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EFFECT OF EXOGENOUS SUPPLY OF SALICYLIC ACID ON IN VITRO GROWTH AND DEVELOPMENT OF ROSE MICROPROPAGULES

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ABSTRACT

Rose (*Rosa hybrida* L. cv. bush rose) micropropagules were grown on salicylic acid (SA) supplemented MS medium with the objectives to study its effect on growth and multiplication under *in vitro* conditions. Salicylic acid was added in the MS medium prior to autoclaving as well as after autoclaving, through filter sterilization. Change in the mode of addition of SA did not make any significant changes in growth as well as biochemical parameters. Low concentration of SA (5 mg l^{-1}) was proved to be good in terms of higher accumulation of biomass and growth while higher concentration exerted subdued effect. Similarly, various biomolecules *viz.* carbohydrates, proteins, phenols and chlorophylls also shown significant enhancement in the micropropagules grown on the medium supplemented with low concentration (5 mg l^{-1}) of SA. It was concluded that SA at low concentration can be used as potential growth regulator in rose micropropagation. Further, it was noted that mode of application of SA does not make any significant change in growth pattern in rose micropropagules.

INTRODUCTION

Rosa hybrida L. cv. bush rose is an important cultivar of rose grown for its high demands throughout the world. Micropropagation protocol for this cultivar has been reported first by Skirvin and Chu (1979) and since then several workers has studied different tissue culture and commercial aspect in this cultivar (Hasegawa, 1979; Bressan *et al*., 1982; Curir *et al*., 1986; Valles and Boxus, 1987; Horn *et al*., 1988; Horn, 1992). However, at odds, these results failed to achieve high multiplication rate and further much improvement in growth.

Role of SA in growth and development of plants (both *in vitro* and *in vivo*), ethylene biosynthesis, respiration and stomatal behavior has been reported (Raskin 1992). Recently SA has identified as an important signaling molecule during pathogen attack in plants (Lu *et al*., 2016). Role of SA in abiotic stress like chilling, heat, heavy metal toxicity, drought and osmotic stress has also been studied (both *in vitro* and *in vivo*) extensively in several plants (Czajkowski *et al*., 2015; Sánchez-Rojo *et al*., 2015; Multu *et al*., 2013; Agami and Mohamed, 2013; Hayat *et al*., 2009; Tirani *et al*., 2013). Potential application of SA as plant growth regulator (PGR) has also been reported previously (Raskin, 1992; Gasper *et al* 1996). Physiological and biochemical functions of plant growing under *in vitro* can be regulated by SA. In potato cultures, it was noted by Czajkowski *et al* (2015) that exogenous supply of SA induces resistance against *Dickeya solani.* Incorporation of SA also resulted in higher secondary metabolite production in *Cistus heterophyllus* (López-Orenes *et al*., 2013). Similarly, Singh *et al.* (2016) has reported better growth and performance in banana micropropagules grown under the influence of SA. High shoot multiplication and andrographolide accumulation in *Andrographis paniculata,* was also reported on SA supplemented medium by Zaheer and Giri (2015). Promotory role of SA under *in vitro* conditions in several plants, like *Chlorophytum borivilianum* (Babel *et al*. 2014), *Ziziphus spina* (Galal, 2012) and *Hibiscus acetocella* (Sakhanokho and Kelly, 2009) has also been investigated. Banana plantlets under the influence of SA, performed better under culture conditions, when challenged to water stress (Bidabadi *et al*., 2012). Beside this, SA can also play important role in induction of somatic embryogenesis which has been shown *Plumbago rosea* and carrot (Komaraiah *et al*., 2004; Hosseini *et al*., 2009). There are two views regarding role of SA in photosynthesis and photosynthetic pigment accumulation. One opinion points to that the SA depress photosynthesis (Moharekar *et al.,* 2003) while others talk about the opposite (Hayat *et al*., 2005; Ghai *et al*., 2002). Regulation of photosynthetic activity is contributed by SA mainly through affecting leaf and chloroplast structure (Uzunova and Papova, 2000), stomatal closure (Melotto *et al*., 2000; Mateo *et al*., 2004) and photosynthetic pigments

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(Fariduddin *et al*., 2003). SA has been shown to promote viability of gel matrix encapsulated shoot buds derived from *in vitro* culture of sunflower (Katouzi *et al*., 2011). The present investigation was aimed to study the promotory effect of salicylic acid on micropropagules of rose under *in vitro* condition to improve its proliferation and multiplication efficiency.

