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Improved *In Vitro* Development of *Talinum portulacifolium via* Exogenous Salicylic Acid Treatment

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ABSTRACT

Salicylic acid (SA)-mediated variations in *in vitro* growth and biochemical attributes were investigated in micropropagules of *Talinum portulacifolium*. Shoots of *T. portulacifolium* were cultured on a standardized multiplication medium supplemented with varying concentrations of SA. The treatments involved two modes of SA incorporation: (a) addition of SA to the medium before autoclaving, and (b) supplementation with filter-sterilized SA after autoclaving. The mode of SA application did not cause any significant difference in growth performance. Lower concentrations of SA exhibited a stimulatory effect on shoot elongation and biomass accumulation, whereas higher concentrations inhibited growth. Moreover, total chlorophyll, protein, carbohydrate, and phenolic contents were significantly enhanced in cultures grown on medium supplemented with a low level of SA. The study concludes that SA, when applied at an optimal low concentration, effectively promotes in vitro growth and enhances biochemical activity in *Talinum portulacifolium* micropropagules, irrespective of its mode of incorporation.

KEY WORDS: Salicylic acid, *Talinum portulacifolium* tissue culture, carbohydrates, proteins, growth parameters.

INTRODUCTION

Salicylic acid (SA) is a naturally occurring plant phenolic compound characterized by an aromatic ring bearing a hydroxyl group and its derivatives. It plays a multifaceted role in regulating various physiological and developmental processes, including growth, ethylene biosynthesis, flowering, respiration, and stomatal movement (Raskin, 1992). In recent years, SA has drawn considerable research attention due to its function as an endogenous signaling molecule that mediates plant defense responses against pathogenic attacks (Lu et al., 2016). Additionally, SA is recognized for its involvement in plant adaptation to diverse abiotic stresses such as drought, temperature extremes, heavy metal toxicity, and osmotic imbalance. The role of SA in conferring both biotic and abiotic stress tolerance under in vitro and in vivo conditions has been well documented across various plant species (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).

Earlier studies have identified SA as a potential plant growth regulator, and its application in plant tissue culture has been substantiated (Raskin, 1992; Gasper et al., 1996). SA influences in vitro plant growth by modulating physiological and biochemical activities. For instance, incorporating SA into culture media has been shown to induce resistance against *Dickeya solani* in potato cultures (Czajkowski et al., 2015). Supplementation of SA in the nutrient medium has also enhanced the accumulation of secondary metabolites in *Cistus heterophyllus* (López-Orenes et al., 2013) and promoted multiple shoot formation and increased andrographolide content in *Andrographis paniculata* (Zaheer and Giri, 2015). Similarly, the exogenous addition of SA improved callus formation, shoot multiplication, and root proliferation in *Ziziphus spina* (Galal, 2012). In bananas, SA application has been reported to improve in vitro plantlet performance under water stress conditions (Bidabadi et al., 2012). A promotory effect of SA has also been observed on the in vitro regeneration of *Hibiscus acetocella* and *H. moschentos* (Sakhanokho and Kelly, 2009). Furthermore, SA has been found to induce somatic embryogenesis in *Plumbago rosea* (Komaraiah et al., 2004) and carrot (*Daucus carota*) (Hosseini et al., 2009). Although SA has been associated with enhanced accumulation of photosynthetic pigments in *Triticum aestivum* and *Brassica napus* (Hayat et al., 2005; Ghai et al., 2002), contrasting findings indicate that its higher concentrations may suppress photosynthesis and trigger oxidative stress in wheat and mung plants (Moharekar et al., 2003).

Talinum portulacifolium, commonly known as *Ceylon spinach* or *fameflower*, is a medicinally important succulent herb belonging to the family Talinaceae. It is widely distributed in tropical and subtropical regions of India and is valued for its traditional therapeutic properties, including anti-inflammatory, antioxidant, and hepatoprotective activities. The plant has

gained attention in recent years due to its potential use in herbal formulations and nutraceutical industries. Despite its importance, large-scale propagation of *T. portulacifolium* through conventional means is limited owing to poor seed viability and low germination rate. Hence, tissue culture technology offers an effective alternative for its rapid multiplication and conservation. In vitro propagation ensures the production of uniform, disease-free, and true-to-type plantlets throughout the year. Several studies have reported successful establishment of micropropagation protocols for *Talinum* species to support large-scale cultivation and sustainable utilization of this valuable medicinal herb. The present study was conducted to evaluate the influence of plant growth regulators on the in vitro regeneration efficiency of *Talinum portulacifolium*.

