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Superoxide dismutase activity and jasmonic acid during *in vitro-ex vitro* transition of pineapple (*Ananas comosus* (L.) Merr.) micropropagated plantlets

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Recent agriculture is characterized by intensive and cleaning productions, which need seeds with high quality in large quantities bonded by *in vitro* culture labs. Nevertheless, *in vitro ex vitro* transition and during acclimatization losses occur due to the death of plantlets unable to survive this abiotic stress. Reactive oxygen species production during jasmonic acid-induced changes of previous transition was demonstrated. The role of superoxide dismutase in regulation of oxidative metabolism signaling in response to environmental stress is analyzed. Pineapple plantlets treated with jasmonic acid showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase, when superoxide dismutase activity showed adequate control against this stress in relation to superior water-use efficiency and survival.

Key words: Environmental stress, water-use efficiency, survival.

INTRODUCTION

The presence of reactive oxygen species (ROS) as superoxide radical (O^{-2}) and hydrogen peroxide (H_2O_2) is associated in plants with the normal biochemistry processes as photosynthesis and respiration (Sejima et al., 2014; Huang et al., 2016). The accumulation and high reactivity has a cytotoxic effect by oxidative damage throughout lipid peroxidation and membrane destruction, protein inactivation and DNA mutation (Pospisil and Prasad, 2014). The reduction oxidation cascades (redox) of photosynthetic and respiratory chains of electron transport do not only provide energy for the metabolism, moreover it generates signals about participation in plant regulation of all the biology aspects at gene expression and the translation including chemistry of the enzymes (Kim et al., 2009). Some antioxidative enzymes as superoxide dismutase (SOD) and peroxidase participate in the ROS metabolism in pathogen infection. In plants, ROS are considered the first defense line against

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oxidative stress (Mittler and Blumwald, 2017). The induction or suppression of ROS production in the leaves is related with the antioxidative enzymatic activity diminishing H_2O_2 (by direct decomposition or oxidation) and O^{-2} (by dismutation) levels (El-Khallal, 2007).

The tissue culture changes some morphological characteristics of plantlets such as chemical composition of epicuticular layer (Preece and Sutter, 1991), form and distribution of stomas, tails and leaves structure (Ziv, 1990); also, physiological characteristics as activities of stomas, roots and leaves functionality. These changes raise the adaptation capacity of some plantlets to external conditions and originate not survival in acclimatization phase of significant number of micropropagated plants (Preece and Sutter, 1991) improved in pineapple using temporary immersion (Gonzalez-Olmedo et al., 2005). In this situation, plantlets do not control the excess of epidermical transpiration considered as principal mortality plants factor when they are transferred into the soil conditions (Durkovic and Misalova, 2009). ROS play a very important role in these adaptation processes as ubiquity response messenger in the stress (Apel and Hirt, 2004).

To supply some of these deficits, the use of plant growth regulators is a common practice (Preece and Sutter, 1991). Jasmonic acid (JA) that acts mainly as signal molecule as plant response against many abiotic and biotic stress (Schilmiller and Howe, 2005; Abdala and Cenzano, 2006), could attenuate these effects in pineapple plantlets during *in vitro-ex vitro* transition and SOD activity could be a biological indicator, whose demonstration is the objective of this work.

MATERIALS AND METHODS

The experiment was carried out with pineapple (*Ananas comosus* (L.) Merr.) micropropagated plantlets according to Daquinta and Benegas (1997) during acclimatization phase. Previous at *in vitro-ex vitro* transition, during *in vitro* rooting phase, a group was growing on medium enriched with Biojas® (a JA formulation) at the dose of 1 $mg.L^{-1}$ established because it was the one that achieved the best effects in a previously tested screening. Another group without Biojas® was used as control.

The variables were determined at the beginning of acclimatization (0 day), 14, 28 and 42 days later than the *in vitro-ex vitro* transition. Ten representative plantlets per treatment were used to choose the leaves analyzed.

