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Influence of sodium salicylate on adventitious organogenesis of a commercial cucumber cultivar

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Abstract Cucumber is an important vegetable crop belonging to the Cucurbitaceae family. An optimized regeneration system is required to address different biotechnological approaches in this species, such as large-scale multiplication, as well as genome editing and genetic transformation techniques. The aim of this study was to investigate the effect of different concentrations of the phenolic compound sodium salicylate (NaSA), a salicylic acid derivative, on *in vitro* callus and shoot regeneration of a commercial cultivar of cucumber. Four-day-old cotyledon explants from the slicing cultivar 'Marketer' were employed. Murashige and Skoog (MS)-derived shoot induction medium containing 0.5 mg L-1 indole-3-acetic acid (IAA) and 2.5 mg L-1 6-benzylaminopurine (BAP) was supplemented with NaSA. Frequency and shoot number were enhanced by 1.5-fold with NaSA at 0.1 µM. Higher salicylate levels led to increased callus formation and decreased shoot regeneration. The application of sodium salicylate at a specific concentration showed a positive trend in *in vitro* adventitious organogenesis of a commercial cucumber cultivar. Some probable mechanisms that may underlie the beneficial effects of salicylic acid/salicylates on *in vitro* regeneration were also discussed.

Keywords: salicylic acid sodium salt, morphogenesis, shoot regeneration, *Cucumis sativus* L., Cucurbitaceae

1. Introduction

Sodium salicylate (sodium 2-hydroxybenzoate; NaSA) is the sodium salt of salicylic acid (SA) (PubChem 2022a). SA (2-hydroxybenzoic acid) is an endogenous growth regulator and a signaling molecule involved in the regulation of various physiological processes related to plant growth and development, as well as in the modulation of plant responses to different abiotic and biotic stresses (Mohamed et al 2020; PubChem 2022b).

Exogenous application of SA may affect a variety of plant processes and attributes, such as photosynthesis and photosynthetic pigments (Khan et al 2003; Maurya et al 2019), respiratory pathways (Khan et al 2003), ethylene biosynthesis (Khan et al 2013), seed germination (Lee et al 2010), flower induction (Cleland and Tanaka 1979), thermogenesis (Rhoads and McIntosh 1992), ion uptake and transport (Harper and Balke 1981; Gondor et al 2016), nitrate reductase activity (Fariduddin et al 2003), protein content (Latif et al 2016), growth parameters (Damalas 2019), biomass production (Maurya et al 2019), plant water relations (Hayat et al 2010), stomatal closure (Khan et al 2003), accumulation of osmolytes (e.g., proline, glycine betaine, polyamines, and soluble sugars) (Khan et al 2014; Madany et al 2020; Shemi et al 2021), secondary metabolite content (e.g., alkaloids, flavonoids, and phenolics) (for review see Nandy et al 2021), allelopathic properties (Shettel and Balke 1983), antioxidant defense system (Maurya et al 2019), and senescence (Rivas-San Vicente and Plasencia 2011). It can also alleviate environmental stresses, such as heat (Khan et al 2013), cold (Saleem et al 2020), drought (Sohag et al 2020), UV-B/C radiation (Li et al 2014; Abrun et al 2016), salinity (Elazab and Youssef 2017), and heavy metals toxicity (Sharma et al 2020), and protect plants from a range of pathogens (Bakker et al 2014). The effect of exogenous SA is influenced by factors such as the plant species, its stage of development, tissue and organ type, mode of application, dose and duration of exposure, environmental and culture conditions, and its endogenous level in the plant (Horváth et al 2007; Janda et al 2014; Kračun-Kolarević et al 2015; Bhutia et al 2018). SA application has been reported to modulate plant functions dose-dependently (Maurya et al 2019). Inadequate levels of SA can also lead to oxidative stress (Gondor et al 2016). SA-like effects have been reported with structurally similar compounds, such as NaSA and acetylsalicylic acid (ASA). Some authors have previously described improved *in vitro* plant regeneration with exogenous SA or ASA. In the present work, it is investigated by applying NaSA.

The cucumber (*Cucumis sativus* L.) is an economically important species belonging to the Cucurbitaceae family, along with melon, watermelon, and squash. Its world production, including gherkins, ranked third among vegetable crops in 2020,

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with 91,258,272 tons (FAOSTAT 2022). This species is an important source of valuable nutrients and bioactive compounds used not only as food but also in cosmetics, therapeutics, and perfumery (Uzodike and Onuoha 2009; Uthpala et al 2020).

