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Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture

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Abstract Embryogenic cell suspension cultures of *Coffea arabica* cv. Caturra Rojo were treated with salicylic acid (SA). Two concentrations, 10^{-12} and 10^{-10} M, had a significant effect on the growth rate of the cell cultures when compared to the control, and this effect was concentration-dependent. These two SA concentrations also had a dramatic effect on both the number of somatic embryos and quality, in terms of embryo size and development. In general, the use of SA had a positive effect on cellular growth and somatic embryogenesis, causing a twofold increase in both processes. The increase in the number of somatic embryos could be a reflection of an increase in the number of embryogenic cells induced with SA treatment.

Keywords Salicylic acid · *Coffea arabica*
Somatic embryogenesis

Abbreviations BA: N⁶-Benzyladenine ·
ASA: Acetylsalicylic acid ·
2,4-D: 2,4-Dichlorophenoxyacetic acid · Kin: Kinetin ·
NAA: α -Naphthaleneacetic acid · SA: Salicylic acid

Introduction

Salicylates belong to a diverse group of plant phenolic compounds usually defined as substances that possess an aromatic ring bearing a hydroxyl group or its functional derivative (Raskin 1992a). Salicylic compounds are ubiquitous in plants (Raskin et al. 1990) and are involved in several biological events, such as flowering (Cleland and Ajami 1974; Kaihara 1981), stomata clo-

sure (Larqué-Saavedra 1979), adventitious root initiation (Kling and Meyer 1983), thermogenesis (Raskin et al. 1987, 1989; Dat et al. 2000), inhibition of ethylene biosynthesis (Huang et al. 1993; Leslie and Romani 1986), induction of multiple stress tolerance (Senaratna et al. 2000), protection of nitrate reductase from degradation (Jain and Srivastava 1981), resistance to pathogens and the biosynthesis of pathogenesis-related (PR) proteins (in the presence or absence of pathogenic organisms) (Raskin 1992b). In some cases SA suppresses certain resistance mechanisms, such as the synthesis of phytoalexins (Ponchet et al. 1983) or proteinase inhibitors (Doares et al. 1995). It also inhibits abscisic acid-induced abscission (Apte and Laloraya 1982).

Roustan et al. (1989) reported that SA at a concentration of 50 μ M stimulated somatic embryogenesis in *Daucus carota*, while in *Pelargonium* \times *hortorum* Baily somatic embryogenesis was stimulated by 20 μ M SA (Hutchinson and Saxena 1996). In both cases there was a twofold increase in the number of embryos. However, Nissen (1994) found that SA, when used at a concentration of 100 μ M, did not stimulate somatic embryogenesis in carrot but inhibited it. Similar results were obtained in somatic embryogenesis of *Medicago sativa* when SA concentrations were lower than 5 μ M (Meijer and Brown 1988). In both of these investigations, ethylene biosynthesis increased.

In *Coffea* spp. several model systems have been reported for somatic embryogenesis induction. In vitro somatic embryogenesis of *Coffea canephora* was first reported by Staritsky (1970), who described the induction of callus tissue from orthotropic internodes. Herman and Haas (1975) subsequently obtained somatic embryogenesis in *Coffea arabica* from callus cultures derived from leaf explants. Söndahl and Sharp (1977, 1979) developed a two-phase experimental protocol for somatic embryogenesis from leaves of *C. arabica* var. Bourbon. Dublin (1981) reported somatic embryogenesis from leaf explants of Arabusta using a medium supplemented with cytokinins but without auxins. Yasuda et al. (1985) induced embryogenic calli from *C. arabica* leaf explants

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using 5 μM BA; white and friable calli were initiated after 16 weeks, and somatic embryos were obtained 4 weeks later. Somatic embryogenesis in coffee is very different from somatic embryogenesis in other species. In carrot, the process takes only a few days (Krikorian and Smith 1991), while the best results in coffee are obtained after several weeks. The group of Söndahl demonstrated the latter when, after several weeks, a small number of somatic embryos were produced in a process called low-frequency somatic embryogenesis; this was followed by the production of somatic embryos on top of the brown tissue, which was called high-frequency somatic embryogenesis (Söndahl and Sharp 1977).

For transformation and genetic improvement programs, it is desirable to have a fast and efficient protocol by which to produce coffee somatic embryos. In the investigation reported here, we examined the effect of SA on cellular growth and somatic embryo production in cell suspension cultures of *C. arabica* cv. Caturra Rojo.