MATERIALS AND METHODS

***Establishment of cultures*:** Shoot cultures of *Rosa hybrida* L. cv. bush rose were established according to the protocol described by Carelli and Echeverrigaray (2002). Healthy plants were procured from local nursery and maintained in green house of college. Mature nodal segments, containing axillary buds, were selected, cut and rinsed with tap water to remove the dust particles followed by two washes with detergent and then sterilization by dipping in 70% ethanol for one min. Further sterilization of explant was done by treatment with 1% Sodium hypochlorite for 10 min which was followed by three to four time washing with sterile distilled water. Aseptic inoculation of explant was done on the standard Murashige and Skoog's (1962) medium containing 3.0 mg l^{-1} BAP, 0.01 mg l^{-1} NAA, 0.8% agar and 3.0% sucrose. After initial establishment of cultures, Regular sub- culturing was done every three weeks interval. Cultures were kept under standard growth room conditions maintained at 28±2°C temperature and a 16 h light/8h dark cycle providing 45µ mol $m^{-2}s^{-1}$ photon flux density.

Experimental design: We tried a range of salicylic acid concentration (5.0-100 mg l^{-1}) to study its regulatory effects. SA was added into standard shoot multiplication medium along with the PGR mentioned as above. Two different ways were adopted to incorporate the SA. In first approach, SA was incorporated in the nutrient medium prior to autoclaving (pre-autoclaving) while in the second case, filter sterilized SA was added in the medium following the autoclaving (post – autoclaving), when the temperature of medium was brought down to about 50°C. Each culture bottle contained ca.50 ml of semi-solid medium and they were caped tightly to avoid contamination. The pH of medium was always adjusted to 5.8 before autoclaving. Each culture bottle was aseptically inoculated with a cluster of single shoot (ca. 1.5 cm) and kept under standard growth room conditions already mention above. The SA treated shoots were further sub-cultured on the same SA containing fresh medium at a gape of every three weeks up to six cycle *i.e.* a period of 126 days. All the above treatments were repeated thrice and three replicates were set for each experiment. At the end of experiment the micropropagules were taken out of culture bottle and were subjected to measurement of various growth parameters and biochemical analysis.

Measurement of growth parameters

Total shoot number, average shoot length and the biomass production in terms of fresh and dry weight was measured. For measurement of biomass (fresh weight and dry weight), propagules obtained from each treatment were taken out and the fresh weight was measured using an electronic top pan balance. For dry weight calculation, after measuring the fresh weight those fresh shoots were kept in an oven at 62˚C for 48 hrs for drying.

Biochemical analyses

Chlorophyll contents: The chlorophyll contents was calculated as per the method described by Arnon (1949). For this, 500 mg of shoots (grown on SA containing medium) were weighed and grounded in pestle and mortar with 80 % acetone under dark conditions. Extracts were centrifuged at 10,000 rpm and the supernatant was used to measure absorbance on spectrophotometer (UV-Vis Shimadzu, Japan) at three different wavelengths (663, 652 and 645 nm). Concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using following formulae:

$$\text{Total Chlorophyll (mg g}^{-1}) = \frac{20.2\times A_{645} + 8.02\times A_{663}}{a\times 1000\times W} \times V$$

$$\text{Chlorophyll a (mg g}^{-1}) = \frac{12.7\times A_{663} - 2.69\times A_{645}}{a\times 1000\times W} \times V$$

$$\text{Chlorophyll b (mg g}^{-1}) = \frac{22.9\times A_{645} - 4.68\times A_{663}}{a\times 1000\times W} \times V$$

V = Volume of the extract in ml
W = Fresh weight of the sample (leaf) in g
a = Length of light path in cell (1 cm)