In vitro regeneration in cucumber has been established and can be achieved through organogenesis and somatic embryogenesis. The most frequently used explants are cotyledons (Chee 1990; Selvaraj et al 2007; Miguel 2017), hypocotyls (Ziv and Gadasi 1986; Chee 1990; Grozeva and Velkov 2014), and leaves (Malepszy and Nadolska-Orczyk 1983; Lou and Kako 1994; Seo et al 2000). Regeneration from protoplasts (Jia et al 1986; Trulson and Shahin 1986; Burza and Malepszy 1995) and suspension cultures has also been reported (Malepszy and Solarek 1986; Bergervoet et al 1989; Kreuger et al 1996). However, regeneration in cucumber is still not optimal (Wang et al 2015) and is highly dependent on the genotype (Wehner 1981; Punja et al 1990; Miguel 2021a,b). An efficient and reproducible regeneration system is fundamental to address different biotechnological approaches, such as secondary metabolite production, pathogen elimination, germplasm conservation, and large-scale multiplication, as well as genome editing and genetic transformation technologies for functional genomics and genetic improvement.

Sodium salicylate (NaSA) is a derivative of salicylic acid (SA). Exogenous application of these phenolic compounds may affect a range of physiological processes in plants. Unlike SA, the influence of NaSA on *in vitro* regeneration has not been explored. The present investigation aimed to evaluate the effect of different levels of sodium salicylate on adventitious organogenesis of a commercial cucumber cultivar to improve its efficiency.

2. Materials and Methods

2.1. Plant material and in vitro regeneration

Cucumber seeds of the commercial cultivar 'Marketer' (Semillas Fitó S.A., Barcelona, Spain) were the starting material. It is a medium-cycle cultivar with vigorous growth, a monoecious flowering habit, and dark green cylindrical fruits 20-23 cm long. It is of good quality for the fresh market and tolerant to Cucumber Mosaic Virus (CMV) (Borrego 1989). Obtaining axenic explants and *in vitro* regeneration followed the methodology previously described by Miguel (2021a). Four-day-old cotyledons with proximal and distal ends excised by 1-2 mm deep were used as explants. They were cultivated for three weeks on MS (Murashige and Skoog 1962) derived shoot induction medium containing 0.5 mg L-1 indole-3-acetic acid (IAA) and 2.5 mg L-1 6-benzylaminopurine (BAP) and supplemented with sodium salicylate (0.0, 0.1, 1.0, and 10.0 µM; Sigma–Aldrich, St. Louis, MO, USA). Callus frequency (CF) and callus extension index (CEI) were then determined. The CF (mean ± SE) is the proportion of explants with the callus on the explant cut surface, and the CEI (mean ± SE) corresponds to the extent of callus on the cut surface and was graded as follows: 0 = absence of callus; 1= trace of callus; 2 = callus on less than half; 3 = callus on half or more; 4 = callus covering the entire extent. Organogenic structures were then cultured for 2 weeks on shoot development and elongation medium containing 0.2 mg L-1 kinetin. At the end, an evaluation of shoot regeneration frequency (SRF) and shoot number index (SNI) was made. SRF (mean ± SE) is the proportion of explants forming shoots, and SNI (mean±SE) was scored according to the number of shoots per explant as follows: 0 = absence; 1 = one shoot; 2 = two shoots; 3 = three or more shoots. Individualized shoots were then rooted on a hormone-free MS medium, and within 3 to 4 weeks, the plantlets were ready for acclimatization (data not shown). *In vitro* culture media were solidified with 0.8% (w/v) agar and adjusted to pH 5.7 before autoclaving. Incubation was performed in a culture chamber at 26 ± 2 ºC under a 16L:8D h photoperiod. The light was provided by cool-white fluorescent lamps (Grolux, Sylvania) at a photon fluence rate of 90 µmol m-2 s-1.

2.2. Data analysis

The experiment was a single-factor design with four levels of sodium salicylate (NaSA) arranged in a completely randomized design. Twelve replicate flasks with six explants each were used in each treatment, except for 10.0 µM NaSA with six replicate flasks. The R statistical computing environment (version 4.0.4; R Core Team 2021) was used to perform all statistical analyses. Non-linear regression analyses were applied to the data to compare treatment means. The R packages 'stats' (R Core Team 2021), 'COMPoissonReg' (Sellers et al 2019), and 'pscl' (Jackman 2020) were used to conduct logistic regression, COM-Poisson regression and Hurdle regression, respectively. The goodness of fit of the models was assessed using the Akaike Information Criterion (AIC; Akaike 1973) and the Bayesian Information Criterion (BIC; Schwarz 1978). The significance level was set at $P \leq 0.06$ for shoot variables and at $P < 0.05$ for all other analyzes.