Materials and methods

Plant material and culture conditions

Embryogenic cell suspension cultures of *Coffea arabica* cv. Caturra Rojo were maintained and embryogenesis was initiated as previously described by Quiroz-Figueroa et al. (2001). Briefly, leaves of in vitro somatic plantlets of *C. arabica* cv. Caturra Rojo were cut, avoiding the mid-vein and edges. Explants were placed on callus induction medium [S1; MS basal salts (Murashige and Skoog 1962), 29.6 μM thiamine-HCl, 550 μM myo-inositol, 210 μM L-cysteine, 87.7 mM sucrose, 4.5 μM 2, 4-D, 9.2 μM Kin and Gelrite (0.1% w/v), pH 5.8]. Secondary-type creamish-like calli were transferred to fresh medium every 4 weeks. Embryogenic suspension cultures were established by transferring aliquots (1–2 g) of this creamish-like callus, which was used as the starting material, to 50 ml of liquid medium S1 (medium S1 without Gelrite) in 250-ml Erlenmeyer flasks. These suspensions were maintained on a gyratory shaker at 100 rpm and 25 ± 2 C in the dark. Subculturing occurred every 2 weeks in 50 ml of fresh medium. After 1 week the suspension cultures, maintained in S1 medium, were transferred to the embryogenesis induction medium [S3; MS inorganic salts at one-half ionic strength (except for KNO_3 which was increased 2 \times normal strength), 29.6 μM thiamine-HCl, 550 μM myo-inositol, 210 μM L-cysteine, 87.7 mM sucrose, 0.27 μM NAA, 2.3 μM Kin, pH 5.8]. The culture conditions consisted of a low inoculum density (0.25 g of tissue), 250-ml Erlenmeyer flask, and 100 ml of medium, with the culture being maintained on a gyratory shaker at 100 rpm and 25 ± 2 C in the dark. The medium was changed every 4 weeks.

Salicylic acid assays

SA, in concentrations varying from 10^{-12} M to 10^{-6} M (in steps of 10^{-2} M) (Larqué-Saavedra and Gutiérrez-Coronado 2000) was first filter-sterilized through a 0.45- μm Millipore filter, then added to the propagation medium, pH 5.8. The cultures were maintained for 28 days, with samples being taken at day 14 and day 28 of culture. To evaluate the effect of SA on somatic embryogenesis, we transferred 14-day-old tissues maintained in propagation medium supplemented with 10^{-12} M SA to induction medium supplemented with various concentrations of SA (10^{-12} to 10^{-6} M at 10^{-2} M steps). After 6 weeks, somatic embryos at different stages of development (globular/heart and torpedo/cotyledonary) were counted under a dissecting microscope.

All experiments consisted of three replicates of each treatment and were repeated at least three times. Data were analyzed by analysis of variance, and the means were compared by the least significant differences (LSD) test at $P=0.05$.

Results and discussion

To answer the question of whether SA affected the growth rate of cultured cells, we added SA to the medium during the period of culture. SA was indeed found to influence the growth rate of the cultured cells and the effect was dose-dependent. After 14 days (Fig. 1A), cultures treated with 10^{-12} M and 10^{-10} M SA grew on average 40% more than the control. Relative growth decreased as the concentration of SA increased. The maximum relative growth, at day 28 (Fig. 1B), was obtained using a concentration of 10^{-12} M SA, at which time the increase was 70% over the control. In general, the higher concentrations did not have a significant effect on cellular growth.

Carswell et al. (1989) reported that micromolar concentrations of ASA increased colony formation from protoplast cultures prepared from an embryogenic suspension culture of inbred elite maize. However, the ASA did

