the response to salinity. The objective of this study was to isolate, sequence, and identify differentially expressed genes in the saline stress-tolerant banana genotype, as well as to associate these genes with their physiological and biochemical mechanisms.

MATERIALS AND METHODS

Micropropagated banana plantlets (*Musa* sp.) of Tap Maeo and Berlin genotypes, which are tolerant to and sensitive to salinity, respectively, were acclimated for 25 days in a hydroponic system prior to the experiments. The plants were grown in a nutrient solution containing 742.86 $mg.L^{-1}$ soluble fertilizer (3% N, 11% P_2O_5, 38% K_2O, 4% MgO, 11% S, 0.025% B, 0.004% Mo, 0.01% Cu-EDTA, 0.025% Zn-EDTA, 0.07% Fe-EDTA, and 0.04% Mn-EDTA) and 840.00 $mg.L^{-1}$ calcium nitrate (15.5% N and 19.0% Ca). The salt treatment began after the acclimation period by adding 50 $mol.m^{-3}$ NaCl. The treatment without NaCl was used as the control.

The plants were analyzed considering the time of the expression of the events to be evaluated.

At the beginning of the saline treatment (time zero) and after 24 and 48 hours, five plants in each treatment group were collected. The roots were frozen in liquid nitrogen to extract the total RNA and for the DDRT-PCR analysis. Seven days after the initiation of the treatments, five plants in each treatment were collected to evaluate membrane

lipid peroxidation and the H^+ATPase activity in the membrane. After fifteen days of treatment, the leaf area was measured, and five plants were collected to analyze the Na^+ and K^+ concentrations.

The experiment was carried out in a completely randomized design with two levels of salt (0 and 50 $mol.m^{-3}$ NaCl), two cultivars (Tap Maeo and Berlin) making a total of 4 subplots. Each subplot contained 10 plants of each genotype, with a total of 40 plants. The experiment was repeated 3 times. The data set was submitted to analysis of variance (ANOVA) and mean values were compared using Tukey's test ($P < 0.05$).

The leaf area (LA) was determined using a leaf area meter (Li-Cor, Inc., Lincoln, Nebraska, USA). The analysis of the Na^+ and K^+ concentrations was performed using flame photometry.

The plasma membrane vesicles were isolated by differential centrifugation of the root primordial. The ATPase activity of the plasma membrane was determined by measuring the Pi liberated during ATP hydrolysis, according to the colorimetric method described by Fiske and Subbarrow, with the modifications (FAÇANHA; DE MÉIS, 1998). Vanadate (membrane ATPase inhibitor) and nitrate (V-ATPase inhibitor) were used as inhibitors.

The total RNA was extracted from the roots using TRIzol. For DDRT-PCR, a 1 µg aliquot of total RNA was used as a standard to obtain cDNA and for the subsequent PCR amplification. The cDNA synthesis reactions were performed at 50°C for 50 minutes. The PCR reactions were performed using combinations of anchor primers (A_1, A_2, A_3, or A_4) with random primers (B_1, B_2, or B_3). The amplification conditions were as follows: 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 42°C for 1 minute, and 72°C for 1 minute, as well as a final elongation stage at 72°C for 5 minutes. PCR was performed at least three times for each primer pair. The PCR-amplified products were separated on 1.5% agarose gels. The genes induced by salt were defined when the expression was 1.5 fold greater than that of the control, as quantified by the PCR product. The nucleotide sequences of each clone were determined using an automatic DNA sequencer (ABI PRISM 377; Applied Biosystems, Foster City, California, USA), using the Big Dye Terminator kit (Applied Biosystems). The BlastX program (ALTSCHUL et al. 1997) was used to compare the obtained sequences with homologous sequences in the databases of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast), the Institute for Genomic Research (TIGR; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi), and the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/search/blast). Multiple sequence alignments were performed using ClustalW software (http://www.ebi.ac.uk) (THOMPSON et al., 1994), and a phylogenetic tree was constructed using the neighbor-joining method with MEGA 3.1 software (KUMAR et al., 2004). The reliability of the tree was assessed by bootstrap analysis with 1500 simulations and edited using MEGA 3.1 software.