3. Results

3.1 Frequency and extension of callus

Results presented as mean ± SEM (Table 1), with significance determined as $P < 0.05$. Callus formation occurred within the first two weeks of culture, starting at the cut ends of the cotyledon explant and scoring 100% frequency. The application of 0.1 µM sodium salicylate (NaSA) produced the lowest callus extension (1.22 ± 0.05), with significant differences from the other treatments that did not differ from each other. Callus extension scores lay between the arbitrary values of 1: traces of callus, and 2: callus in less than half, at explant cut, ends.

3.2 Frequency and number of shoots

Mean results ± SEM are showed in Table 1. Shoots formed from callus within 2-4 weeks of culture, mostly at the proximal end of the cotyledon explant. The highest frequency (45.83 ± 5.91) and shoot index (0.38 ± 0.06) corresponded to 0.1 µM NaSA, the lowest level, with differences compared to the NaSA-free control ($P = 0.06$), and the treatments with NaSA ($P < 0.05$), which did not differ from each other ($P < 0.05$). It represented a 1.5-fold increase compared to the control.

Table 1 Effect of adding sodium salicylate to MS-derived shoot induction medium containing 0.5 mg L-1 IAA and 2.5 mg L-1 BAP on in *vitro* callus and shoot regeneration from cotyledon explants of a commercial cultivar of *Cucumis sativus* L. Data presented as mean ± standard error of the mean (SEM).

Sodium salicylate		Callus regeneration frequency (%)[p]	Callus extension index[q]	Shoot regeneration frequency (%)[r]	Shoot number index[s]
µM	µg L-1				
0.0	0	100	1.46 ± 0.06[a]	30.56 ± 5.47[b]	0.26 ± 0.05[b]
0.1	16	100	1.22 ± 0.05[b]	45.83 ± 5.91[a]	0.38 ± 0.06[a]
1.0	160	100	1.54 ± 0.06[a]	23.61 ± 5.04[b]	0.23 ± 0.06[b]
10.0	1601	100	1.43 ± 0.08[a]	20.00 ± 6.86[b]	0.14 ± 0.05[b]

[p,q]Data obtained after three weeks of culture on shoot induction medium.
[r,s]Data obtained after two weeks of culture on shoot development and elongation medium.
[q]COM-Poisson regression, [r]logistic regression, and [s]Hurdle regression used to analyze the data. Mean values followed by different letters in the same column are significantly different: [q]$P < 0.05$; [r,s]$P \leq 0.06$).

4. Discussion

The effect of sodium salicylate (NaSA) on *in vitro* regeneration of a commercial cucumber cultivar was studied. NaSA is the sodium salt of salicylic acid (SA) (PubChem 2022a) that dissociates to produce SA in a solution (Palmer et al 2019). SA is a growth regulator and a signaling molecule of phenolic nature ubiquitously distributed in plants (Raskin et al 1990; Mohamed et al 2020). Its basal levels vary greatly among species, even within the same family (Raskin et al 1990; Rivas-San Vicente and Plasencia 2011). Exogenous application of SA and its derivatives (salicylates) may generate various metabolic and physiological responses in plants and affect their growth and development. The effect of some of these compounds on *in vitro* regeneration has been reported in various plant species. Shoot regeneration of cucumber (cv. Poinsett 76) from cotyledon explants increased with 30 µM SA, whereas other levels at 10-50 µM SA were detrimental (Vasudevan et al 2006). In cucumber (cv. Zhongnong 18), root formation using hypocotyl explants improved at specific SA levels (50 and 100 µM), not differing from the control (no SA) at 10 µM (Dong et al 2020). In melon (*Cucumis melo* L.), callus and shoot formation from cotyledon explants increased with SA and acetylsalicylic acid (ASA) at 50-200 µM, while higher levels (300-1000 µM) had an inhibitory effect (Shetty et al 1992). Shoot regeneration of *Hibiscus acetosella* and *H. moscheutos* from shoot meristem explants improved with 500 µM SA. In comparison, 1000 µM SA did not differ from the control (no SA) (Sakhanokho and Kelley 2009). Somatic embryogenesis in callus culture of *Astragalus adsurgens* Pall. derived from hypocotyl segments increased with 75-200 µM SA, while 300 and 400 µM SA had no positive effect; SA levels did not affect callus growth, except for 400 µM SA, with a negative impact (Luo et al 2001). Somatic embryogenesis of ten chir pines (*Pinus roxburghii* Sarg.) genotypes using shoot apical dome explants from mature trees was enhanced by applying SA at 7.2 µM (Malabadi et al 2008). In suspension cultures of arabic coffee (*Coffea arabica* L.), SA at 10–6 µM had a positive effect on somatic embryogenesis (Quiroz-Figueroa et al 2001). Based on the literature review, applied SA/salicylates act in a dose-dependent manner. It can be stimulatory, causing no relevant changes, or inhibit *in vitro* regeneration. The results of the present investigation are consistent with these findings.

