In vitro carbohydrate stress: salicylic acid increases soluble invertase activity in *Pistacia vera* L. *in vitro* plantlets

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Abstract

The action of salicylic acid (SA) has been well investigated in plant resistance against pathogen attacks but its role may be extended to a more global anti-stress plant cell strategy. The expression of defense-related functions may be also enhanced by elevated hexose levels. To verify if there exists a relation between these two defense programs, SA effect on soluble acid invertase (EC 3.2.1.26) activity was investigated in *in vitro* grown *Pistacia vera* plantlets from isolated embryonic axes, at different doses of exogenous sucrose. For this purpose embryonic axes were cultivated on Murashige and Skoog medium containing 10, 20, 30, 40 and 50 $g.L^{-1}$ sucrose (control range) with 50 µM SA. After one month of growth, roots and shoots were used for analysis separately. SA treatment significantly enhanced the soluble invertase activity in tissues. The increase was more remarkable in root tissues. Sucrose limitation (10 $g.L^{-1}$) SA-treated plantlets and sucrose osmotic stressed (50 $g.L^{-1}$) SA-treated plantlets have shown very important increases in invertase activity and this was accompanied with a significant raise of total protein in spite of growth reduction. The implication of soluble invertase in the anti-stress strategy of these tissues seems to be important. It is likely that hexose signaling of defense expression may be related to the action of salicylic acid on soluble invertase activity.

Keywords: Pistachio; sucrose; invertase; salicylic acid; *in vitro* stress

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Introduction

Salicylic acid, a natural plant hormone, plays a very important role in the regulation of plant defense mechanisms (Shah and Klessig, 1999) in response to biotic (Dempsey et al., 1999) and abiotic stresses (Janda et al., 2007). Defense activators as salicylic acid (SA) or β-butyric acid (BABA) have been used efficiently at low doses in defense priming experiments on Arabidopsis seedlings (van Hulten et al., 2006; Rajjou et al., 2006) and may be good candidates for studying *in vitro* defense priming to acclimate in vitro plants to various stress conditions of the *in vitro* life and subsequent transplantation.

In vitro culture medium provides carbohydrates for the growth and development of vitroplants and sucrose is used preferentially. The addition of exogenous sucrose to the nutrient media limits the photosynthetic capacity

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of vitroplants (Capellades et al., 1991, Hdider and Desjardins, 1994) and sucrose concentration has a direct effect on the success of acclimatization (Serret et al., 1997). Soluble sugars such as sucrose may interact in the control of reactive oxygen species balance (Couée et al., 2006) and therefore are directly implicated in plant stress defense strategy undoubtedly activated by unfavorable conditions of *in vitro* culture. Sucrose breakdown is done by the action of two enzymes, sucrose synthase and invertase, with hexoses release. Acid invertase (EC 3.2.1.26) activity was stimulated by sucrose feeding *in vitro* as shown by Van Le et al. (2001) on tomato vitroplants, but differentially according to illumination and CO_2 conditions. Herbers et al. (1996) showed that the elevated levels of hexose have been implicated as signals for the induction of a plant defense response. If hexose signaling of defense expression occurs, it is probably related to the increased expression of vacuolar invertase (Blee and Anderson, 1998).

To know if interrelations may exist between the roles of SA and hexoses in the activation of *in vitro* plant defense, we have studied the effects of a low dose of SA on the level of the invertase activity in *Pistacia vera* vitroplantlets obtained from embryonic axis and cultivated in a range of sucrose concentrations from 10 to 50g.L^{-1}.

Materials and Methods

Embryonic axes were excised from seeds of *Pistacia vera* L. var. Qazvin and were cultivated on Murashige and Skoog (1962) medium (MS) containing 10, 20, 30, 40 and 50 g.L^{-1} sucrose with or without 50 µM SA. After one month of culture at 25° C and with 16 h lights (2000 Lux) and 8 h obscurity photoperiod regime, the axes were harvested and used for growth determination. Growth was estimated on the basis of changes in length of roots and shoots. Total protein was determined according to Bradford (1976). Invertase was extracted from roots and shoots separately according the method of Schaffer et al. (1989) with a slight modification. Briefly 100 mg of tissues were extracted in 800 µL of cold 50 mM Hepes-NaOH (pH 7.5) containing 0.5 mM Na_2EDTA, 2.5 mM DTT, 3 mM DETC, 0.5% (w/v) BSA and 1% (w/v) PVP. The homogenized solution was centrifuged at 15300 rpm for 30 min and the supernatant was dialyzed against cold 25 mM Hepes-NaOH pH 7.5 and 0.25 mM Na_2EDTA for 20h. 200 µL of extract was diluted with 600 µL of citrate phosphate buffer pH 5 and the enzyme reaction was started by adding 200 µL of 0.1 M sucrose. The reaction mixture was then incubated at 37° C for 1 hour and stopped by addition of 1 ml Sumner reagent (2,5-dinitrosalysilic acid 1% (w/v) in 0.5 M KOH and 1 M K/Na-tartrate). The reaction mixture was kept for 5 min in a boiling water bath, cooled to room temperature and the color intensity read at 540 nm against a blank that contained boiled enzyme extract. A completely random design was used and an analysis of variance was done for statistical analysis with LSD post test at a level of significance of $p<0.05$.