***Total phenols*:** The phenol contents was measured as per the method described by Mahadevan (1975) using Folin Ciocalteu's reagent. For this purpose, 500 mg of shoots (grown on SA containing medium) were weighed and crushed in pestle and mortar in 70 % methanol. The extract was centrifuged at 10,000 rpm for 15 minutes. The clear supernatant was used for quantitative determination of total phenol contents. For each reaction 500 µl methanolic extract was taken in a test tube and to this was added 1.0 ml suitably diluted (1:1 ratio of reagent and DDW) Folin Ciocaltaeu's reagent followed by 2.0 ml of Na_2CO_3 (20% w/v) solution. The test tubes were heated in boiling water bath with interval shaking for about 1.0 min. Tubes were subsequently cooled under running tap water. The blue colored product was diluted to 25 ml by adding DDW and the per-cent transmittance was measured at 650 nm in a spectrophotometer (UV-Vis Shimadzu, Japan). The total phenol concentration in each sample was calculated with the help of already prepared standard curve using different concentrations (10-100 µg) of caffeic acid.

***Total carbohydrates*:** Quantitative estimation of total carbohydrate content was carried out as per the method described by Tandon (1976). SA treated *in vitro* derived propagules were homogenized in 0.1 M phosphate buffer (pH 7.0) and the homogenates were centrifuged at 10,000 rpm for 15 min. For each reaction 15 µl of supernatant was mixed with 4.0 ml of 0.2% Anthrone reagent (in conc. H_2SO_4) and placed in water bath for five minutes. The absorbance was recorded at 610 nm wave length. The total carbohydrate contents were determined using standard curve prepared from various concentrations of glucose.

***Total Protein*:** Quantitative estimation of total protein was performed as per Bradford's method (1976). One ml of the suitably diluted crude tissue extract (the supernatant) was mixed with 5.0 ml of Coomassie Brilliant Blue G–250 dye

(Bradford reagent) and transmittance of the resultant solution (coloured complex) was read with Spectrophotometer (UV-Vis Shimadzu, Japan) at 595 nm. The amount of protein was determined using standard curve prepared from various concentrations of albumin protein.

For all the above analysis, three replicates were used and each reaction was repeated thrice. Suitable blanks were maintained wherever required. Statistical analyses was done to check the validity of data.

RESULTS

In the present study, addition of different concentrations of salicylic acid (SA) in standard rose multiplication medium during culture evoked varied responses. It was also noted that addition of SA prior to autoclaving the MS medium did not show any significant difference in growth parameter *viz.* shoot length, shoot number, fresh weight and dry weight compared to filter sterilized addition of SA post autoclaving. Although little change was observed in few biochemical indices (chlorophyll and phenol) but it did not exerted in any abnormal phenotypic changes. No noteworthy change was observed in total carbohydrate content when mode of application of SA was changed. Rose cultures in multiplication stage, grown on standard MS medium supplemented with prescribed PGRs, were taken as control to compare the results.

At low concentration of SA (5.0 mg l^{-1}), less shoot number and reduced shoot length was recorded. Proportionate increase in shoot number and length was observed with higher concentration of SA (*i.e.* 10 and 25 mg l^{-1}) though still it was recorded less than control plantlets. Further increase in SA concentration showed decline in shoot number and length (Table 1). Similar observation were recorded for total biomass (Fresh weight and dry weight). At low concentration of SA, no significant biomass accumulation was observed. Steady increase in biomass was observed at 10 and 25 mg l^{-1} SA but still it failed to equate with control plantlets. Further increase in SA concentration showed sharp decline in biomass (Table 1). Interestingly, we observed that when SA was added prior to autoclaving, total chlorophyll increased in a gradual way up to 25 mg l^{-1} concentration. Further increase in concentration of SA resulted in sharp decline of total chlorophyll but still this value remained higher than corresponding control propagules. Contrast to this, post autoclaving addition of sterilized SA did not make any significant change in total chlorophyll at 25 mg l^{-1} or less concentrations. Conversely, significant rise in total chlorophyll was recorded at 50 mg l^{-1} concentration of SA in the same treatment condition (Table 2). Steady increase in total carbohydrates, phenol and protein was recorded with preliminary concentrations (5.0 and 10.0 mg l^{-1}) of SA, when added in the medium prior to autoclaving. Incorporation of higher concentration of SA resulted in turn down of all these bio-molecules.