In the current study, the frequency and extension of callus were not influenced by NaSA application, except for 0.1 µM NaSA with lower callus extension. The same NaSA concentration showed a positive trend in both shoot regeneration frequency and shoot number index, with a 1.5-fold increase compared to the control. In data not shown, higher levels of NaSA were studied. At 100 µM NaSA, both shoot regeneration frequency and shoot number index were similar to those at 10.0 µM NaSA. Callus formation decreased at 160 and 800 µM NaSA, and no shoots were formed.

The mechanisms underlying the effects of SA/salicylates on *in vitro* regeneration are complex and remain to be elucidated. Multiple factors and signaling pathways are involved.

Phytohormones are crucial for regulating physiological processes throughout the plant life cycle (Kosakivska et al, 2020). They play a key role in *in vitro* regeneration. Crosstalk of SA with other phytohormones has been described under different growth, development, and stress conditions (for reviews, see Rivas-San Vicente and Plasencia 2011; Koo et al 2020; Hayat et al 2021).

In the study by Torun et al (2020), SA treatments changed endogenous levels of abscisic acid (ABA), cytokinins (CTKs), ethylene, IAA, and jasmonic acid, in leaves of barley (*Hordeum vulgare* L.) cultivars under control and saline conditions. Effects of SA depended on the treatment timing and the cultivar. The ability of SA to mitigate salt stress appears to result from increasing ROS scavenging and antioxidant enzyme activity, which is closely related to changes in endogenous phytohormones. In wheat, Shakirova et al (2003) reported that exogenous SA changed the hormone content in ABA, IAA, and CTKs in seedlings

during germination with rapidly reversible changes, increased cell division and root cell extension, plant growth, and yield. Under salinity, SA treatment lessened changes in phytohormones levels and alleviated salinity-damaging effects on seedling growth.

Auxins and cytokinins are two of the most critical factors for tissue culture (Szechyńska-Hebda et al 2007). The balance between endogenous auxin and cytokinin signaling is crucial for *de novo* organ regeneration (Su and Zhang 2014, and references therein). In a recent study, SA treatments promoted adventitious root formation in cucumber explants through competitive inhibition of the auxin conjugation enzyme CsGH3.5 and increased the level of free IAA (Dong et al 2020). GH3 family genes have been extensively identified and characterized in several plant species (reviewed by Liao et al 2015), including cucumber (Wu et al 2014). They may be responsible for cellular auxin homeostasis via the conjugation of auxin (mostly IAA) to amino acids (Liao et al 2015, and references therein). The crosstalk between SA and auxin is also known to balance plant defense and growth (Zhong et al 2021). Besides, SA and IAA share a common precursor (chorismate), the end product of the shikimate pathway (Pérez-Llorca et al 2019). Little is known about the interaction between cytokinin and SA signaling pathways. Its role in *in vitro* regeneration remains to be explored. However, its involvement in other processes has been reported, but the mechanisms underlying its effects are complex. In *Arabidopsis thaliana*, it was suggested that cytokinin up-regulates plant immunity by elevating SA-dependent defense responses and in which SA, in turn, feedback inhibits cytokinin signaling (Argueso et al 2012). Studies in japonica rice (*Oryza sativa* subsp. japonica) showed that cytokinins acted synergistically with SA to trigger defense gene expression (Jiang et al 2013). In barley, SA treatments reduced the total endogenous cytokinin content in the leaves of two of the three cultivars and increased it in the third. Under saline conditions (150 and 300 mM NaCl), SA treatments generally increased total CK levels, particularly at 300 mM salt (Torun et al 2020). Therefore, if SA can interact with cytokinins, it is likely that it may also influence the *in vitro* regeneration process.

