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CHANGES OF MORPHOGENETIC PATTERNS OF PLANTS CULTIVATED *IN VITRO* UNDER THE INFLUENCE OF SALICYLIC ACID EMPLOYED AS A TRIGGER OF ANTIOXIDANT DEFENCE MECANISMS IN CABBAGE PLANTS

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Abstract

Salicylic acid is a stress-signal molecule that is involved in the modulation of growth and development of plants. There is evidence that this stress-signal molecule has a positive impact on the antioxidant defence system, but few studies have been done on determining the most effective concentration and its impact on the growth and development of cabbage plants cultivated in vitro. In the present experiment, we assessed the impact of three different concentrations of salicylic acid (1 mM, 0.5 mM and 0.1 mM) on seed germination indexes, shoot initiation and proliferation, root development, as well as physiologic traits (phenolic and chlorophyll content). It has been found that a concentration of 1mM SA inhibited both seed germination and shoot development, which is probably related with suppression of GA-mediated pathway. Instead, the addition of 0.1 mM SA stimulated shoot proliferation rate, shortened the time for shoot initiation and increased shoot and root elongation. Our results provide the foundation for further studies related to the plant`s agronomic performances when cultivated in the field.

Key words*: shoot, roots, regeneration, defence-related process, germination.*

INTRODUCTION

Salicylic acid is widely distributed plant hormone that has positive impact on a large number of physiological processes such as seed germination, plant signalling and plant response to biotic and abiotic stress. One of the major abiotic stresses is drought, which affects crop growth and yield and thus it is a major constraint for plant productivity worldwide (Lefevere, Bauters, & Gheysen, 2020).

Under the pressure of climatic changes, the effects of drought stress are expected to increase more deepening the water crisis and the availability of water for plants. Drought stress adversely affects a variety of vital physiological and biochemical processes in plants (Hasanuzzaman et al., 2012), causing important loss in plant production.

Thus, nowadays the researches are focusing on deciphering whether exogenously-applied SA can reduce drought stress impact on plants. Up to now, various levels of SA have been shown to protect the buds of *Vitis* genotypes during cryopreservation (Pathirana et al., 2015) and to stimulate flowering in *Eleusine coracana* L. (Appu & Muthukrishnan, 2014) and fruit development in *Fragaria* x *ananassa* Duch. (Kazemi, 2013), *Malus domestica* Borkh. (Shaaban et al., 2011), and *Mangifera indica* L. (Ngullie et al., 2014).

The literature shows that SA, alone or in combination with other compounds (such as jasmonic acid), played the role of elicitor of secondary metabolites in various species such as: *Vitis vinifera* L. (Xu et al., 2015), *Bacopa monnieri* L. (Xu et al., 2015), *Silybum marianum* L. (Ahmed et al., 2020), *Carthamus tinctorius* L. (Golkar et al., 2019), and *Hypericum perforatum* L. (Gadzovska et al., 2013).According to literature, SA affects other physiological processes in plants such as growth, photosynthesis, uptake of ions, heat production, flowering and ethylene production (Ghorbani Javid et al., 2011).At the same time, SA was reported to induce an increase of *in vitro* regeneration frequency in Hibiscus plants (Sakhanokho & Kelley, 2009) and to induce water stress tolerance in Satureja hortensis (Yazdanpanah et al., 2011). However,

the efficiency of exogenous SA depends on the species, developmental stage of the plant, the type of application and the concentration of SA (Joseph et al., 2010; Pasternak et al., 2019). The review of literature showed that there are few methods of SA application employed: soaking the seeds prior to sowing, adding to the hydroponic solutions and tissue culture media, irrigating and spraying with SA solution and all revealed the protective role of SA against abiotic and biotic stress agents (Radwan et al., 2008; Sakhabutdinova et al., 2003). The ability of SA to mitigate against hostile effects of salinity in crop plants has been reviewed by Ghorbani Javid et al. (2011).