RESULTS AND DISCUSSION

The salinity reduced the leaf area of both genotypes by different magnitudes (Table 1). In the tolerant genotype, Tap Maeo, the reduction in leaf area was 21.8%, whereas the reduction in the sensitive genotype Berlin was 45.2%.

The leaf area reduction that occurs due to the increase of the levels of salt is a common response in banana and has been previously described in 46 genotypes evaluated by different researchers (GOMES et al., 2004; SILVA et al., 2009; FERRAZ, 2008; WILLADINO et al., 2011); these genotypes exhibited reductions in leaf area that varied from 16.3 to 69.2%. Decreased leaf area is related to constrained CO_2 fixation, which reduces the net assimilation rate and increases the production of ROS through the Mehler reaction (MILLER et al., 2009). The reduction in leaf area is also due to the deviation of energy required for the activation and maintenance of metabolic activities that are associated with salinity tolerance, such as the maintenance of membrane integrity, the synthesis of organic solutes, ion extrusion, and the regulation of ion transport and distribution in various organs and within cells (MUNNS; TESTER, 2010).

The K^+ concentration in the shoots and roots varied with the addition of NaCl to the nutrient solution. Whereas the sodium concentration increased in the roots with increasing salinity but did not differ between the genotypes. In addition in the shoots, while the Tap Maeo genotype demonstrated a small increase in Na^+ concentration the Berlin, sensitive genotype, exhibited an increase of more than 300% (Table 1). This Na^+ distribution pattern, which is characterized by low concentrations of that ion in the shoots is characteristic of salinity tolerance, as observed in 30 banana genotypes exposed to the same conditions (GOMES et al., 2004; GOMES et al., 2005; WILLADINO et al., 2011). The maintenance of ionic homeostasis in the tolerant genotypes requires the extrusion of Na^+

from the cytosol to the vacuole or apoplast through the Na^+/H^+ antiporter (SHI et al., 2002).

The analysis of total RNA revealed fragments corresponding to rRNA 28S and 18S with optical quality ($A_{260/230}$) and density ($A_{260/280}$) ratios of approximately 1.9 and 1.7, suggesting low contamination with polysaccharides and proteins, respectively, and a high yield of total RNA.

Twelve primer combinations were used. The optimal pattern and resolution clarity of the amplified products were obtained with the A_2B_2 primer pair 48 hours after stress.

The transcripts of all of the samples (T_0, T_1, and T_2) generated by the same pair of primers were applied side-by-side. Twenty-three differentially expressed amplicons were isolated, cloned, and sequenced. The length of the amplicons varied between 300 and 1900 base pairs (bp). The similarity between the sequences obtained here and previously known gene sequences was determined. Similarity was considered to be present when the E values were less than (1e-05), which would therefore be considered significant.

The tolerant variety, Tap Maeo, have the differentially expressed *Musa* 07 cDNA fragment (Table 2) in the roots that have a high homology to *SOS1* gene that encoding SOS1, the Na^+/H^+ antiport.

This cDNA fragment (approximately 500 bp) have an amino acid sequence with 85% homology to the *Salt Overly Sensitive 1* (*SOS1*) gene of the glycophyte *Arabidopsis thaliana* (access number, AF256224) (SHI et al., 2000) (Figure 1) and 81% homology to *Pisum sativum*, *Thellungiella halophile,* and *Brassica napus*.

The salt overly sensitive (SOS) pathway functionally consists of the Na^+ transporter, SOS1, the protein kinase SOS2, and the $Ca2^+$ sensor SOS3, which constitute a functional model that ensures cellular homeostasis in plants that are adapted or tolerant to saline stress (FEKI et al., 2014; KUMAR et al., 2009). The phylogenetic tree of the Na^+/H^+ antiport sequences (SOS1), based on higher plants, animals, fungi, and *Escherichia coli*, indicated two distinct "clusters", as supported by the high bootstrap value, i.e., a vacuole cluster and a plasma membrane cluster, which indicates that *Musa* 07 is a fragment of the Na^+/H^+ antiporter gene (Figure 2).