Table 1 Effect of salicylic acid on in *in vitro* growth of rose micropropagules

Mode of SA application	Salicylic acid concentration mg l^{-1}	No. of shoots Mean ± SD	Length of shoots (cm)±SD	Fresh weight (g)±SD	Dry weight (g)±SD
Pre-autoclaving	0	12.61±3.42^{a}	3.54±0.36^{a}	10.81±3.74^{c}	2.19±0.041^{a}
	5	08.63±1.49^{b}	2.92±0.32^{ab}	6.02±1.47^{b}	1.19±0.022^{b}
	10	14.58±4.11^{a}	3.54±0.45^{c}	7.78±1.29^{ab}	1.66±0.020^{a}
	25	18.24±3.84^{c}	3.66±0.39^{a}	10.12±2.55^{a}	2.03±0.031^{c}
	50	13.95±3.92^{ab}	3.47±0.22^{a}	5.75±1.26^{b}	1.13±0.006^{ab}
	75	07.82±1.14^{c}	1.78±0.20^{b}	3.64±0.73^{a}	0.62±0.009^{c}
	100	05.90±0.94^{a}	1.69±0.31^{c}	3.12±0.18^{c}	0.53±0.012^{a}
Post-autoclaving (Filter sterilized)	0	13.67±4.41^{a}	3.61±0.34^{a}	11.43±2.38^{a}	2.31±0.012^{a}
	5	07.88±2.20^{c}	2.80±0.21^{c}	07.25±1.73^{a}	1.47±0.020^{b}
	10	11.51±3.41^{c}	3.48±0.33^{ab}	08.77±2.09^{c}	1.58±0.015^{a}
	25	15.91±3.43^{b}	4.08±0.51^{c}	11.46±2.28^{b}	2.31±0.029^{ab}
	50	08.58±3.49^{ab}	3.62±0.37^{a}	07.92±1.17^{a}	1.28±0.006^{c}
	75	07.47±2.14^{a}	3.14±0.20^{ab}	03.88±0.41^{b}	0.62±0.004^{a}
	100	05.87±2.12^{c}	2.13±0.11^{c}	02.87±0.22^{ab}	0.42±0.003^{a}

Means followed by the same letters are not significantly different according to Duncan's multiple range test at p < 0.05, n = 9. SD is Standard Deviation

Table 2 Effect of salicylic acid on chlorophyll contents in rose micropropagules grown under *in vitro* conditions

Mode of SA application	Salicylic acid concentration mg l^{-1}	Total chlorophyll content (mg g^{-1} FW± SD)	Chlorophyll a content (mg g^{-1} FW± SD)	Chlorophyll b content (mg g^{-1} FW± SD)
Pre-autoclaving	0	0.36±0.0114^{a}	0.18±0.0142^{ab}	0.16±0.0117^{a}
	5	0.28±0.0321^{c}	0.13±0.0098^{c}	0.14±0.0112^{c}
	10	0.43±0.0315^{ab}	0.21±0.0078^{a}	0.22±0.0114^{ab}
	25	0.39±0.0124^{c}	0.22±0.0095^{a}	0.21±0.0117^{c}
	50	0.24±0.0168^{b}	0.11±0.0043^{b}	0.12±0.0098^{b}
	75	0.26±0.0097^{a}	0.14±0.0022^{c}	0.13±0.0085^{a}
	100	0.22±0.0110^{ab}	0.11±0.0018^{a}	0.12±0.0078^{c}
Post-autoclaving (Filter sterilized)	0	0.38±0.0041^{a}	0.19±0.0110^{a}	0.18±0.0108^{ab}
	5	0.18±0.0042^{c}	0.09±0.0178^{ab}	0.10±0.0085^{c}
	10	0.15±0.0039^{ab}	0.08±0.0047^{a}	0.09±0.0047^{b}
	25	0.13±0.0026^{c}	0.07±0.0019^{c}	0.07±0.0042^{c}
	50	0.14±0.0019^{a}	0.08±0.0014^{a}	0.08±0.0018^{a}
	75	0.08±0.0021^{b}	0.04±0.0011^{b}	0.03±0.0014^{b}
	100	0.08±0.0032^{b}	0.04±0.0031^{ab}	0.05±0.0033^{a}