The plant tissue culture *in vitro* is one of the most effective experimental models in the investigation of various aspects related to the structure and functions of the plant cell and tissues. The formula of basic culture medium provides all nutrients, energy and water necessary for plantlets, organs, tissues or cells growth and the regulation of developmental generally requires the addition of plant growth regulators (PGRs). Successful culture strictly depends on a selection of appropriate PGRs and their concentration and combination with other PGRs. The most commonly used PGRs are auxins and cytokinins. Auxins mediate cell division while cytokinins mediate cell differentiation (Moubayidin et al. 2009). However, cultured plant tissues are also influenced by gibberellins (GAs), brassinosteroids (BRs), abscisic acid (ABA), salicylic acid (SA), jasmonates (JAs) and interactions among them (Gaspar et al., 1996; Phillips and Garda, 2019).

Thus, in our experiment we assessed the impact of three different concentration of salicylic acid (1 mM/l, 0.5 mM/l and 0.1 mM/l) on seed germination indexes, shoot initiation and proliferation, root development as well as physiologic traits (phenolic and chlorophyll content).

MATERIALS AND METHODS

Seeds of *Brassica oleracea,* CT genotype, were used in the experiments developed at Vegetable Research and Development Station Bacau, in The Laboratory of Tissue Culture *in vitro*, aiming to determine the impact of exogenous application of SA.

The biological material was surface sterilised by immersion in mercuric chloride solution ($HgCl_2$) 0.1% for 10 minutes, followed by repeated washing with sterile distilled water.

The sterile seeds were cultivated on Murashige et Skoogmedium (1962) supplemented with 30 g/l sucrose and solidified with 8.0 g/l of agar. The pH was adjusted to 5.8 prior to the addition of the agar.

Three different concentrations of salicylic acid (1 mM, 0.5 mM and 0.1 mM) were added to media and autoclaved at 121°C (1.06 kg/cm^2) for 25 min. Germination indexes were recorded and the one-week-old seedlings were used as source of explants, namely apexes and hypocotyls.

The excised explants were cultivated on the same previously mentioned variants: V0 - control without SA, V1 - 1 mM SA, V2 - 0.5 mM SA, V3 - 0.1 mM SA.

Cultures were then incubated at 26±1°C, a 16-h photoperiod, and 5000 lx light intensity. Repeated sub cultures were done at an interval of 30 days, and day to day observation was carried out to note the responses. The newly formed shoots exhibited the ability to form roots on the same nutritive medium used for shoot micropropagation, which allows the separation of rooted plantlets and the continuation of microclonation process.

The rooted plantlets were transferred to hydroponics conditions in Erlenmeyer flasks 30 mL for acclimatization, while the newly formed shoots were transplanted in new media for their further development. The pots with the hydroponic solution (that contained Previcur - propamocarb clorhidrat 530 g/l + + fosetil de aluminiu 310 g/l in a concentration of 0.15%) were covered with clear bags to provide 100% relative humidity. They were placed in an acclimatization room under a 16/8 h photoperiod at 20-23°C.

The acclimatized plants were planted in a potting mixture of sterilized sand + vermiculite (1:1 ratio) in plastic cups, hardened in a mist chamber (80% relative humidity) for 2 weeks before transfer to green house.

Biometric measurements including plant height, shoot length and root length were recorded 60 days after culture initiation.

Chlorophyll content was determined using Lichtenthaler (1987) protocol. Fresh plant samples (0.1 g) were homogenized and extracted with 80% acetone. The samples were centrifuged for 10 min at 3,530 rpm in the Hettich Universal Centrifuge 320 | 320 R. After collecting the supernatant (SN), the absorbance at 470, 647 and 663 nm was read using UV-VIS spectrophotometer and the concentrations of Cla, Clb and Car were quantified using the following formulas:

$$Cl_a(mg/L) = 12{,}25 * Abs\ 663\ nm - 2{,}79 * Abs\ 647\ nm \quad (1)$$

$$Cl_b(mg/L) = 21{,}50 * Abs\ 647\ nm - 5{,}10 * Abs\ 663\ nm \quad (2)$$

$$Car\ (mg/L) = \frac{1000 * Abs\ 470\ nm - 1{,}82 * Cl_a - 85{,}02 * Cl_b}{198} \quad (3)$$

All variants we tested in three replications and the results were analysed using ANOVA.