This same antiporter is also involved in the distribution of Na^+ between the shoots and the root (PARDO, 2010) by restricting xylem loading with the Na^+ ion, thereby preventing its transportation to the shoots (SUN et al., 2010; SHI et al., 2002). This altered distribution of Na^+ prevents toxic concentrations of this cation in the cytoplasm of shoot cells of the tolerant genotype Tap Maeo (Table 1), thereby protecting the photosynthetic apparatus. The cDNA clone *Musa* 23, which is differentially expressed in the Tap Maeo genotype, exhibited 73% similarity to the amino acid sequence of the *SOS2* gene in *Arabidopsis* (access number AF237670.1) (Table 2). Greater expression of SOS2 is responsible for better salinity tolerance response in different genotypes under salt stress (CHAKRABORTY et al., 2012). The *SOS2* gene encodes a serine/threonine protein kinase with either a regulatory or second catalytic domain, with a regulatory-catalytic interaction within the *SOS2* gene. The protein kinase SOS2 interacts with a Ca^{2+}-binding protein similar to calcineurin, SOS3, which responds to an increased Ca^{2+} concentration in the cytosol and triggers the interaction between SOS2 and SOS3 (FEKI et al., 2014).

The SOS2/SOS3 complex activated by saline stress is responsible for the phosphorylation and activation of SOS1, the Na^+/H^+ antiporter, which is present in the membrane (MILLER et al., 2009). Therefore, the SOS2 and SOS3 proteins are components of the SOS pathway. The SOS pathway ensures the ionic homeostasis of the Tap Maeo genotype through the extrusion of Na^+ from the cytoplasm to the apoplast or vacuole. The Na^+/H^+ antiporter is a secondary transporter that depends on the activity of ATPases the primary transporters. ATPases are responsible for the electrochemical gradient that ensures the energization of the Na^+/H^+ antiporter (VITART et al., 2004). The plants of the Tap Maeo genotype exhibited an increase of more than 20% in the activity of ATPase and PPase, ensuring sodium extrusion, and the sensitive genotype demonstrated a decrease in the activity of these proton pumps (Figure 3).These significant biological markers indicate that activation of the SOS pathway is a fundamental mechanism for saline stress tolerance of banana.

The cDNA fragment *Musa* 26 encodes betaine aldehyde protein dehydrogenase (BADH, EC 1.2.1.8), enzyme that catalyzes the oxidation of betaine aldehyde to glycine betaine (GB). GB plays important role in stress tolerance that include the stabilization of complex proteins and membranes *in vivo*, protection of the transcriptional and translational machinery, reduction of ROS accumulation and peroxidation of membrane lipids, and as a molecular chaperone in the refolding of enzymes (GIRI, 2011; CHEN; MURATA, 2011). The peroxidation of membrane lipids was monitored indirectly through the production of malondialdehyde (MDA), a byproduct of lipid peroxidation. The Tap

Maeo genotype did not exhibit variations in the MDA concentration when subjected to the stress, however, in the Berlin genotype, the accumulation of Na^+ caused an increase in the concentration of MDA (Figure 3).

The ability of tolerant varieties to maintain low concentrations of MDA, as well as MDA accumulation in susceptible varieties, indicates that variations in lipid peroxidation of membrane can be used to evaluate the effect of salt stress on cell, once the membrane is the first site to perceive this stress. Another cDNA fragment, the *Musa* 10 clone, was identified and exhibited 71% similarity to the amino acid sequence of the ascorbate peroxidase (APX) gene of *Vigna unguiculata* (access number U61379.1). APX is the most important enzyme associated with the elimination of H_2O_2 from the cytosol and chloroplasts (BARBOSA et al., 2014), preventing the oxidative damage. The excessive production of H_2O_2 and other ROS (O_2^-, OH^-, and $^1O_2$) is cytotoxic (FOYER; SHIGEOKA, 2011) and frequently occurs in plants subjected to biotic or abiotic stress (MITTLER, 2006). Tolerant genotypes respond to such stress with an increase in the activity of antioxidant enzymes, preventing and avoiding damage to nucleic acids, proteins and membrane lipid peroxidation, thus maintaining redox homeostasis (AZOOZ et al., 2009). The differences on the biochemistry and molecular markers highlights the importance of the antioxidant system as a mechanism of salt stress tolerance in banana.