Soluble proteins extraction, involving enzyme, was carried out using the same procedure. 0.25 g of macerated leaves in liquid nitrogen was aggregated at Tris-HCl buffer 01 M, pH 7.5, with 0.1 $mmol.L^{-1}$ EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 15 mM mercap-toethanol (ME, 1:4) (p:v). Further, 10% polyvinyl polypyrrolidone (PVPP) with respect to fresh weight was added. Homogeneous suspension was centrifuged at 15000 g during 20 min. The supernatant was used as enzymatic extract and to quantify soluble proteins according to Bradford (1976) expressed as mg $Prot.g^{-1}$ fresh weight (FW) referred to Bovine Serum Albumin (BSA) standard curve.

Reaction mixture to determine SOD (EC 1.15.1.1) activity comprised 20 µL of enzymatic extract, 1 mL potassium phosphate (KOH), 50 $mmol.L^{-1}$ buffer, pH 7.6, 0.1 $mmol.L^{-1}$ EDTA, 0.01 $mmol.L^{-1}$ cytochrome C, 0.05 $mmol.L^{-1}$ xanthin, 0.03 unities of xanthin oxidase (EC 1.2.3.22) (SIGMA). Mixture xanthin-xanthin oxidase was used as superoxide radicals source using just as cytochrome C method (550 nm) (extinction molar coefficient 340 = 21.1 $(mmol.L^{-1})^{-1}cm^{-1}$) (Mc Cord and Fridovich, 1969) in spectrophotometer (Pharmacia, LKB). Reaction time was 3 min, enzymatic activity was expressed as µmol of superoxide by $min.g^{-1}$ FW and specific activity was expressed as µmol superoxide by $min.mg^{-1}$ Prot.

Leaf D from the same plantlets from two treatments was used for physiological evaluations realized at the beginning of acclimatization phase and after 14, 28 and 42 days. Photosynthesis (µmol CO_2 $m^{-2}.s.^{-1}$) and transpiration total (mmol H_2O $m^{-2}.s^{-1}$) were measured using CIRAS-2 (Portable System of Photosynthesis, Europe, PP Systems, UK) equipment connected to universal cuvette PLC6 2.5 cm^2. The water-use efficiency (WUE) was estimated at these variables as relationship between photosynthesis and transpiration total.

Survival was estimated as percentage as relationship between the number of alive plantlets in each moment of evaluation and the total number per treatment at the beginning (0 day).

The Statistical Package for Social Sciences (Version 11.5 for Windows, SPSS Inc.) was used to perform statistical significance range test for bi-factorials comparisons or Student's t-test for comparison of two conditions, both at 5% were evaluated using two-way analysis of variance (ANOVA) followed by Tukey's Multiple significance. Normal distribution and homogeneity of variances were evaluated with Kolmogorov-Smirnov and Levene tests, respectively. Some data were mathematically transformed for statistical analyses. Discrete quantitative variables were transformed according to $y' = SQR(y)$ or $y' = SQR(0.5 + y)$. Percentage variables were transformed according to $y' = 2 \arcsin(SQR(y/100))$.

RESULTS AND DISCUSSION

The *in vitro-ex vitro* transition of plants provokes an abiotic stress to them and one of the responses to this situation is related to ROS such as superoxide anion, hydrogen peroxide, etc. At high concentrations, ROS cause abnormalities and in extreme cases may result to cell death of plant tissues (Kim et al., 2009). SOD is the first in plant defense system to transform the superoxide anion into H_2O (Kim et al., 2009).

Figure 1 shows the results of SOD activity determined under effects of 1 $mg.L^{-1}$ and without JA. The results of Figure 1 showed no differences between two groups on the SOD activity in all the evaluated moments. However, in control plantlets, the values of the activity of this enzyme were different at the initial as much as at final evaluation. Plantlets treated with JA increased the enzyme activity of SOD from the first 14 days.

In this period, the same plantlets registered higher soluble protein content than control group (Figure 1 B). Only on the 28 day evaluation, this variable was higher in plantlets not treated with JA. At the end of acclimatization for both groups, this variable decreases to the lowest values of the experiment due to the reduction in the synthesis of these biomolecules, the translocation to other organs or degradation as a consequence of environmental conditions under which the plantlets were grown.