RESULTS AND DISCUSSIONS

Under the cultivation techniques tested in our experiment, a concentration of 1 mM SA delayed the seed germination with almost 3 days and the plants exhibited root deficiencies, from lack of roots till very small and abnormal development (Figure 1).

Figure 1. Plants with deficiencies in root development on variants with 1 mM SA

Instead, a concentration of 0.1 mM exhibited similar results with the control variant, the germination rate and shoot growth being positively influenced by the presence in the culture medium of SA (Figure 2). An increased germination percent at low SA concentrations and a decreased percent at higher levels, was reported also in other experiments on carrots, cucumbers, and wheat (Rajasekaran, 2002).
Regarding the morphogenetic reaction of explants, apexes were more suitable for cultivation, new shoots rapidly appeared at the base of the explant and shoot multiplication and elongation was also promoted by the addition of 0.1 mM SA.

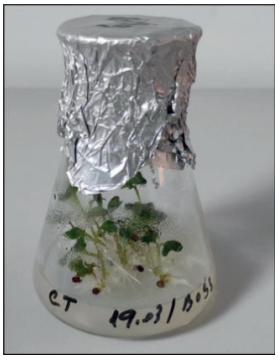


Figure2. Plants with normal root development on variants with 0.1 mM SA

Hypocotyls generated mainly roots and at one bottom of explants, shoots that evolved normally in fully grown plants (Figure 3).
The percentage of shoots producing explants was 93.5% in elicitor-free media and media with reduced concentrations of SA, while significantly lower value for explant response was observed for 1 mM SA (72%).

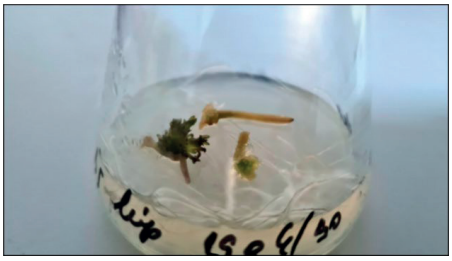


Figure 3. Morphogenetic reaction of hypocotyls on variants with 0.1 mM SA

On variants with high concentration, salicylic acid suppressed growth of explants and they gradually died (Figure 4). The main effect is exposed by roots that are highly affected by the concentration of SA in culture media. This effect was observed in many crops. For example, in *Arabidopsis*, low SA concentrations are not only effective in increasing root growth, but also decrease K^+ leakage from cells due to acute salt stress, whereas high SA concentrations not only inhibit root growth, but also have no impact on K^+ leakage (Pasternack, 2019).

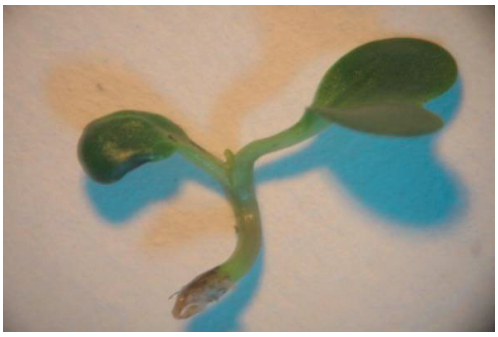


Figure 4. Failure of the plants to develop roots on media with high concentration of SA