Results

Effects of SA on growth of embryonic axis, invertase activity and total protein content of *Pistacia vera* L. plantlets are shown in Figs I, II and III respectively. The findings suggest that SA treatment significantly enhanced the soluble invertase activity in tissues under study. This increase was more remarkable in root tissues. Moreover, sucrose limitation (10 g.L^{-1}) SA-treated plantlets and sucrose osmotic stressed (50 g.L^{-1}) SA-treated plantlets have shown very important increases in invertase activity. This increase was accompanied by a significant raise in total protein despite growth reduction.

Discussion

Sucrose concentration of the medium influenced the growth of embryonic axes, under illumination conditions that were relatively low (2000 lux) and therefore photosynthesis limiting. In these conditions the growth was sucrose dose dependent. A higher growth was measured in samples grown in MS medium containing 30 and 40 g.L^{-1} of sucrose. On the other hand, in embryonic axis cultivated in sucrose limitation condition (10 g.L^{-1}) or sucrose excess condition (50 g.L^{-1}) where sucrose acted as osmotic agent, the growth was severely reduced and more pronounced in the roots (Fig. I).

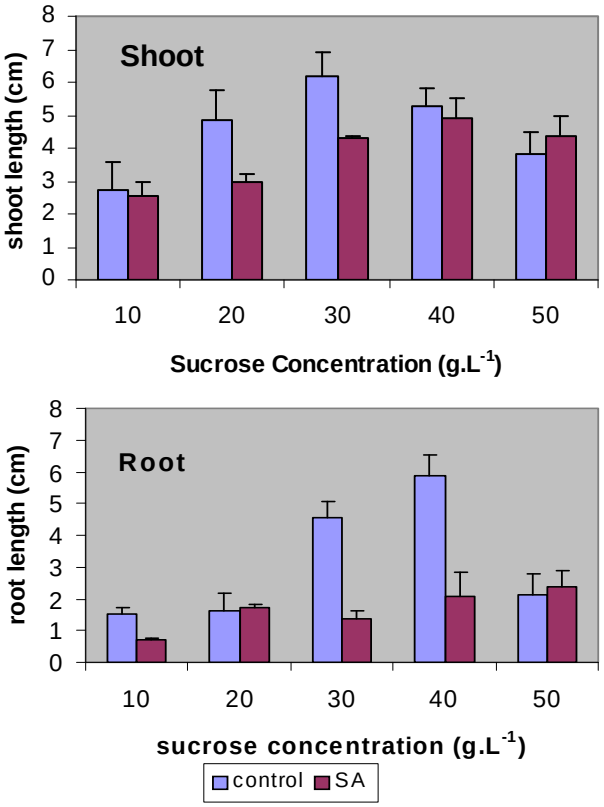

Fig. I. Effect of SA (50µM) on growth of embryonic axis in MS medium after 30 days of culture (Data are mean ± SE of 4 replicates)

SA affected the growth of plantlets and the effect was dose dependent. Fifty µM was chosen for this experiment since in a previous study (non published data) on a range of concentrations of SA, this dose proved to be the minimum dose to have an effect on pistachio growth. When SA was added to the medium, shoot elongation was affected in the case of 20 and 30 $g.L^{-1}$ sucrose which are the sucrose conditions that have supported the best growth in control. With regard to roots, SA strikingly reduced the roots growth of plantlets grown on 30 and 40 $g.L^{-1}$. With SA treatment, a significant inhibition of root growth was also obtained for sucrose limitation stressed plantlets (Fig. I). It may be remarked that SA had apparently no effect on the growth of sucrose osmotic stressed plantlets. In a previous work on tomato SA-treated plantlets, Tari et al. (2002) have shown an osmotic SA effect. In sucrose osmotic stressed *Pistacia* plantlets, the osmotic effect of SA was probably suppressed by the osmotic effect of sucrose and this explains the absence of difference between SA treated plantlets and control for this condition only. Tari et al. (2002) showed that this osmotic effect suggests an increase in soluble sugars and this correlates with findings about *Camellia sinensis* cuttings (Haddad Kaveh et al, 2004) and by the present results on invertase activity in *Pistacia* (Fig. II).