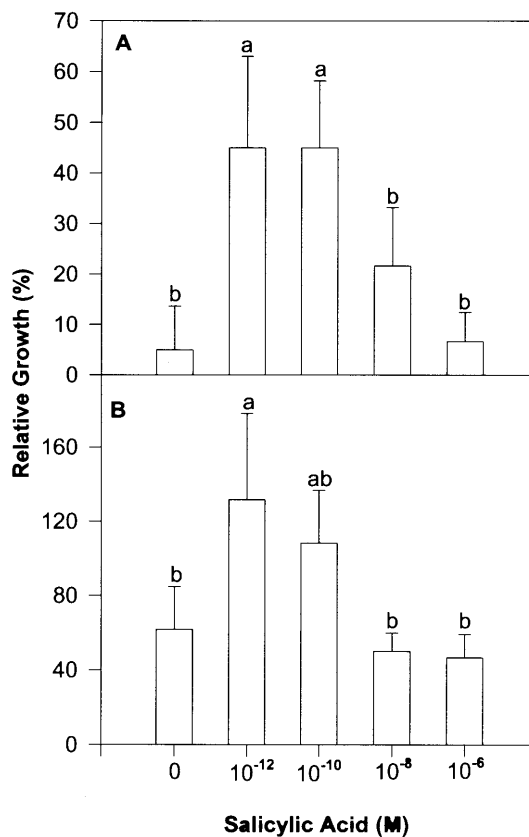


Fig. 1 Effect of SA on the relative growth of *Coffea arabica* cell culture after 14 days (A) and 28 days (B) in culture. Different letters indicate that differences are significant at $P=0.05$. Relative growth is based on the increment in weight on the determined day with respect to the control (without SA) at day 0 and is expressed as a percentage

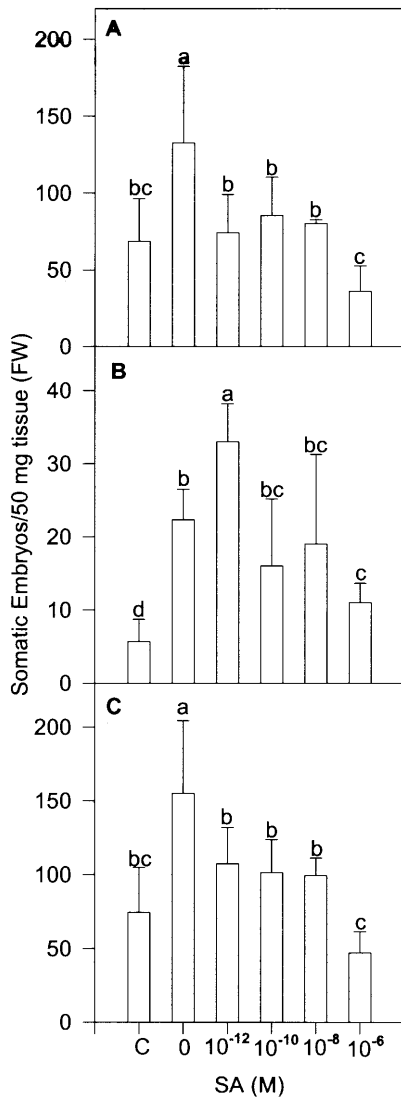


Fig. 2A–C Effect of SA on somatic embryo production of *C. arabica* cell culture. Tissues maintained in propagation medium with SA (10^{-12} M) until day 14 were transferred to induction medium supplemented with different concentrations of SA. Mean numbers of somatic embryos at the globular/heart stage (A), torpedo/cotyledonary stage (B) and total (C) were taken after 6 weeks of embryogenesis induction. Treatment C Propagation and induction tissues without SA. Different letters indicate that differences are significant at $P=0.05$

not promote the growth rate of protoplast-derived cell colonies. These data are in agreement with our observation that at micromolar concentrations there is no effect on the growth rate of the embryogenic coffee tissues (Fig. 1). However, the response can be dependent on the species, since in pearl millet (*Pennisetum americanum*) micromolar concentrations of ASA enhanced callus growth on a dry weight basis. There was also a significant reduction in ethylene production in the presence of ASA, which may be responsible for the enhancement in the growth of embryogenic cultures (Pius et al. 1993).

After 14 days, cellular suspensions of coffee cultured on the propagation medium containing 10^{-12} M SA were

transferred to embryogenic induction medium either containing no SA or supplemented with different concentrations of SA. The number of somatic embryos at different stages of development (globular/heart and torpedo/cotyledonary) and the total number of embryos were determined after 6 weeks. The highest number of somatic embryos at the globular/heart stages was obtained when the SA was not present in the induction medium (Fig. 2A); this was an exception as none of the SA concentrations had a significant effect on the number of somatic embryos formed when compared to the control. In contrast, all treatments (Fig. 2B) had significant effects on the number of somatic embryos at the torpedo/cotyledonary stages relative to the control. The 10^{-12} M SA treatment had a greater effect in terms of somatic embryo number (Fig. 2B) and quality (measured as embryo size and the absence of malformations) (Fig. 3C). However, SA had no effect on the capacity of the embryos to generate plantlets (data not shown).