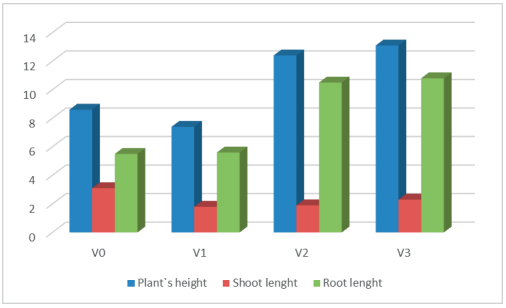


Figure5. Biometric measurements on *Brassica* plants cultivated on media supplemented with different concentration of SA

Our results support the results of Mateo et al., 2006, that concluded that when exogenous SA concentration is higher than 1 mM, plants usually tend to oxidative burst and cell death. Inhibitory effect of high levels of SA has been reported also on somatic embryogenesis in other crops, too. Roust et al. (1989) found that 7 mg l^{-1} SA was optimal for somatic embryo production in carrot suspension cultures, but higher levels (35 mg l^{-1}) were toxic. In 2001, Luo et al. reported that *A. adsurgens* Pall. Plants exhibits a decrease in somatic embryogenesis at levels of SA that are exceeding 24 mg l^{-1}. According to Behzad et al. (2014), a concentration of 2.0 and 5.0 mg l^{-1} of SA, completely inhibited microspore embryogenesis of *Brassica* plants. Although initial divisions were observed, these failed to proceed further into fully developed MDEs. They concluded that the exact toxic level of SA may be genotype-dependent.

SA in concentration of 0.1 and 0.5 mM shortened the time for shoot initiation, increased shoot and root elongation, the results being higher than the control variant (Table 1).

Table 1. Effects of different concentrations of SA in MS medium for multiple shoot induction at *B. oleracea* L. after 60 days of culture - means ±SE

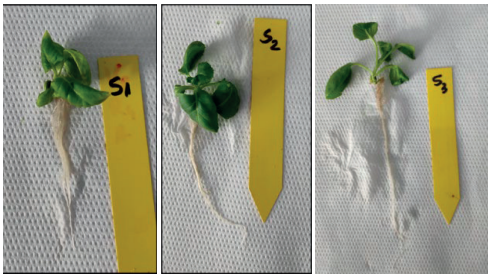
Variant	% of explant showing response	Average no. of shoots
V0 - Control	95.3	35.6±1.5
V1 - 1 mM SA	72.0	17.0±0.5
V2 – 0.5 mM SA	97.4	44.0±0.5
V3 - 0.1 mM SA	98.8	48.9±2.9

As illustrated in the graphic below (Figure 5), the results obtained proved that 0.1 mM SA promoted the best growth of shoots and roots.

The results indicated highly significant differences in shoot tips performance, demonstrating noticeable effects of SA over organogenesis of cabbage plants grown *in vitro*. A lower concentration of SA promoted both the adventitious shoot formation and their further development in fully grown plants (Figure 6).

Figure 6. Newly formed shoots on variants with 0.1 mM SA

The rooted plantlets (Figure 7) were transferred to the hydroponics conditions and it resulted in more than 95% survival of plantlets.



(a) (b) (c)

Figure7. Rooted plants during first week of hardening: (a) - V1 - 1 mM SA; (b) - V2 - 0.5 mM SA; (c) - V2 - 0.1 mM SA

The total chlorophyll content was enhanced by the addition of the SA elicitor in a concentration of 0.1 mM to the culture media as compared to control. The supplementation of media with SA in higher concentration resulted in similar or a slightly lower Chl a content than in controls (Table 2) d with SA. Our results support other plant studies that had indicated the role of SA in enhancing the photosynthetic pigment content and photosynthesis in different crops (Khan et al., 2003; Khodary, 2004).

