Article

# Salicylic acid induces tolerance to cryogenic stress in *Solanum tuberosum*

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## Abstract

A common problem in cryogenic processes is survival at ultra low temperature (-196 °C). Based on the physiological effects of salicylic acid (AS) on the signaling of stress tolerance responses, the objective was to evaluate the effect of AS on increasing survival and on the growth of potato buds undergoing cryogenics. Microplants of the advanced clone 06-27 of the Germplasm Bank *in vitro* of the Potato Program in Metepec State of Mexico, Mexico, were incubated for 28 days in AS (0, $10^{-6}$ and $10^{-5}$ M) and subsequently subjected to cryogenic process. Effect of AS in plant regeneration: a significant increase in survival was observed in plants pretreated with AS $10^{-5}$ and $10^{-6}$ M with respect to the control (1.66-2.04 times respectively). Fresh weight, stem length and root significantly increased in microplants pretreated with AS with respect to the control. Cryogenesis: both concentrations of $10^{-5}$ and $10^{-6}$ M AS induced significantly greater survival (2.17-3.21 times respectively) of explants to cryogenics. Plants preincubated in AS and regenerated after being subjected to cryogenics for 1 h significantly increased root length. The control showed callus formation, absent in the plants treated with AS. The AS-cryogenic combination favored the development of plants subjected to methods that employ ultra-low temperatures in potatoes for the purpose of germplasm cryopreservation or cryotherapy to obtain virus-free materials.

**Keywords:** *in vitro* regeneration, potato, ultra low temperature.

Reception date: June 2019
Acceptance date: October 2019

## Introduction

An important factor for food security is the adequate conservation of available genetic resources and the preservation of plant diversity. The conservation of genetic resources can be done by short or long term strategies. One of these long-term strategies is the use of cryogenic procedures, which involve the storage of plant materials such as corn (*Zea mays*) beans, yam (*Sphenostylis stenocarpa*), cowpea (*Vigna unguiculata*), peanut bambara (*Vigna subterranea*), soybean (*Glicine max*), oil palm (*Elaeis guineensis*), cocoa (*Theobroma cacao*), coconut (*Cocos nucifera*), avocado (*Persea americana*), mango (*Mangifera indica*) and coffee (*Coffea* spp.) among other species (Sánchez-Chiang and Jiménez, 2010).

This storage is done at ultra low temperatures (-196 °C) using liquid nitrogen (NL) (Day *et al*., 2008; Hamilton *et al*., 2009). At this temperature all cellular functions are suspended and therefore the tissue can be stored without changes or deterioration for long periods (Kaczmarczyk *et al*., 2012). In *Solanum tuberosum*, various cryogenic methods have been successfully tested, such as encapsulation-dehydration, vitrification and D-cryoplaque **(**Hao e*t al*., 2002; Halmagyi *et al*., 2005; Hirai, 2011; Yamamoto *et al*., 2015). Wang *et al*. (2006), proposed the use of cryogenic methods for the long-term conservation of potato accessions and for the elimination of viruses.

However, the physiological response to cryogenics will depend on the genotype, with the optimization of methods to increase survival (Rivera *et al*., 2008) being necessary. During cryogenics cell tissues are susceptible to various types of stress such as mechanical, osmotic and temperature, which lead to oxidative stress. (Baek and Skinner, 2012). Exposures to extreme temperatures such as cold (0-20 °C) or freezing (<0 °C) result in decreased survival (Chinnusamy *et al*., 2007), producing oxidative damage (Horvath *et al*., 2007).

The formation of reactive oxygen species (EROS) in cryogenics can occur during several of the steps involved, (Kaczmarczyk *et al*., 2012). It has been shown that the addition of exogenous antioxidants such as ascorbic acid, vitamin E and glutathione in blackberry and citrus fruits during cryopreservation, resulted in greater survival (Wang and Deng, 2004; Uchendu *et al*., 2010). Plants have enzymatic and non-enzymatic antioxidant systems to resist stress, enzymatic such as catalase (CAT), peroxidase (POX), super oxide dismutase (SOD) and non-enzymatic such as ascorbic acid, glutathione and α tocopherol (Gill and Tuteja, 2010).