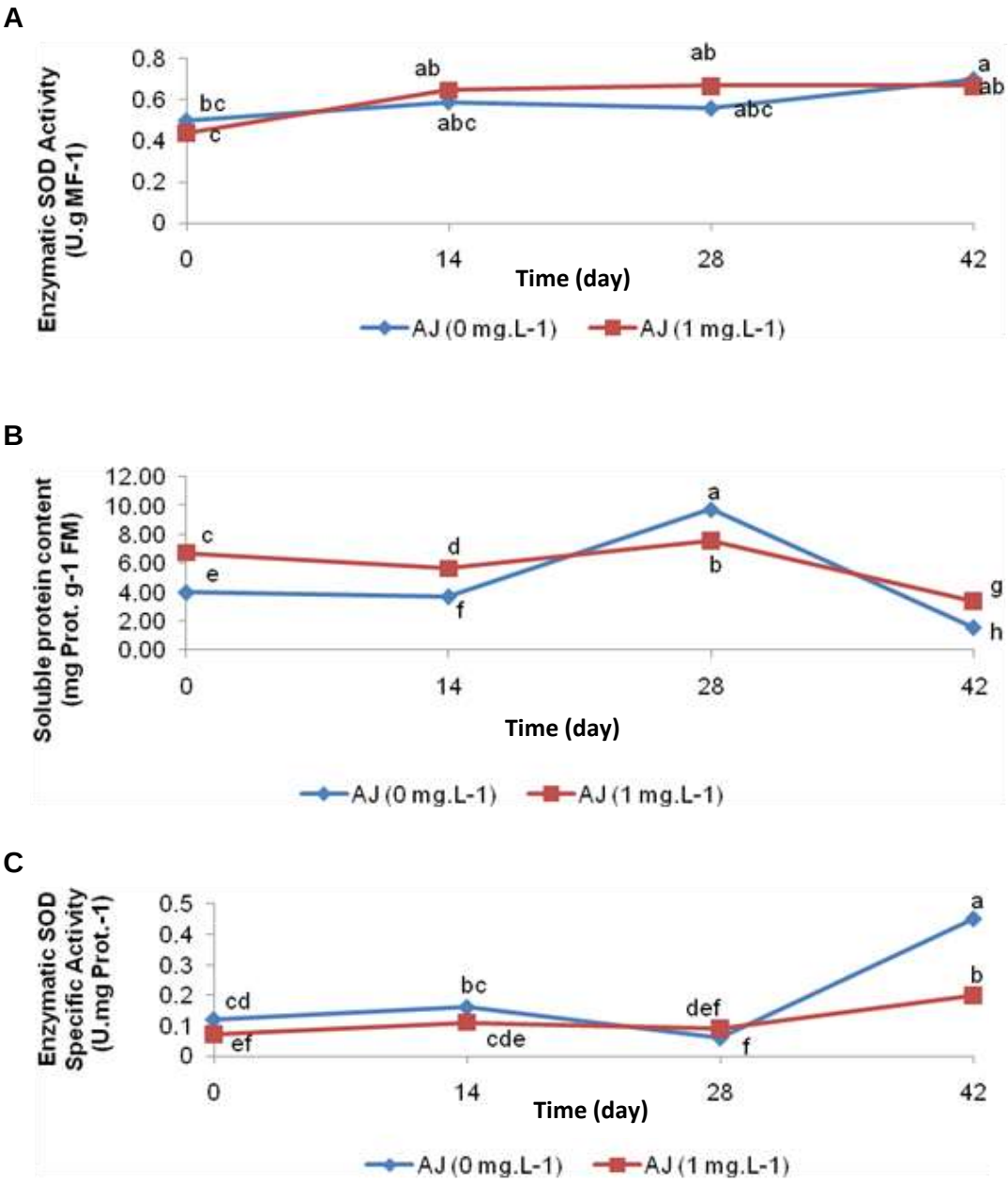

Figure 1. Effect of jasmonic acid on the SOD activity (A) (SE= ±0.02 U.g MF^{-1}), SOD specific activity (C) (SE= ±0.02 U.mg $Prot^{-1}$) and soluble protein content (B) (SE=±0.36 mg $Prot.g^{-1}$ MF), of *Ananas comosus* cv MD-2 plantlets in acclimatization conditions. Means with different letters indicate signification (ANOVA, Tukey test, $p \leq 0.05$). Each datum represents the mean for n=6. One unit (U) corresponds to 1 µmol of superoxide by minute.