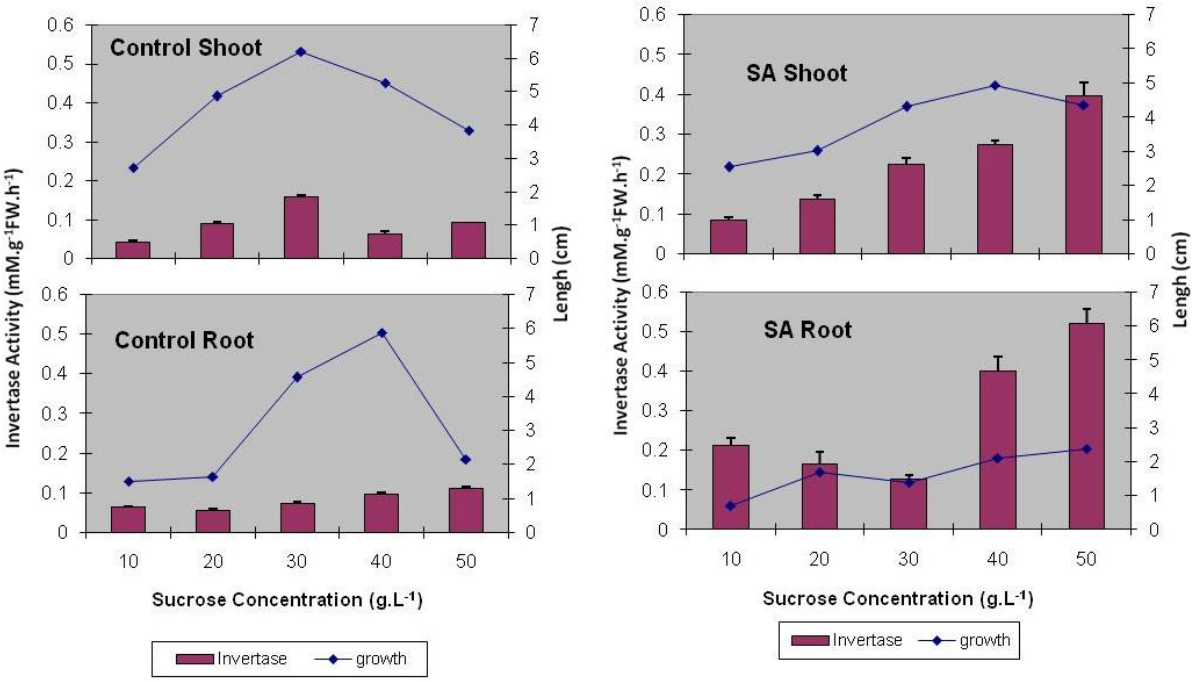

Fig. II. SA effect on growth and invertase activity of *Pistacia vera* L. plantlets cultivated