Means followed by the same letters are not significantly different according to Duncan's multiple range test at p < 0.05, n = 9. SD is Standard Deviation

Though these values still stayed higher than their respective controls (Fig. 1, Fig.2 and Fig. 3). Contrary to this, addition of SA after autoclaving did not confirm the responses similar to that when SA was incorporated before autoclaving.

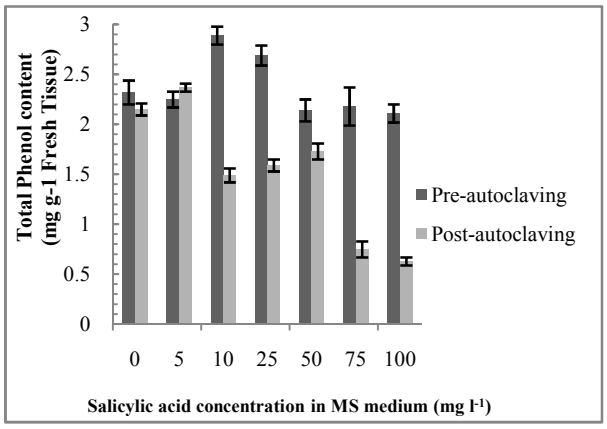

Figure 1 Effect of salicylic acid on total phenol contents in rose micropropagules grown under *in vitro* conditions

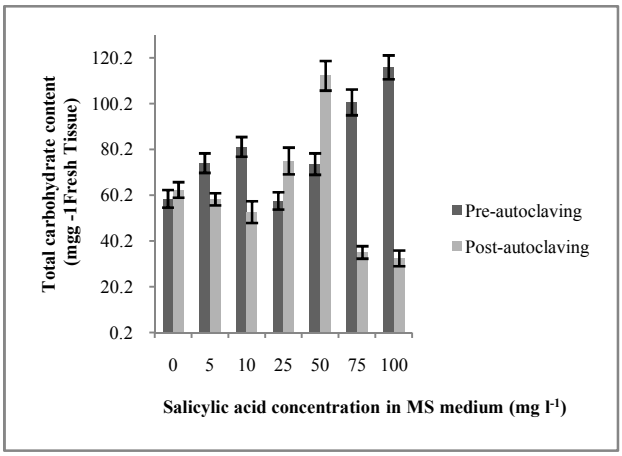

Figure 2 Effect of salicylic acid on total carbohydrate contents in rose micropropagules grown under *in vitro* conditions

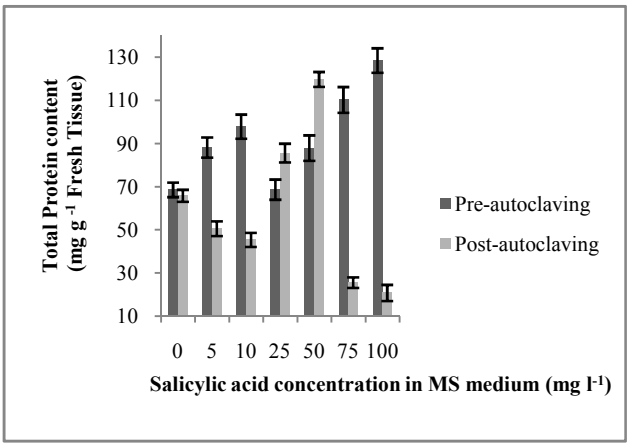

Figure 3 Effect of salicylic acid on total phenol contents in rose micropropagules grown under *in vitro* conditions

Though at low concentration of SA, slender increase in chlorophyll content was recorded which again was decreased at higher concentration and remained lesser than corresponding control. It was noted that with increasing concentrations of SA (post -autoclaving), carbohydrate content also increased. In précised observations, mode of addition of SA did not affected growth parameters (Shoot number, shoot length, fresh weight and dry weight) but biochemical parameters showed varied response. It was also observed that primary low concentrations of SA (*i.e.* 5.0 and 10.0 mg l^{-1}), irrespective of its method of treatment, apathetically affected both growth as well as biomolecules accretion. At the same instance, add to SA concentration to a moderate level improved the growth conditions but this again showed negative trend beyond optimum concentration.