MATERIALS AND METHODS

Shoot cultures of *Talinum portulacifolium* were initiated following the method outlined by Banerjee and Langhe (1985). After successful culture establishment, shoot multiplication was carried out on Murashige and Skoog (1962) medium supplemented with 3.0 mg l^{-1} BAP, 0.01 mg l^{-1} IAA, 0.8% agar, and 3.0% sucrose. The cultures were routinely subcultured at three-week intervals. All cultures were maintained under controlled growth room conditions at 28 ± 2 °C with a 16 h light/8 h dark photoperiod and a light intensity of approximately 45 µmol m^{-2} s^{-1}. To evaluate the regulatory influence of salicylic acid (SA), a concentration range of 5.0–100 mg l^{-1} was tested by supplementing SA into the standard shoot multiplication medium containing the above-mentioned growth regulators.

Two approaches were used for SA incorporation: (a) addition of SA before autoclaving (pre-autoclaving), and (b) addition of filter-sterilized SA to the medium after autoclaving, once the temperature was lowered to approximately 50 °C (post-autoclaving). Each culture vessel contained about 50 ml of semi-solid medium, and bottles were sealed tightly to prevent contamination. The pH of all media was adjusted to 5.8 prior to autoclaving. A single shoot cluster (approximately 1.5 cm in length) was aseptically inoculated into each bottle and maintained under the same growth conditions described above. SA-treated shoots were subcultured onto freshly prepared SA-supplemented media at three-week intervals for six successive cycles, totaling 126 days of culture. All treatments were conducted in triplicate, with three replicates per experiment. At the end of the experimental period, the micropropagules were removed from the culture vessels for assessment of growth parameters and subsequent biochemical analyses.

Measurement of growth parameters

Total shoot number, mean shoot length, and biomass production (expressed as fresh and dry weight) were evaluated for each treatment. To determine biomass, micropropagules from all treatments were carefully removed from the culture vessels, and their fresh weight was recorded immediately using an electronic top-pan balance. For estimation of dry weight, the same shoot samples were placed in a hot air oven maintained at 62 °C for 48 hours to achieve complete drying. The dried samples were then weighed to obtain the final dry biomass values.

Biochemical analyses

Chlorophyll contents: The estimation of chlorophyll pigments was carried out following the procedure of Arnon (1949). For this purpose, 500 mg of shoot tissue obtained from SA-treated cultures was homogenized using a mortar and pestle in 80% acetone under dark conditions to prevent pigment degradation. The homogenate was then centrifuged at 10,000 rpm, and the resulting supernatant was collected for spectrophotometric analysis. Absorbance readings were recorded at 663, 652, and 645 nm using a UV–Vis spectrophotometer (Shimadzu, Japan). The concentrations of chlorophyll *a*, chlorophyll *b*, and total chlorophyll were subsequently calculated using the 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 total phenolic content was estimated following the method of Mahadevan (1975) using Folin–Ciocalteu's reagent. For this analysis, 500 mg of shoot tissue obtained from SA-treated cultures was homogenized in 70% methanol using a mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 minutes, and the clear supernatant was collected for quantitative determination. For each assay, 500 µl of the methanolic extract was mixed with 1.0 ml of appropriately diluted Folin–Ciocalteu's reagent (1:1 ratio of reagent and double-distilled water), followed by the addition of 2.0 ml of 20% (w/v) sodium carbonate solution. The reaction mixture was heated in a boiling water bath for about one minute with intermittent shaking and then cooled under running tap water. The resulting blue-colored complex was made up to 25 ml with double-distilled water, and absorbance was measured at 650 nm using a UV–Vis spectrophotometer (Shimadzu, Japan). The total phenolic content of each sample was determined using a standard calibration curve prepared with varying concentrations (10–100 µg) of caffeic acid.