It has been reported that salicylic acid participates in signaling antioxidant activity in potatoes under extreme temperatures (Mora-Herrera *et al*., 2005; Aguilar-Camacho *et al*., 2016). Salicylic acid (AS) induces an increase in antioxidant enzymes during periods of pre-acclimatization at low temperatures in treated plants (Sasheva *et al*., 2010; López-Delgado *et al*., 2018). Mora-Herrera and López-Delgado (2006), found that AS induces freezing temperature tolerance (-6 °C) in potatoes. AS has also been tested in cryogenics in various species, inducing tolerance to freeze stress (Wang and Valkonen, 2009b; Li *et al*., 2011; Pathirana *et al*., 2016).

In embryonic axes of Persian lilac (*Melia azedarach* L.) they reported that the AS significantly increased the viability percentages after being subjected to cryopreservation (Bernard *et al*., 2002). The objective of this work was to test the potential effect of AS in increasing survival and growth responses in potato buds undergoing cryogenic process.

## Materials and methods

*Solanum tuberosum* L. plants of advanced clone 06-27 with late blight tolerance of the *in vitro* Germplasm Bank of the INIFAP Potato Physiology and Biotechnology Laboratory in Metepec State of Mexico, Mexico, were selected based on their low tolerance to cryogenesis observed in preliminary laboratory work. The plants were incubated for 28 days in MS propagation medium (Murashige and Skoog, 1962) under treatment of AS, 0, $10^{-6}$ and $10^{-5}$ M (Mora-Herrera *et al*., 2005; Aguilar-Camacho *et al*., 2016). Nodal buds (1-2.5 mm) of these microplants were evaluated under the following conditions:

a) Effect of AS on plant regeneration after treatment: twenty buds were subcultured to MS medium without AS and incubated for 15 days, to assess survival, fresh weight, plant height and root length. Survival was assessed considering oxidation and turgidity.
b) Cryogenics: twenty buds were subcultured to MS medium without AS and incubated for 3 days, to be subjected to cryogenics. The cryogenic method used was Dehydration-cryoplate (D-cryoplate) (Yamamoto *et al*., 2015, modified by Arizaga *et al*., 2017) which consists of the following steps.

1) a volume of 2µL of sodium alginate (2 % w/v sodium alginate/0.4 M sucrose in a basal solution of MS medium), was placed in each of the 10 wells of a cryoplate (7 x 37 x 5 mm).
2) each of the buds were transferred individually to each of the wells of the cryoplate, to later cover them with the sodium alginate solution.
3) the cryoplate with the buds was covered with a sterile sheet of BEMCOT paper (7X30 mm).
4) 1 mL of calcium chloride solution (0.1 M calcium chloride/0.4 M sucrose in basal MS solution) was added to cover the cryoplate. Polymerization of sodium alginate/calcium chloride was completed after 15 min at room temperature, removing excess calcium chloride solution.
5) the cryoplate with the buds and the adhered paper were transferred to a loading solution (SC) (2 M glycerol/1 M sucrose in basal MS solution), for 45 min, the excess SC was removed.
6) the cryoplate with the buds and the attached paper were transferred to a petri dish with 35 g of silica gel for the dehydration process, for 90 min at 24 °C.
7) after dehydration, the cryoplates were transferred to cryotubes and immersed directly in liquid nitrogen (NL) for 60 min.
8) the cryoplates were removed from the NL and transferred to cryotubes with 2 mL of sucrose solution (1 M in basal MS medium) for 15 min at room temperature.
9) The buds attached to the Bemcot paper were removed from the cryoplate and placed in petri dishes with MS medium for 24 h.

10) the sodium alginate capsule was subsequently removed leaving the bud exposed, which was subcultured to fresh MS medium.
11) 15 days after the subculture, the survival of the regenerated plants was evaluated, considering those that resumed their growth alive; as well as callus formation and root length (n= 8-20).

Statistical analysis was performed through analysis of variance (Anova) and Duncan test (Duncan, 1955) in all experiments, using the Statgraphics Centurion XVI program. The established level of reliability was $p< 0.05$. The experiments were performed in triplicate.