It is known that SA operates through many targets to mediate its many effects on plant processes (Klessig et al 2016). A variety of SA-binding proteins (SABPs) have been identified in plants (Klessig et al 2016; Pokotylo et al 2019). Their wide range of affinities for SA, together with the varying SA levels in subcellular compartments, tissues, developmental stages, and following an environmental stimulus, provides great flexibility and involves multiple mechanisms by which SA can exert its effects (Klessig et al 2016).

Plant tissue culture conditions can cause stress that affects the protocol performance. It may also lead to genetic and epigenetic alterations (Pacheco et al 2008). Exogenous application of SA and salicylates may generate protective effects against a variety of biotic and abiotic stresses. Citrus exposed to NaSA in laboratory and field experiments alleviated heat, cold, and disease stresses (Mann et al 2011). Applied NaSA attenuated the adverse effects of salt stress on wheat (*Triticum aestivum* L.) and stimulated growth by increasing the photosynthetic rate and retarding dark respiration (Al-Hakimi 2001). The toxicity of the heavy metals Pb and Cu in duckweed (*Lemna gibba* L.) and cadmium (Cd) in maize (*Zea mays* L.) were reduced by exogenous NaSA (Duman et al 2010; Gondor et al 2016).

Stresses generated by *in vitro* culture, such as those resulting from excess ethylene, reactive oxygen species (ROS), and (poly) phenols (Miguel 2021a), may affect regeneration efficiency (Seong et al 2005; reviewed by Szechyńska-Hebda et al 2007; Klimek-Szczykutowicz et al 2022), could be mitigated by SA/salicylates. Ethylene biosynthesis decreased in mung bean (Lee et al 1999) and in two barley cultivars under 300 mM NaCl stress (Torun et al 2020) when exposed to SA. In cucumber seedlings, a SA treatment stimulated the antioxidant activity of the enzymes ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), guaiacol peroxidase (GPX), glutathione reductase (GR), and superoxide dismutase (SOD), decreased electrolyte leakage, H2O2 concentration, and thiobarbituric acid-reactive substances (TBARS), enhancing heat tolerance (Shi et al 2006). In another study, SA inhibited CAT and APX activities while increasing SOD, peroxidase (POD), DHAR, and GR activities, decreased ROS levels and lipid peroxidation, and alleviating manganese toxicity in cucumber plants (Shi and Zhu 2008). Total polyphenol content in shaken cultures of watercress (*Nasturtium officinale* R. Br.) decreased with applied NaSA (Klimek-Szczykutowicz et al 2022).

In some reports, the efficacy of applied SA/salicylates increased under stress conditions. For example, in *in vitro* propagation of three Cavendish banana (*Musa acuminata* Colla) cultivars, NaSA treatments under non-saline conditions were generally less effective than under salinity (120 mM NaCl) on the vegetative and physiological traits evaluated (Elazab and Youssef 2017).

In addition, other reported plant processes and attributes, particularly with NaSA application, may also influence regeneration efficiencies, such as changes in photosynthetic pigment content (Elazab and Youssef 2017; Klimek-Szczykutowicz et al 2022) and net photosynthetic and respiration rates (Al-Hakimi 2001), beneficial ion uptake and transport (Al-Hakimi and Hamada 2001; Elazab and Youssef 2017), enhanced growth (Al-Hakimi 2001; Elazab and Youssef 2017) and biomass parameters (Bhambhani et al 2012; Klimek-Szczykutowicz et al 2022), improved plant water relations (Al-Hakimi 2001), changes in secondary metabolite content (Silja and Satheeshkumar 2015; Klimek-Szczykutowicz et al 2022), and so forth.

Differences between the effects of exogenously applied SA and NaSA on plants have been reported, such as on bud formation, growth parameters, heavy metal tolerance, plant immune response, total phenolic content, and antioxidant activity (Flores-Tena 1993; Zhang et al 2001; Gondor et al 2016; Karn et al 2022). It is still unclear why SA and NaSA differ in their effects.

The findings and considerations of the present study can be further explored and extended to other cultivars and species. Genetic, molecular, and physiological studies will help to understand the role of salicylic acid/salicylates in *in vitro* plant regeneration.

5. Conclusions

From the present study, it is concluded that *in vitro* shoot regeneration of a commercial cucumber cultivar was enhanced by adding sodium salicylate to the induction medium. These findings are an added value to address different biotechnological approaches in this economically important species, such as large-scale multiplication and culture-based genetic transformation.

Conflict of Interest

The author declares no conflicts of interest.

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