***Total carbohydrates*:** Quantitative estimation of total carbohydrates was performed following the procedure described by Tandon (1976). In vitro–derived propagules treated with SA were homogenized in 0.1 M phosphate buffer (pH 7.0), and the resulting homogenate was centrifuged at 10,000 rpm for 15 minutes. For each assay, 15 µl of the supernatant was combined with 4.0 ml of 0.2% anthrone reagent prepared in concentrated sulfuric acid (H_2SO_4). The reaction mixture was then heated in a boiling water bath for five minutes to allow color development. After cooling, absorbance was measured at 610 nm using a UV–Vis spectrophotometer. The total carbohydrate concentration in each sample was calculated from a standard calibration curve constructed with known concentrations of glucose.

***Total Protein*:** Quantitative estimation of total protein was carried out following the method of Bradford (1976). For this purpose, 1.0 ml of appropriately diluted crude tissue extract (supernatant) was mixed with 5.0 ml of Coomassie Brilliant Blue G-250 dye solution (Bradford reagent). The absorbance of the resulting blue-colored complex was measured at 595 nm using a UV–Vis spectrophotometer (Shimadzu, Japan). The protein concentration in each sample was calculated using a standard calibration curve prepared with known concentrations of bovine serum albumin (BSA).

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 investigation, the supplementation of salicylic acid (SA) into the standard *Talinum portulacifolium* multiplication medium resulted in notable alterations in the growth behavior of *T. portulacifolium* micropropagules under in vitro conditions. The mode of SA incorporation, whether added before or after autoclaving, did not significantly influence the physical growth parameters such as shoot number, shoot length, fresh weight, and dry weight, which remained nearly comparable. However, the biochemical attributes, particularly total chlorophyll and phenolic contents, exhibited considerable variations depending on the mode of SA application, whereas total carbohydrate content remained largely unaffected between the two treatments. Control cultures grown on standard MS medium supplemented with prescribed plant growth regulators (PGRs) but without SA served as a reference.

At a lower concentration of SA (5.0 mg l^{-1}), both shoot number and shoot length were reduced compared to the control. A gradual increase in SA concentration up to 10 and 25 mg l^{-1} led to a moderate improvement in these parameters, although the values did not surpass those of the control. Further elevation of SA concentration beyond 25 mg l^{-1} caused a marked decline in growth, indicating inhibitory effects (Table 1). A similar pattern was recorded for biomass accumulation, where fresh and dry weights increased slightly at intermediate SA concentrations (10 and 25 mg l^{-1}) but decreased sharply at higher levels, suggesting toxicity at elevated concentrations (Table 2).

When SA was incorporated before autoclaving, total chlorophyll content showed a gradual increase with rising SA concentrations up to 25 mg l^{-1}, after which a decline was observed, though levels remained comparable to the control. Conversely, when filter-sterilized SA was added after autoclaving, chlorophyll content remained nearly unchanged up to 25 mg l^{-1} but exhibited a substantial increase at 50 mg l^{-1} (Table 3). The total phenolic, carbohydrate, and protein contents followed a similar pattern, with an initial rise at lower SA levels (5.0 and 10.0 mg l^{-1}) followed by a decline at higher concentrations, though still exceeding control values (Table 3).

Post-autoclaving incorporation of SA showed a less pronounced effect on biochemical parameters compared to pre-autoclaving addition. While a slight increase in total chlorophyll was observed at 5.0 mg l^{-1}, higher concentrations led to a rapid decline below control levels. In contrast, total carbohydrate content exhibited a steady increase with higher SA concentrations when added after autoclaving. Overall, it was inferred that the mode of SA incorporation had minimal influence on physical growth parameters, though it substantially affected biochemical traits. Lower concentrations of SA (5.0–10.0 mg l^{-1}) had inhibitory effects on both growth and biochemical responses, whereas moderate levels improved growth performance before again exerting adverse effects beyond the optimum concentration.