## Results and discussion

### Effects of AS on plant regeneration after treatment.

After the treatment of AS in the microplants treated with AS $10^{-6}$ and $10^{-5}$ M, physiological responses were observed such as greater survival, root length and plant height. A significant increase in survival of 2.04 and 1.66 times respectively compared to the control (Figure 1).

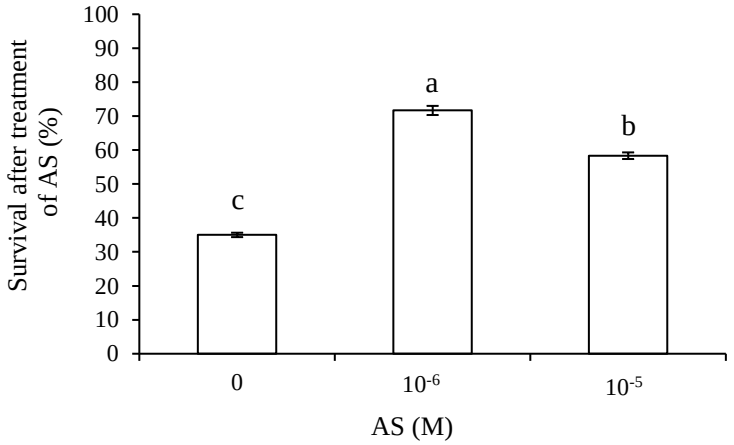


**Figure 1. Effect of AS on the survival of potato clone 06-27 after treatment.** Variables evaluated 15 days after subculture. Data obtained from 3 experiments (n= 20). Data analyzed with Anova and Duncan test ($p< 0.05$). Different letters indicate significant differences from the control.

Similar effects on increased survival by AS have been found in various species when used in different concentrations, such as in *Astragalus adsurgens* (1.64 times) (Luo *et al*., 2001) and *Coffea arabica* (1.57 times) (Quiroz-Figueroa *et al*., 2001).

Microplants pretreated with AS $10^{-6}$ and $10^{-5}$ M showed a significant increase in root length of 3.28 and 2.87 times respectively, as well as in plant height of 2.60 and 1.87 times compared to the control (Figures 2a and 2b).

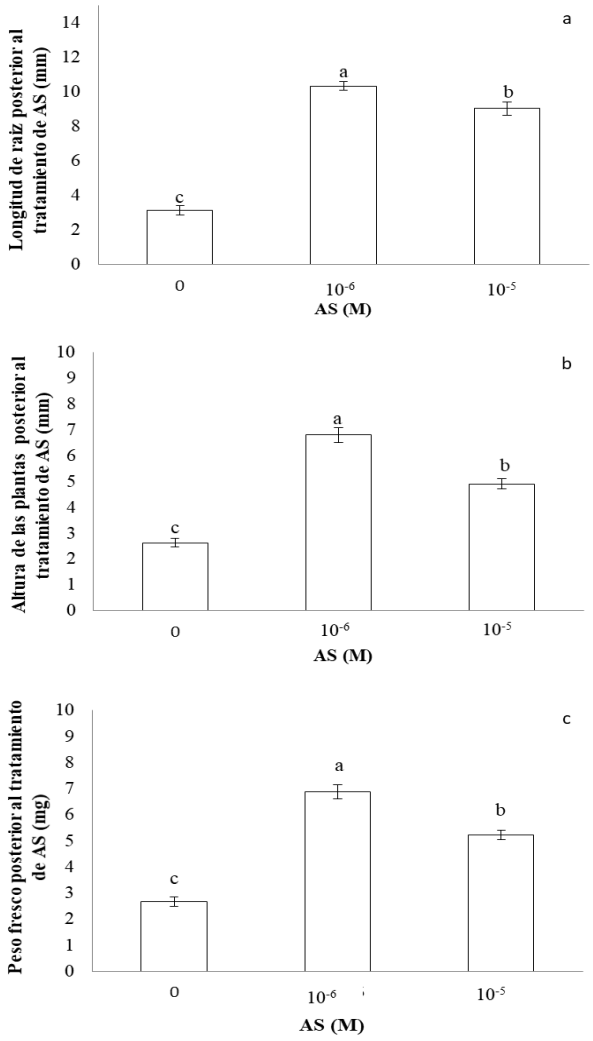


**Figure. 2 Effect of AS on the growth of potato clone 06-27 after treatment. a) root length; b) plant height; and c) fresh weight.** Variables evaluated 15 days after subculture. Data obtained from 3 experiments (n= 20). Data analyzed with Anova and Duncan test ($p< 0.05$). Different letters indicate significant differences from the control.