In summary, salinity tolerance in *Musa* involves a set of at least two simultaneous mechanisms. These mechanisms include the activation of the SOS system, which ensures the extrusion of Na^+ from the cytoplasm and the activation of the antioxidative system, in particular the increase in the synthesis of the enzyme APX and glycine betaine.

TABLE 1 - Leaf area (LA), Na^+ and K^+ concentrations ($g.kg^{-1}$), and K^+/Na^+ ratio in shoots (S) and roots (R) of banana genotypes grown for 15 days with or without 50 mol m^{-3} NaCl in the nutrient solution

Genotype	Treatment (mol m^{-3} NaCl)	LA (cm^2)	Na^+		K^+		K^+/Na^+	
			S	R	S	R	S	R
Tap Maeo	0	3.26 a	3.7 c	4.2 b	31.2 a	41.4 a	8.4	9.8
	50	2.53 b	5.9 b	6.2 a	29.7 a	40.9 a	5.0	6.6
Berlin	0	2.79 b	3.4 c	3.8 b	32.3 a	40.6 a	9.5	10.7
	50	1.26 c	16.5 a	6.9 a	30.0 a	39.9 a	1.8	5.8
		LA Reduction (%)	Na^+ Increase (%)		Reduction (%)			
					K^+		K^+/Na^+	
Tap Maeo		21.8	59.5	47.2	4.6	0.2	49.5	32.7
Berlin		45.2	385	81.6	7.1	1.7	89.5	45.8

Data indicated by different letters within each column exhibit significant difference at the 5 % probability level according to Tukey's test

TABLE 2 - Catalog of cDNA detected by differential display in banana roots*.

N° cDNA fragment	Primer combinations	PCR Fragment size (bp)	Access number	Best homology to database	Organism	Identity of aminoacid (%)
Musa 07	A_2B_2	500	AF256224.1	putative Na+/H+ antiporter SOS1	*Arabidopsis thaliana*	85
Musa 10	A_2B_2	390	U61379.1	cytosolic ascorbate peroxidase	*Vigna unguiculata*	81
Musa 23	A_2B_2	430	AF237670.1	serine/threonine protein kinase SOS2	*Arabidopsis thaliana*	73
Musa 26	A_2B_2	450	AF359282.1	betaine aldehyde dehydrogenase (BADH)	*Suaeda liaotungensis*	72

* Of the sequences obtained, four exhibited a high degree of similarity to amino acids sequence of genes whose functions are already known, nine displayed low similarity, and ten sequences were not similar

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Musa_07   --LG-
IYAAFARTAFKGDSQKSLHHFWEMVAYIANTLIFILSGVVIAEGILDSDKIAYQG
AtSOS1
MTLGMFYAAFARTAFKGDSQKSLHHFWEMVAYIANTLIFILSGVVIAEGILDSDKIAYQG
      ** .**********************************************************

Musa_07   -IHGDFFFCYTFTSNYRVFVVGVLYPLLCRFGYGLDWKESIILVWVG----------
AtSOS1
NSWRFLFLLYVYIQLSRVVVVGVLYPLLCRFGYGLDWKESIILVWSGLRGAVALALSLSV
        :*. * .:  ** ************************** *
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FIGURE 1 - Comparison of amino acid sequence between *Musa* 07 clone of *Musa* sp. with the *SOS1* gene of *A. thaliana* (AF256224). Sequences labeled with colons, asterisks, periods, and dashes represent those with similar amino acids, identical amino acids, different amino acids, and a lack of amino acids, respectively. The ClustalW program was used to generate the alignment (THOMPSON et al., 1997).