Table 1. Effect of salicylic acid on in *in vitro* growth of *Talinum portulacifolium* 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	19.48 ± 2.35	6.74 ± 0.45	16.94 ± 2.35	3.83 ± 0.050
	5	12.43 ± 1.22	4.69 ± 0.23	6.43 ± 1.29	1.45 ± 0.020
	10	23.34 ± 3.15	4.93 ± 0.11	9.18 ± 1.21	2.10 ± 0.011
	25	28.14 ± 3.69	6.67 ± 0.21	11.93 ± 1.65	2.60 ± 0.012
	50	14.96 ± 2.98	3.13 ± 0.15	5.44 ± 0.96	1.10 ± 0.006
	75	11.86 ± 1.05	2.00 ± 0.11	4.43 ± 0.23	0.50 ± 0.021
	100	9.16 ± 1.04	1.94 ± 0.14	3.58 ± 0.12	0.77 ± 0.009
		19.87 ± 5.32	5.84 ± 0.21	16.64 ± 1.29	3.65 ± 0.032
Post-autoclaving (Filter sterilized)	0	9.95 ± 2.12	3.55 ± 0.19	7.18 ± 1.91	1.44 ± 0.012
	5	20.78 ± 4.65	5.03 ± 0.32	7.99 ± 3.45	1.78 ± 0.010
	10	26.94 ± 4.21	5.63 ± 0.42	13.74 ± 5.20	3.04 ± 0.021
	25	12.49 ± 3.64	3.71 ± 0.21	4.68 ± 2.87	0.97 ± 0.006
	50	11.23 ± 1.23	3.49 ± 0.13	3.55 ± 3.65	0.71 ± 0.001
	75	7.99 ± 1.09	2.32 ± 0.62	3.44 ± 0.21	0.62 ± 0.002
	100	19.48 ± 2.35	6.74 ± 0.45	16.94 ± 2.35	3.83 ± 0.050
SE		4.63	0.97	1.45	4.63
CD5%		14.54	2.78	4.62	14.54
CD1%		17.09	4.39	6.60	17.09
CV		36.25	38.62	50.71	36.25

SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation

Table 2. Effect of salicylic acid on chlorophyll contents in Talinum portulacifolium 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	0.30 ± 0.0050	0.13 ± 0.0025	0.14 ± 0.0026
	5	0.38 ± 0.0242	0.20 ± 0.0150	0.19 ± 0.0120
	10	0.44 ± 0.0254	0.23 ± 0.0024	0.19 ± 0.0110
	25	0.46 ± 0.0283	0.26 ± 0.0114	0.20 ± 0.0133
	50	0.28 ± 0.0234	0.16 ± 0.0082	0.12 ± 0.0054
	75	0.29 ± 0.0082	0.14 ± 0.0043	0.10 ± 0.0038
	100	0.29 ± 0.0050	0.12 ± 0.0011	0.14 ± 0.0026
		0.25 ± 0.0049	0.12 ± 0.0143	0.13 ± 0.0043
Post-autoclaving (Filter sterilized)	0	0.23 ± 0.0050	0.13 ± 0.0124	0.11 ± 0.0026
	5	0.20 ± 0.0047	0.08 ± 0.0010	0.12 ± 0.0016
	10	0.18 ± 0.0031	0.07 ± 0.0008	0.11 ± 0.0031
	25	0.19 ± 0.0023	0.08 ± 0.0007	0.11 ± 0.0011
	50	0.30 ± 0.0050	0.13 ± 0.0025	0.14 ± 0.0026
	75	*	*	*
	100	*	*	*
SE		0.0079	0.0042	0.0049
CD5%		0.0229	0.0134	0.0157
CD1%		0.0310	0.0170	0.0208
CV		5.59	6.01	6.26

*** Inadequate sample tissue** ***SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation***

Table 3. Effect of salicylic acid on total phenol, carbohydrates and protein contents in *Talinum portulacifolium* micropropagules grown under *in vitro* conditions

Mode of SA application	Salicylic acid concentration mg l^{-1}	Total Phenol content (mg g^{-1} Fresh tissue)± SD	Total carbohydrate content (mgg $^{-1}$Fresh tissue)± SD	Total Protein content (mgg $^{-1}$Fresh tissue)± SD
Pre-autoclaving	0	1.22 ± 0.04	78.72 ± 3.38	72.72 ± 2.83
	5	1.58 ± 0.08	98.65 ± 3.91	93.85 ± 4.39
	10	2.14 ± 0.26	110.71 ± 5.05	107.86 ± 5.47
	25	1.93 ± 0.11	79.02 ± 3.29	66.83 ± 4.37
	50	1.34 ± 0.17	104.14 ± 4.34	95.94 ± 6.98
	75	1.33 ± 0.14	134.74 ± 5.53	123.90 ± 5.98
	100	1.30 ± 0.10	162.02 ± 4.94	144.54 ± 5.58
		1.26 ± 0.06	69.07 ± 2.78	60.78 ± 3.88
Post-autoclaving (Filter sterilized)	0	1.52 ± 0.07	64.14 ± 3.19	55.02 ± 2.54
	5	0.55 ± 0.01	50.22 ± 10.20	42.91 ± 4.49
	10	0.64 ± 0.05	103.46 ± 5.63	91.63 ± 3.46
	25	0.80 ± 0.02	168.54 ± 6.29	153.41 ± 5.20
	50	1.22 ± 0.04	78.72 ± 3.38	72.72 ± 2.83
	75	*	*	*
	100	*	*	*
SE		0.0657	8.5665	7.8975
CD5%		0.190	22.2436	21.3542
CD1%		0.335	32.3412	30.6512
CV		09.55	17.32	16.25