The results obtained in this research are of high interest, because greater root development is important for the establishment in both in vitro and ex vitro conditions of any species, because it is directly related to the absorption of nutrients and their It also induces an increase in growth (Ruiz, 2000; Selles *et al*., 2003; Callejas-Rodríguez *et al*., 2012).

This coincides with that reported by Larqué-Saavedra and Martin-Mex (2007), since they found an increase in density and root length of plants treated with AS. According to Sakhabutdinova *et al*. (2003), AS treatments can increase cell division in seedling roots by increasing their overall growth, the above may be related to the effect of AS on the increase in root length observed in this work in potato microplants (Figure 2a).

Other works that agree with the results obtained in this investigation corresponding to the effects of AS in root length and plant height (Figure 2a, 2b), have been reported in different crops such as Hibiscus (varieties *acetocela* and *moscheutos*) (Sakhanokho and Kelley, 2009), corn (Gunes *et al*., 2007), soy (Gutiérrez-Coronado *et al*., 1998) and wheat (Shakirova *et al*., 2003). Pre-treated microplants with AS $10^{-6}$ and $10^{-5}$ M showed a significant increase in fresh weight of 2.58 and 1.96 times respectively, compared to the control (Figure 2c). Paez-García *et al*. (2015), mention that the application of AS induces an increase in biomass due to a greater development in the radical system, which leads to a better absorption of water and nutrients.

## Cryogenesis

The microplants that resumed their growth from the treatments of AS $10^{-6}$ and $10^{-5}$ M showed an increase in cryogenic survival of 3.21 and 2.17 times respectively, compared to the control (Figure 3).

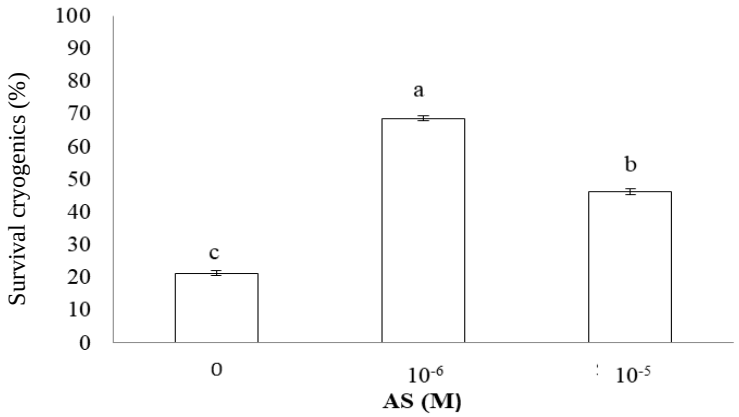


**Figure 3. Effect of AS on the survival of potato clone 06-27 after cryogenesis. Variables evaluated 15 days after subculture.** Data obtained from 3 experiments (n= 8-20). Data analyzed with Anova and Duncan test ($p< 0.05$). Different letters indicate significant differences from the control.

These results contrast with the values obtained by Rivera *et al*. (2008), who obtained up to 52% survival in the absence of pretreatment of AS. The observed results may be related to a reduction in oxidative stress due to AS, as has been demonstrated in potatoes under different types of stress, such as greater survival under thermotherapy (up to 2 times) (Aguilar-Camacho *et al*., 2016).

Higher tuber weight in plants infected with phytoplasma (1.88 times) (Sánchez-Rojo *et al*., 2011), especially greater survival at low temperatures (1.77-2.35 times) (Mora-Herrera *et al*., 2005; López-Delgado *et al*., 2018). After the cryogenic process, the absence of callus was observed in the microplants from both AS treatments while the control showed 68% callus formation without stem bud formation (Figure 4).