Table 2. Effects of different concentrations of SA in MS medium on carotenoids and chlorophyll content of newly formed plants

Variant	Cla (mg/L)	Clb (mg/L)	CAR (mg/L)
V0 - Control	4.49	4.22	0.44
V1 - 1 mM SA	4.12	3.61	0.49
V2 - 0.5 mM SA	4.28	4.24	0.46
V3 - 0.1 mM SA	4.67	4.25	0.43

CONCLUSIONS

The results show that a concentration of 1mM SA inhibited both seed germination and shoot development, which is probably related with suppression of GA-mediated pathway. The markedly negative influence of this concentration of SA on the multiplication rate, shoot formation, and quality of plant tissue registered in our study strengthen the results obtained in other species, where a level of SA above 1 mM is considered high and likely to negatively regulate plant development and growth. The conclusions of our experiment underline the fact that SA highly influences root development starting at seed germination. Also, the cultivation of plants on medium culture with SA often lead to a dwarf phenotype of plants. The shoots are smaller but more robust and the quantity of chlorophyll is increased. This may be explained by a shift of plant response from growth to defence pathways induced by exogenous SA application. Instead, the addition of 0.1 mM SA stimulated shoot proliferation rate, shortened the time for shoot initiation and increased shoot and root elongation.

The content in phenolic and chlorophyll was higher in plants regenerated on this variant also, which indicates the beneficial effect of addition of 0.1mM SA on defence related processes.

Our results provide the foundation for further studies related to the plant`s agronomic performances when cultivated in the field.

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REFERENCES

Ahmed, H.S., Moawad, A.S., AbouZid, S.F., & Owis, A.I. (2020). Salicylic acid increases flavonolignans accumulation in the fruits of hydroponically cultured Silybum marianum. *Saudi Pharmaceutical Journal*, 28, 593–598.

Appu, M., Muthukrishnan, S. (2014). Foliar application of salicylic acid stimulates flowering and induce defence related proteins in finger millet plants. *Univers. J. Plant Sci.*, 2, 14–18.

Behzad A., Mehran E. Shariatpanahi, Jaime A. (2014). Teixeira da Silva Efficient induction of microspore embryogenesis using abscisic acid, jasmonic acid and salicylic acid in *Brassica napus* L *Plant Cell Tiss Organ Cult* 116:343–351, DOI 10.1007/s11240-013-0408-x

Gadzovska, S.; Maury, S.; Delaunay, A.; Spasenoski, M.; Hagège, D.; Courtois, D.; Joseph, C. (2013). The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphtodianthrones and phenylpropanoids in *Hypericum perforatum* L. *Plant Cell Tissue Organ Cult*, 113, 25–39

Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA (1996). Plant hormones and plant growth regulators in plant tissue culture. *In vitro Cell Dev Biol Plant* 32:272–289.

Golkar, P.; Taghizadeh, M.; Yousefian, Z. (2019). The effects of chitosan and salicylic acid on elicitation of secondary metabolites and antioxidant activity of safflower under in vitro salinity stress. *Plant Cell Tissue Organ Cult.*, 137, 575–585.

Javid, M. G., Sorooshzadeh, A., Moradi, F., Modarres Sanavy, S. A. M., & Allahdadi, I. (2011). The role of phytohormones in alleviating salt stress in crop plants. *Australian Journal of Crop Science*, 5(6), 726-734.

Hasanuzzaman, M., Nahar, K., Alam, M. M., & Fujita, M. (2012). Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by modulating the

antioxidant defense and glyoxalase system. *Australian Journal of Crop Science*, 6(8), 1314-1323.

Kazemi, M. (2013). Foliar application of salicylic acid and calcium on yield, yield component and chemical properties of strawberry. *Bull. Env. Pharmacol. Life Sci*, 2(11), 19-23.

Khan, W., Prithiviraj, B., & Smith, D. L. (2003). Photosynthetic responses of corn and soybean to foliar application of salicylates. *Journal of plant physiology*, 160(5), 485-492.

Khodary, S. E. A. (2004). Effect of salicylic acid on the growth, photosynthesis and carbohydrate metabolism in salt stressed maize plants. *Int. J. Agric. Biol*, 6(1), 5-8.