*** Inadequate sample tissue *SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation***

DISCUSSION

Salicylic acid (SA) is recognized as a key signaling molecule that regulates various physiological and developmental processes in plants, including growth, defense, and responses to both biotic and abiotic stresses (Hayat et al., 2010; Yusuf et al., 2012; Rivas-San Vicenta and Plasencia, 2011). Only a limited number of studies have explored the regulatory influence of SA on micropropagule development, shoot morphogenesis, and somatic embryogenesis under in vitro conditions (Babel et al., 2014; Komaraiah et al., 2004; Galal, 2012; Ram et al., 2013). Hence, the present investigation aimed to examine the promotory role of exogenously applied SA on the in vitro growth and development of *Talinum portulacifolium* micropropagules.

Previous reports have demonstrated the use of filter-sterilized SA to study its modulatory effects in tissue cultures of *Cistus heterophyllus* and *Rosa hybrida* (López-Orenes et al., 2013; Ram et al., 2013). Similar approaches were adopted by Handro et al. (1997) and Bidabadi et al. (2012) to study stem elongation and water stress tolerance under in vitro conditions. Conversely, other researchers incorporated SA into the medium before autoclaving for various in vitro studies (Komaraiah et al., 2004; Sakhanokho and Kelley, 2009; Galal, 2012). Notably, Babel et al. (2014) provided one of the few comparative analyses of these two methods of SA incorporation.

In the present study, the mode of SA application—whether added before or after autoclaving—did not significantly affect shoot growth or biomass accumulation in *T. portulacifolium*. However, biochemical parameters such as total phenols, chlorophyll pigments, proteins, and carbohydrates showed some variation between treatments, likely due to pH alterations caused by the addition of SA after autoclaving. Based on these findings, it can be inferred that incorporating SA prior to autoclaving is more suitable for achieving consistent physiological responses.

The positive influence of SA on shoot proliferation and biomass production under in vitro water stress conditions has previously been reported in *Talinum portulacifolium* (Bidabadi et al., 2012). In our results, lower concentrations of SA enhanced shoot number, length, and biomass accumulation, whereas higher concentrations had inhibitory effects. This observation aligns with Babel et al. (2014), who reported similar trends in *Chlorophytum borivilianum*. Hosseini et al. (2009) suggested that elevated SA levels promote ethylene synthesis, a growth-retarding hormone that hampers somatic embryogenesis in carrot cultures—a mechanism that may also explain the growth inhibition observed here. Comparable outcomes have been described in *Ziziphus spina-christi* tissue cultures, where low SA levels promoted shoot proliferation, while higher doses were detrimental (Galal, 2012). Likewise, Hayat et al. (2005) noted that low SA concentrations increased pigment synthesis, biomass, leaf number, and nitrate reductase activity in wheat seedlings, but excessive levels suppressed growth.

In *Chlorophytum borivilianum*, Babel et al. (2014) observed that lower SA concentrations enhanced photosynthetic pigments, total carbohydrates, phenols, and biomass, whereas higher doses reduced them. A similar pattern was evident in our study: lower SA concentrations improved total chlorophyll, carbohydrate, protein, phenol, and biomass levels, while higher concentrations resulted in reduced growth and biochemical accumulation. Supporting this, Khodary (2004) reported increased carbohydrate and pigment levels in maize plants subjected to salt stress and low SA treatment. Collectively, these findings suggest that low concentrations of SA stimulate in vitro growth and enhance key physiological and biochemical activities in *Talinum portulacifolium*, whereas higher concentrations exert inhibitory effects on both growth and metabolism.

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