As a consequence of the behaviour previously analyzed in Figure 1A and B, the enzymatic SOD specific activity also varied (Figure 1C). Changes registered in this variable are in agreement with the concentration of soluble proteins quantified in the plantlets (Figure 1B) and they deserve a proteomic study of each moment of evaluation. During the transition moment, plantlets treated with JA showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase (Aragón et al., 2010), which was expressed since the specific activity of SOD lightly increased at the end of evaluation against high increase observed in plantlets without JA in relation to the variable content of soluble protein and therefore with enzymatic specific activity, since the enzymatic activity was the same in both treatments (Figure 1A).

Table 1. Effects of Jasmonic acid (1 mg.L^{-1}) on water use efficiency (mmol CO_2 mol^{-1} H_2O) of MD-2 hybrid pineapple plantlets (*Ananas comosus* (*n* = 40)) in acclimatization conditions.

Treatment	Days after acclimatization			
	0	14	28	42
Without Biojas®	0.61[b]	3.35[a]	6.46[b]	17.29[a]
Biojas®	1.44[a]	3.62[a]	8.37[a]	14.05[a]
SE	0.15	0.12	0.44	0.86

Means within columns followed by the same letters are not significantly different (Student't Test, $p \leq 0.05$).

At the end of acclimatization, the moment where the reduction of metabolic activity and growth rate is observed frequently, among other reasons due to substrate exhaustion, environmental and nutritional factors that resulted to be restrictive. The specific activity of SOD increased for both groups. The increase was higher in the control plantlets because they had a higher enzymatic activity and low protein concentration. This demonstrated the anti-stress effects induced by the JA on pineapple plantlets of this experiment.

Normally, plantlets are stressed during the *in vitro-ex vitro* transition due to changes on environmental conditions such as light and relative humidity (Kozai et al., 2000). As a result, plantlets suffer from abiotic stress that is frequently manifested through dehydration and photo-oxidation (Preece and Sutter, 1991) that provokes changes in the electron transfer chain and thus in redox systems. Light reactions are the most important source of ROS in illuminated mesophyllic cells. Jasmonates induced the degradation of chloroplast proteins, among them ribulosebiphosphate carboxylase/oxygenase subunits (Agrawal et al., 2002). JA through the same mechanisms might have reduced the generation of ROS such as superoxide anion (O^{-2}) that has the capacity to cause oxidative damage to proteins, DNA and lipids.

Low generation of ROS (presumably O^{-2}) in plantlets treated with JA ensures their good growth. Higher ROS production can cause a retarded growth in plants as it was observed in transgenic potato plants with an elevated ROS production by the over expression of chloroplastic Cu/Zn SOD (Kim et al., 2009).

Forty two days after acclimatization, both groups increased the specific activity of SOD with a marked difference among them where control plantlets had higher values. This final moment of acclimatization corresponds to stress factors that provoke metabolic changes as previously analyzed. It is known that early stimulation of antioxidant enzymes during the C3 to CAM change is accompanied by the increase in ROS generation. That is supported by molecular induced analysis during 30 to 40 h treatment with salinity in *M. crystallium* leaves, showing that genes related to stress and antioxidant proteins are among the first to be induced (Kore-eda et al., 2004; Niewiadomska and Borland, 2008).

As we all know, plant will trigger the production of ROS in response to stress. They have a dual effect which is based on their overall cellular amount in plant. If kept in low level, they can function as signaling molecules to transmit information from metabolism to trigger appropriate cellular defense/acclimation response to environmental changes (Mittler, 2017).

Using data not shown on transpiration and photo-synthesis, the water-use efficiency was calculated as shown in Table 1.