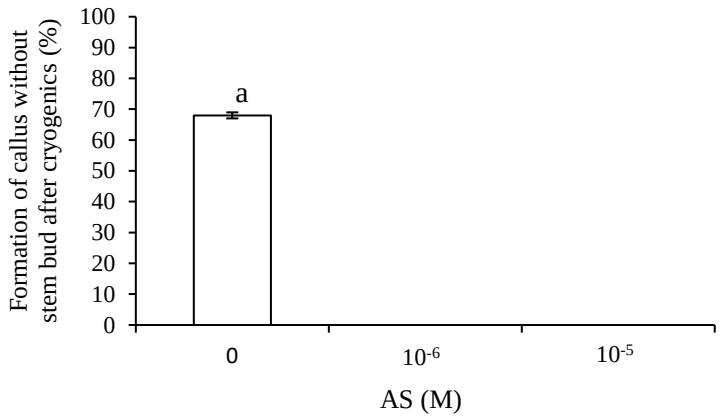


**Figure 4. Effect of AS on the development of potato clone 06-27 after cryogenesis. a) Callus formation without stem bud development; and b) Root formation.** Evaluations made 15 days after the cryogenic process. Data obtained from 3 experiments (n= 8-20). Data analyzed with Anova and Duncan test ($p< 0.05$). Different letters indicate significant differences from the control.

During plant regeneration after cryogenic techniques, direct regeneration without callus formation is very desirable, because it ensures the genetic stability of the plants obtained (Reed, 2008). The results in this investigation demonstrate the potential of AS to increase survival in cryogenesis in potato genotypes that have low survival in these processes, in addition to preventing callus formation in regeneration, since the risk of somaclonal variation is reduced, favoring conservation of phenotypic and genotypic characteristics of plant material.

In addition to the absence of callus in the regenerated explants, a significant increase in root length was obtained in plants pretreated with AS $10^{-6}$ and $10^{-5}$ M of 3.35 and 2.36 times respectively after cryogenesis (Figure 5).

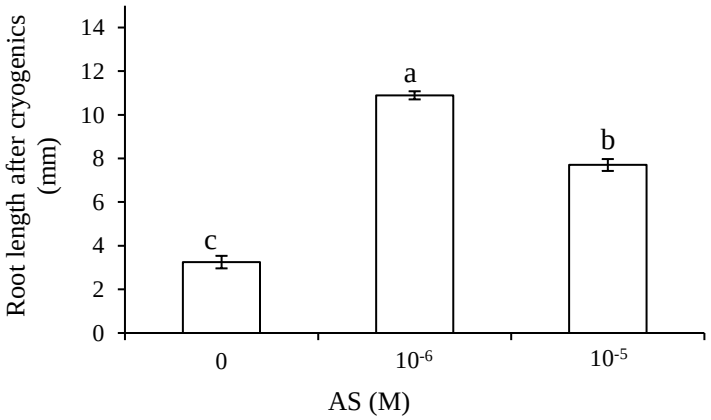


**Figure 5. Effect of AS on the development of potato clone 06-27 after cryogenesis. a) callus formation without stem bud development; and b) root formation.** Evaluations made 15 days after the cryogenic process. Data obtained from 3 experiments (n= 8-20). Data analyzed with Anova and Duncan test ($p< 0.05$). Different letters indicate significant differences from the control.

Compared to the control, the success of optimal regeneration after exposure to liquid nitrogen is based on an adequate physiological and morphological state of the explant that was subjected to the cryogenic method (Engelmann *et al*., 2008).

Pretreatments with AS can be an important tool for a better survival to cryopreservation or cryotherapy techniques in potatoes, in genotypes with low survival at ultra low temperatures since it is a signaling molecule of stress tolerance induction and plant growth regulator (Horvath, 2007), also involved in the development of roots in conditions of stress due to low temperatures (Huang and Villanueva, 1993; Melkonian *et al*., 2004).

## Conclusions

In this investigation, the effect of AS was observed as an inducer of tolerance to ultra-low temperatures used in cryogenic processes in potato plants that have low survival to cryogenics, increasing survival rates.

In addition to this there is also a promoter effect of AS in the development of explants subjected to the cryogenic process since a significant increase in the development of the roots of these explants after their regeneration was obtained. AS avoided callus formation, which is an important factor in maintaining genetic stability in crops such as *Solanum tuberosum*.

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