Joseph, B., Jini, D., & Sujatha, S. (2010). Insight into the Role of Exogenous Salicylic Acid on. *Asian Journal of Crop Science*, 2(4), 226-235.

Lefevere, H., Bauters, L., & Gheysen, G. (2020). Salicylic acid biosynthesis in plants. *Frontiers in plant science*, 11, 338.

Luo, J. P., Jiang, S. T., & Pan, L. J. (2001). Enhanced somatic embryogenesis by salicylic acid of Astragalus adsurgens Pall.: relationship with H2O2 production and H2O2-metabolizing enzyme activities. *Plant Science*, 161(1), 125-132.

Mateo, A., Funck, D., Mühlenbock, P., Kular, B., Mullineaux, P. M., & Karpinski, S. (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *Journal of Experimental Botany*, 57(8), 1795-1807.

Moubayidin, L., Di Mambro, R., & Sabatini, S. (2009). Cytokinin–auxin crosstalk. *Trends in plant science*, 14(10), 557-562.

Murashige, T. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol plant*, 15, 473-497.

Ngullie, C. R., Tank, R. V., & Bhanderi, D. R. (2014). Effect of salicylic acid and humic acid on flowering, fruiting, yield and quality of mango (Mangifera indica L.) cv. KESAR. *Advance Research Journal of Crop Improvement*, 5(2), 136-139.

Pasternak, T., Groot, E. P., Kazantsev, F. V., Teale, W., Omelyanchuk, N., Kovrizhnykh, V., ... & Mironova, V. V. (2019). Salicylic acid affects root meristem patterning via auxin distribution in a concentration-dependent manner. *Plant Physiology*, 180(3), 1725-1739.

Phillips, G. C., & Garda, M. (2019). Plant tissue culture media and practices: an overview. *In Vitro Cellular & Developmental Biology-Plant*, 55, 242-257.

Radwan, D. E. M., Lu, G., Fayez, K. A., & Mahmoud, S. Y. (2008). Protective action of salicylic acid against bean yellow mosaic virus infection in Vicia faba leaves. *Journal of plant physiology*, 165(8), 845-857.

Rajasekaran, L. R., Claude, A. S., & Caldwell, D. (2002). Stand establishment in processing carrots–Effects of various temperature regimes on germination and the role of salicylates in promoting germination at low temperatures. *Canadian Journal of Plant Science*, 82(2), 443-450.

Roustan, J. P., Latche, A., & Fallot, J. (1989). Effect of salicylic and acetylsalicylic acid on ethylene production and somatic embryogenesis in carrot (*Daucus carota* L.) cell suspensions. *Comptes Rendus de l'Academie des Sciences Serie 3 Sciences de la Vie* (France).

Sakhabutdinova, A. R., Fatkhutdinova, D. R., Bezrukova, M. V., & Shakirova, F. M. (2003). Salicylic acid prevents the damaging action of stress factors on wheat plants. *Bulg J Plant Physiol*, 21, 314-319.

Shaaban, M. M., El-Aal, A. M. K. A., & Ahmed, F. F. (2011). Insight into the effect of salicylic acid on apple trees growing under sandy saline soil. *Research Journal of Agriculture and Biological Sciences*, 7(2), 150-156.

Xu, A., Zhan, J. C., & Huang, W. D. (2015). Effects of ultraviolet C, methyl jasmonate and salicylic acid, alone or in combination, on stilbene biosynthesis in cell suspension cultures of *Vitis vinifera* L. cv. Cabernet Sauvignon. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 122, 197-211.

Yazdanpanah, S., Baghizadeh, A., & Abbassi, F. (2011). The interaction between drought stress and salicylic and ascorbic acids on some biochemical characteristics of Satureja hortensis. *African Journal of Agricultural Research*, 6(4), 798-807.