JA reduced the transpiration of treated plantlets only at the beginning of acclimatization with significant difference, which can be attributed to stomata conductance. It can be supposed that JA induced stomata closure, as previously has been informed by other authors (Creelman and Mullet, 1997; Evans, 2003). Exogenous MeJA does not appear to antagonize ABA-induced stomatal closure, although the ability of MeJA to regulate stomatal apertures remains controversial (Montillet et al., 2013). Recently, it has been proposed that 12-OPDA (a JA precursor), rather than MeJA, acts in promotion of stomatal closure (Savchenko et al., 2014 but Han et al. (2018) demonstrated the negative regulation of stomatal development.

In this study, the last evaluations of the transpiration recorded similar values in both treatments, which decreased in each evaluation with respect to previous study. At the same time, photosynthesis did not change between the treatments but increased during the experiment, but without significant differences in the last three evaluations. All these joined by the low transpiration rate in plantlets treated with JA to perform their photosynthesis with the higher water-use efficiency (Table 1) especially at the beginning of acclimatization which is the most critical moment of the acclimatization process. WUE was also significantly higher in plantlets treated with Biojas® after 28 days of acclimatization.

JA increased WUE by reducing the transpiration rate without a marked difference on photosynthesis in respect to control plantlets. The low WUE of these plantlets at the beginning of acclimatization is as a result of the incapacity of the plantlets to control excessive water loss through transpiration. In general, the WUE increased during acclimatization in both groups because the plants improved their control on transpiration rate. WUE is the resultant compromise between the maximum o photosynthesis and the minimum transpiration to improve the plant quality (Cernusak et al., 2007), as shown in this

Table 2. Effects of Jasmonic acid (1 mg.L^{-1}) on survival (%) of MD-2 hybrid pineapple plantlets (*Ananas comosus* (*n* = 90)) in acclimatization conditions.

Treatment	Days after acclimatization			
	0	14	28	42
Without Biojas®	100^{a}	98^{ab}	94^{b}	94^{b}
Biojas®	100^{a}	98^{ab}	96^{ab}	96^{ab}

Means followed by the same letters are not significantly different (ANOVA, Tukey Test, p≤ 0.05). Data were transformed according to y´= 2 arcsin (SQR(y/100)).

experiment where plantlets treated with Biojas® comprised intrinsically of the capacity to suffer tolerance to the abiotic stress caused by acclimatization conditions and increased the survival as shown in Table 2.

The higher levels of survival were in line with the efficiency of the methodology used according to Yanes et al. (2000). Nevertheless, plantlets treated with JA reduced the losses by the death of plantlets during 42 days, due to 94% of survival in control which was statistically different while 96% in JA treatment was similar. Another datum that resume the productive value of application BioJas® to save 2% of plantlets during *in vitro-ex vitro* transition in relation to the expression of SOD activity to suffer tolerance to the effects of this abiotic stress, added to the knowledge on the physiology of *ex vitro* pineapple (*A. comosus* var. MD-2) as CAM or C3 regulated by the environmental conditions (Aragón et al., 2012). JA acts mainly as signal molecule as plant response against this abiotic stress and SOD activity could be a biological indicator if studied in line with the performed by Avila et al. (2017) with Ethrel®48 treatment to increase pineapple flowering.

It is known that temperature increased (in this case from 23 to 29°C) during the transition. The influence of temperature on the production of plants can be direct, on the growth of the plant altering its physiology, or indirectly by varying the humidity, the quantities of minerals absorbed by the plant and its transport. Whatever the influence of the thermal increase is in this transit, the results of the application of Biojas® favoured the relationships of the metabolic processes, perhaps as demonstrated by Cejas et al. (2012) in other thermal management required to improve productivity in new climatic scenarios. It would then be a tool to apply in predictive studies (Lobell and Asseng, 2017). Pineapple plantlets treated with JA showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase, when superoxide dismutase activity showed adequate control against this stress related to superior water-use efficiency and survival.

Thus, based on these results, this study could show the molecular, hormonal, and histological changes that are present right after Biojas® application, providing new insights into how pineapple acclimatization occurs under natural conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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