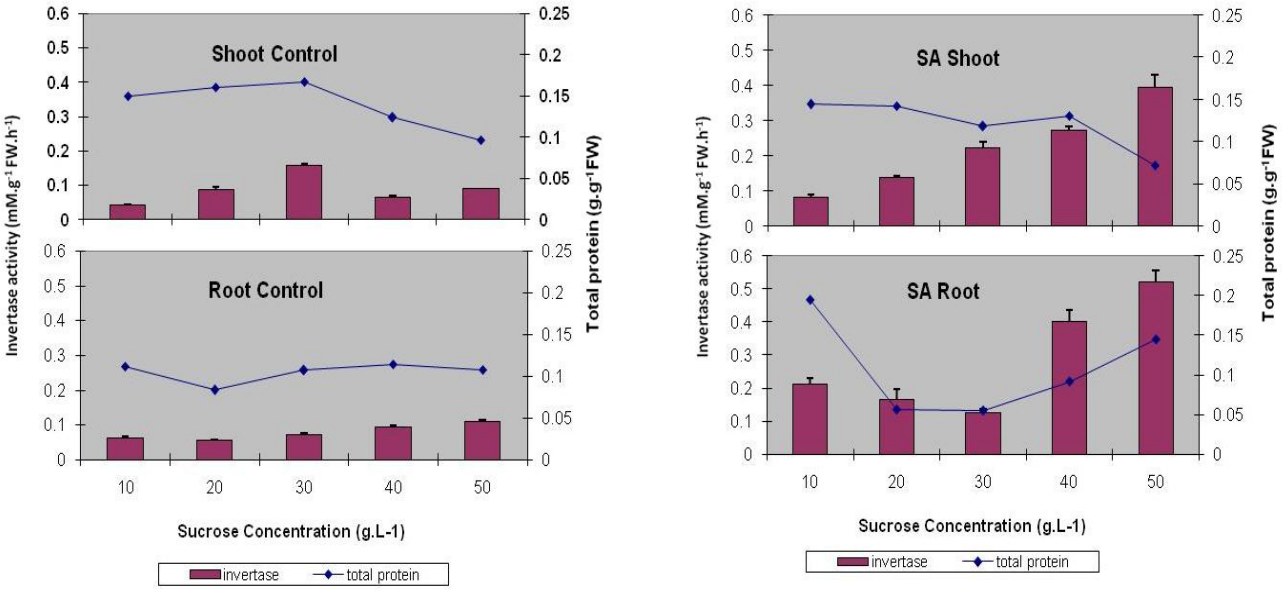

Fig. III. Effect of SA on total protein content in *Pistacia vera L*. plantlets cultivated on MS-medium with various concentration of sucrose for 30 days of culture (Data are mean ± SE of 4 replicates)

In *Pistacia* plantlets, the level of invertase activity was low in root and shoot control samples. A slight stimulation of the enzyme activity has been noted in shoots of plantlets having grown with 30 $g.L^{-1}$ sucrose. One may suggested that the increase of hexoses level that has been inevitably produced as a result of this elevation of invertase activity has probably been used to sustain the shoot growth in this condition. This suggestion is based on the fact that there appeared a good concordance between the curve of shoot growth and the variations in enzyme activity.

Exogenous supply of 50 µM SA has stimulated invertase activity in all conditions of culture. Tae Kyung et al (2011) who tested SA on isolated tomato leaves during *Botrytis cinerea* infection did not observe this stimulatory effect of SA on the invertase activity contrary to jasmonic acid but the doses they used were higher and this should certainly be considered in the analysis of results.

Our SA effect is particularly important in stressed tissues and is more pronounced in root samples. In roots, variations in the level of invertase activity were not related to growth. Therefore, we can suppose that hexoses that are produced in SA condition may have been employed for other metabolism needs than the production of cell wall in growth. They may take part in the synthesis pathways of special proteins such as defense proteins and their synthesis may have been triggered by SA. This supposition seems to be confirmed with the results concerning the dosage of total protein (Fig. III). In SA-treated roots of stressed plantlets with 10 $g.L^{-1}$ and 50 $g.L^{-1}$ sucrose in the medium, the very significant enhancement of invertase activity (3 and 5 fold respectively) occurred with an elevation of total protein. With these results we can say that in condition of stress the plantlets seem to be more responsive to salicylic acid. Nutrient stress was triggered by very low concentration of sucrose or osmotic stress due to the high sucrose concentration in the medium. These two conditions amplified the stimulatory effect of SA on the activity of soluble invertase. Giving exogenous SA to the plantlets may have enhanced the endogenous level of SA as noted in Seo et al (1995). We may suppose that in our case the level of SA in the tissues has been elevated but probably not in the same extent between the different sucrose conditions. High levels of endogenous SA were also notified in response of several types of stress (wounding, ozone, pathogen attack) (Zhang and Klessig, 1998; Leon et al., 1995). So the amplified response in invertase activity of stressed SA-treated plantlets

may be explained by the likely higher increase of endogenous SA in these particular stress conditions. In fact the regulation of the endogenous concentration of the hormone SA is complex and closely regulated and the regulation may even depend on the activity of invertase as described In Leclere et al (2008) showing a parallel between decrease in cell wall invertase activity and increased SA content.

When SA was given to stressed plantlets in the case of sucrose limitation or in the case of sucrose excess, the response of invertase was specially high suggesting that the defense system of this plantlets has been stimulated. Combined effects of SA and sucrose induced stresses were amplified. Because of the SA activation of invertase, hexose levels may have been considerably enhanced in these plantlets. One may expect that, to provide for hexose production triggered by SA, plantlets cultivated on a limited sucrose medium have to synthesize sucrose, i.e., they have to work in an autotrophic way to a greater extent. So one may suppose that effective stimulation of defense by a low dose of SA associated with nutrient carbohydrate stress may offer better possibilities for the plantlets to withstand new stresses of transfer to *ex vitro* conditions and this hypothesis remains to be verified.

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