When cellular suspensions were maintained in propagation medium supplemented with 10^{-12} M SA and then transferred to induction medium without SA, the cells produced the greatest total number of somatic embryos (Fig. 2C). In *Medicago sativa*, the addition of millimolar concentrations of SA to the differentiation medium resulted in a reduction in the number of somatic embryos, which parallel a substantial increase in ethylene production (Meijer and Brown 1988). In embryogenic suspensions of *Daucus carota*, somatic embryogenesis was stimulated in the presence of SA and ethylene production was inhibited (Roustan et al. 1989). In contrast, Nissen (1994), using embryogenic suspensions of the same species as Roustan et al. (1989), determined that the presence of low concentrations of SA stimulated the production of ethylene and inhibited the somatic embryogenesis process. In explants of geranium, the presence of ASA during a 3-day period of somatic embryogenesis induction caused a twofold increase in the number of somatic embryos and enhanced synchronization of embryo development when compared to the control (Hutchinson and Saxena 1996). This is very similar to the results presented here.

Figure 3A shows the morphological aspects of embryogenic cellular suspensions produced in the absence of SA (control). The induction of the embryogenic process in the absence of SA in cultures previously grown in the presence of 10^{-12} M SA during the maintenance phase produced a more homogeneous response (Fig. 3B). The presence of 10^{-12} M SA (Fig. 3C) and 10^{-10} M SA (Fig. 3D) during the induction phase of somatic embryogenesis resulted in a better development of the somatic embryos. In addition, the embryos were more abundant and larger than those produced in the absence of SA (Fig. 3A, B) or in the presence of higher concentrations of SA (Fig. 3E, F).

In our investigation, picomolar concentrations of SA had a positive effect on coffee embryogenic cellular suspension cultures, resulting in a twofold increase in cellular growth and somatic embryogenesis as well as en-