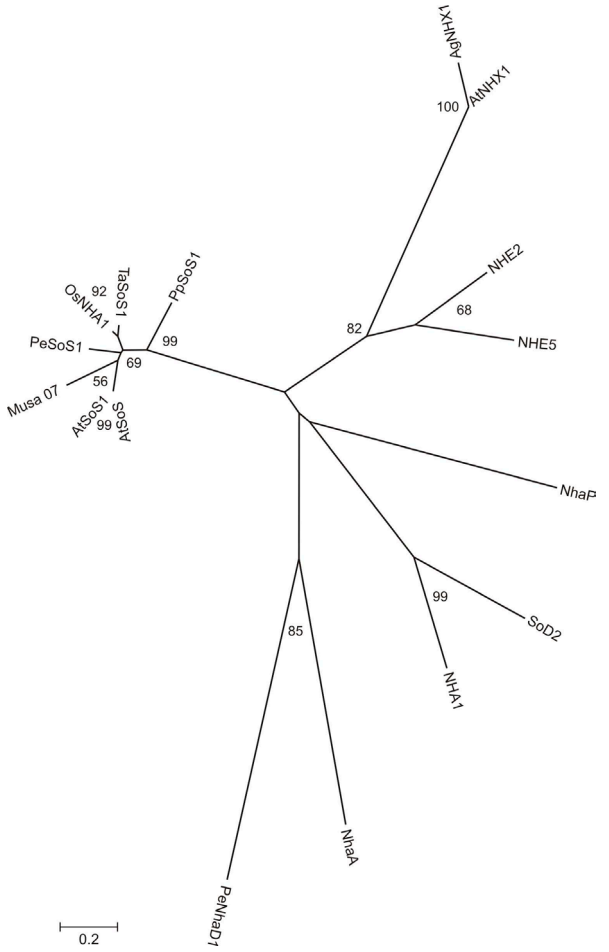

FIGURE 2 - Musa 07 clone position in the phylogenic tree of Na+/H+ antiporter. Multiple alignments of the sequences were performed with CLUSTALW, and a phylogenic tree was built using the neighbor-joining method with the MEGA 3.1 program. The accession numbers (in parentheses) and the Na+/H+ antiporter sources are as follows: AgNHX1 (AB038492), Atriplex gmelini; AtNHX1 (AF510074), AtSOS1 (AF256224), and AtSOS1 (AY062746), Arabidopsis thaliana; NHE2 (AAD41635) and NHE5 (AAC98696), Homo sapiens; OsNHA1 (AY328087), Oryza sativa; TaSOS1 (AY326952), Triticum aestivum; PpSOS1 (AJ564258), Physcomitrella patens; PeNhaD1 (AJ561195), Populus euphratica; NhaP (BAA31695), Pseudomonas aeruginosa; NHA1 (NP-013239), Saccharomyces cerevisiae; SOD2 (CAA77796), Schizosaccharomyces pombe; NhaA (P13738), Escherichia coli; and PeSOS1 (DQ517530), Populus euphratica

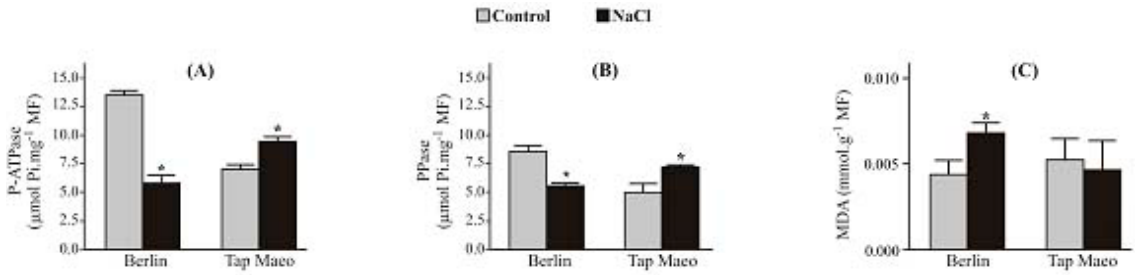

FIGURE 3 - Hydrolytic activity of P-ATPase and PP_iase and MDA concentration in roots of banana genotypes grown for 7 days with or without 50 mol m^{-3} NaCl in the nutrient solution.

Significant difference between the means of the treatments according to F test (0,05%).

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