DISCUSSION

Role of salicylic acid growth and development in plants, plant-microbes interaction and in combating stresses has been established (Hayat *et al.,* 2010; Yusuf *et al.*, 2012; Rivas-San Vicenta and Plasencia, 2011). Application of SA in better growth of plants under *in vitro* conditions has also been reported by few workers (Singh *et al.,* 2016; Babel *et al.*, 2014; Komaraiah *et al.,* 2004; Galal, 2012; Ram *et al.,* 2013). We, in the present study has attempted to envisage the regulatory role of exogenously supplied SA on *in vitro* growth and development of rose micropropagules. López-Orenes *et al.* (2013) used filter sterilized SA in medium to study regulating role of SA in *Cistus heterophyllus* cultures. Similar way was adopted by Ram *et al.* (2013) in *Rosa hybrida* culture. Post autoclaving addition of SA in the medium to study its role in stem elongation and water stress has also been reported (Handro *et al.* 1997; Bidabadi *et al.,* 2012). Opposing to this a lot of workers used SA prior to autoclaving of medium for different studies under *in vitro* conditions (Komaraiah *et al.,* 2004; Sakhanokho and Kelley, 2009; Galal, 2012). A few report of comparison of mode of application of SA under *in vitro* conditions are also available (Singh *et al*., 2016; Babel *et al.*, 2014). We here report that the mode of application of SA in the growth medium did not have much influence on shoot growth and biomass production in rose micropropagules during culture conditions. Although biochemical parameters like chlorophyll pigments, phenols, protein and carbohydrates showed significant changes and this may be due to change of pH of growth medium which was changed due to addition of SA after sterilization of medium. These observations support the hypothesis that like other synthetic growth regulators, SA can be added in the medium prior the autoclaving to induce the growth without any harmful effect. In case of banana, positive effect of SA in growth of micropropagules under *in vitro* condition (Singh *et al*., 2016) and in response to water stress (Bidabadi *et al.,* 2012) has been reported. Similar results were recorded in micropropagules of *Chlorophytum borivilianum* by Babel *et al.* (2014). In our study, low concentration of SA resulted in increased growth of rose propagules in terms of shoots number, shoot length and biomass. However, higher concentrations of SA negatively affected these parameters. Our results are in accordance with report of Singh *et al.* (2016) and Babel *et al.* (2014) where the same observation were recorded. High level of SA also induce ethylene biosynthesis in cell, which interfere and retard the growth (Hosseini *et al.*, 2009). In the current study, this may the reason behind depression of growth with increasing concentration of SA in the growth medium. The comparable results were reported by Galal (2012) in *in vitro* cultures of the tree *Ziziphus spina christi* where high concentration of SA hampered the growth. Likewise in wheat also the low concentration of SA resulted in promoted the

growth while higher concentration adversely affected the growth (Hayat *et al.*, 2005). In case of banana, when micropropagules were cultured on medium supplemented with low level of SA, photosynthetic pigments, total phenol, total carbohydrates and biomass increased significantly while, the higher concentration resulted in depression of growth (Singh *et al.* 2014). In present case also the total chlorophyll, carbohydrates, protein, phenol and biomass increased with lower level of SA, at the same time as the higher concentration resulted in miserable growth and less accumulation of these biomolecules. In maize, higher carbohydrates and chlorophyll accumulation in low SA treated plant was observed (Khodary, 2004). It can therefore, be concluded that lower concentrations of SA under *in vitro* condition in rose resulted into improvement of growth and enhance important physiologically biochemical activities while higher concentration hold back the growth.

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