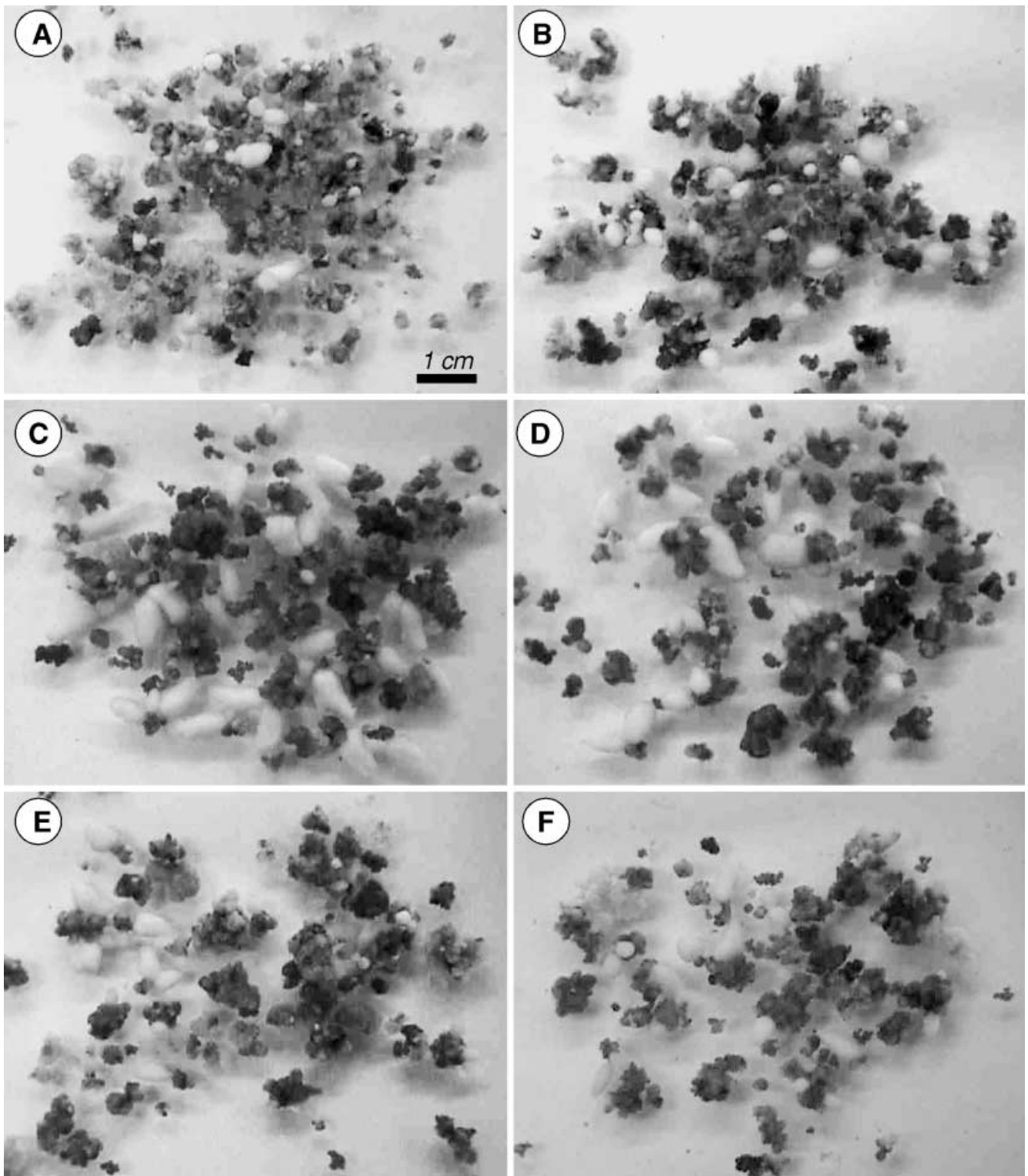


Fig. 3A–F Morphology of somatic embryos from *C. arabica* cell culture induced with SA. **A** Without SA in both propagation and embryogenesis induction steps [0/0]. **B** Induction of the embryogenic process in the absence of SA in cultures grown previously in the presence of 10^{-12} M [0/12]. **C** Induction with 10^{-12} M SA in cultures previously grown in 10^{-12} M SA [12/12]. **D** Induction

with 10^{-10} M SA in cultures previously grown in 10^{-12} M SA [10/12]. **E** Induction with 10^{-8} M SA in cultures previously grown in 10^{-12} M SA [8/12]. **F** Induction with 10^{-6} M SA in cultures previously grown in 10^{-12} M SA [6/12]. Photographs correspond to somatic embryos obtained in the experiment reported in Fig. 2 and are representative samples taken from single flasks

hancing synchronization. The increased number of somatic embryos could be a consequence of the greater number of embryogenic cells or, as has been previously suggested, to the inhibition of ethylene synthesis (Roustan et al. 1989). On the other hand, the effect of SA at higher concentrations could be due to the possibility that phenolic compounds are able to change the organization of the membrane (Jain and Srivastava 1981).

Prior to this work, and to the best of our knowledge, SA has never been tested at these very low (picomolar) concentrations for its physiological effect, other than in the case of the activation of the systemic acquired resistance pathway in bean (De Meyer et al. 1999). Our results, together with those of other investigators, suggest that SA could be acting as a morphoregulator signal, which supports the hypothesis that SA is functioning as a growth regulator (Raskin 1992b).

The presence of contradictory evidence makes it difficult to explain the mechanism of SA action in the somatic embryogenesis responses observed. The question remains as to whether or not SA directly affects cellular differentiation. However, a possible mechanism could involve ethylene production, since SA at concentrations between 10 μM and 100 μM stimulated carrot somatic embryogenesis and strongly inhibited the production of ethylene by blocking the enzymatic conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene (Leslie and Romani 1986; Roustan et al. 1989). This effect is dose-dependent, as low concentrations of SA stimulated rather than inhibited ethylene production in other carrot somatic embryogenesis systems (Nissen 1994). This effect is also species-dependent. For example, SA concentrations of 5–20 μM in *M. sativa* inhibited somatic embryogenesis but did not inhibit ethylene biosynthesis (Meijer and Brown 1988).

Browning of the tissues, caused by an excessive accumulation of phenolic compounds, is necessary for the somatic embryogenesis process in coffee (Quiroz-Figueroa et al. 2001). Similar observations have been reported by other authors (de Garcia and Menéndez 1987; Neuenschwander and Baumann 1992; Van Boxtel and Berthouly 1996; Menéndez-Yuffá and de Garcia 1997). It is possible that these phenolic compounds act as signals to induce the differentiation process. An alternative explanation is the possibility that, due to the chelating properties of these compounds, some inhibitors present in the embryogenic cultures are inactivated.

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