IJPSR (2018), Volume 9, Issue 12 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 March, 2018; received in revised form, 18 June, 2018; accepted, 02 July, 2018; published 01 December, 2018

SALICYLIC ACID-INDUCED MODULATION OF GROWTH AND METABOLISM OF A MEDICINAL PLANT *MENTHA SPICATA* L.

M. Kundu [1], S. Halder [2] and A. Bhattacharjee [*1]

Plant Physiology and Biochemistry Section [1], UGC Centre for Advanced Study, Department of Botany, Burdwan University, Burdwan - 713104, West Bengal, India.
PG Department of Botany [2], Darjeeling Government College, Darjeeling - 734101, West Bengal, India.

Keywords:

Salicylic acid, Plant growth regulator, Medicinal plant, *Mentha spicata*, Antioxidants, Growth and biochemical attributes

Correspondence to Author:
Prof. Aloke Bhattacharjee

Professor,
Plant Physiology and Biochemistry Section, UGC Centre for Advanced Study, Department of Botany, Burdwan University, Burdwan - 713104, West Bengal, India.

E-mail: alokebc@yahoo.co.in

ABSTRACT: Salicylic acid is a phenolic plant growth regulator found in plants with various important physiological roles. This investigation was aimed to study the effect of exogenous application of salicylic acid on the growth, metabolism and health status of *Mentha spicata*, an aromatic medicinal plant having several monoterpenes and antioxidants which show various kinds of medicinal properties. In the present work, some growth and biochemical parameters were recorded at 30 and 60 days after treatment with different concentrations of salicylic acid (0, 100, 200 and 300 µg ml^{-1}). Results clearly revealed that salicylic acid particularly at 200 µg ml^{-1} concentration significantly enhanced most of the growth and some of the biochemical attributes as well as the antioxidant property when compared with that of control plants. It can be concluded that exogenously applied salicylic acid at particular doses can enhance the biomass production as well as the medicinal potential of *M. spicata* as evidenced from some reliable physiobiochemical parameters.

INTRODUCTION: First and certainly the oldest system of human health care is herbal medicine. Herbs are used for the treatment of human ailments by almost all civilizations and cultures. In recent years, both in developed and developing countries, the demand of medicinal plants has increased rapidly. Mint (*M. spicata*) is one of the most commonly used essential oil bearing medicinal herbs. India is the largest mint oil producer, with an annual production of essential oil of 15,000 - 20,000 tons [1]. The genus *Mentha* under the family Lamiaceae includes 18 species and 11 named hybrids [2].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(12).5294-00

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(12).5294-00

Plant extracts of mint species are used in cosmetic industry, food industry, pharmaceutical industry and are generally considered safe to use [3, 4, 5]. This plant especially the leaves contains several monoterpenes including menthol, and carvone is the main volatile component [6]. Various kinds of medicinal properties of this plant include: carminative, antispasmodic, anti-vomitting, anti-hysteric, anti-irritant, antibronchities, antioxidative, antifungal action *etc*.

The plant needs profuse foliar biomass production for utilizing this biomass as potential source of essential oil which are mainly used for treatment of various human-ailments. Like some traditional growth regulators, salicylic acid (SA), an established non-traditional plant growth regulator (NTPGR) is reported to enhance the biomass production as well as to increase the quality of essential oil in plants [7, 8, 9].

Salicylic acid, a phenolic compound, is widely found in higher plants and reported to perform various important physiological roles. This NTPGR is presently well established as a potent endogenous signaling molecule [10, 11]. It renders plant resistance and tolerance to biotic and abiotic stresses [12, 13, 14]. Several studies also revealed the growth promoting effect of SA [15, 16]. Moreover, exogenous application of SA has been reported to be effective in inducing secondary metabolite formation [17, 18, 19].

Keeping in mind the ever-increasing demand of essential oil of mint and the growth promoting effect of SA, the present study was aimed to examine whether foliar application with different concentrations of SA could enhance the health status and foliar yield attributes of mint, which might lead to its oil productivity as leaves are the main source of mint oil. Thus, the prime objective of this work was to analyse the role of SA on augmentation of the plant biomass with concomitant enhancement of the general vigour status of our experimental aromatic medicinal plant, which may reflect in its oil productivity.

MATERIALS AND METHODS: The fresh plant samples were collected from North Bengal University campus, West Bengal, India. The plant was authenticated by Dr. A. Mukherjee, Professor, Department of Botany, The University of Burdwan, Burdwan, West Bengal, India. The voucher specimen [NBU Campus/2014/M106/(BURD)] has been deposited at the Burdwan University Herbarium. Uniform-sized selected explants of the plant material (*M. spicata)* having a few nodes in each planting material were planted in the earthen pots having loamy soil enriched with vermi-compost. Different concentrations (0, 100, 200 and 300 µg ml^{-1}) of aqueous solutions of SA were applied on the plants through foliar spraying after 30 days of planting. Some growth attributes, like plant height, number of branches per plant, number of leaves per plant, leaf area and leaf biomass as well as biochemical parameters like photosynthetic pigments, protein content, phenol content and enzymes like catalase and peroxidase activities were recorded at 30 days after treatment.

Fresh Weight and Dry Weight: Fresh weight was recorded by weighing 10 uniformly growing *Mentha* plants using an electronic balance. After taking fresh weight of such plants from each treatment, the plants were dried at 60 ºC for 72 h using a hot air oven and the dry weight of plants were recorded thereafter.

Leaf Area: It was determined by measuring the maximum length and breadth of all the leaves of a plant and multiplying them with a predetermined factor of 0.72. The factor was calculated by measuring the actual area of all the leaves of a plant separately on graph paper followed by dividing the total leaf area with the summation of length and breadth of all the leaves. This process was sufficiently replicated and the average value was used [20].

Estimation of Total Chlorophyll: Chlorophyll was measured using Arnon's method, 1959 [21]. Two hundred mg of the leaves were weighed, thoroughly homogenized using 80% acetone and centrifuged at 4800 *g* for 20 min. The residue was reextracted and the pooled above solutions were used to measure total chlorophyll content. The spectrophotometer was adjusted at wavelength of 650 nm for chlorophyll.

Estimation of Total Protein: Protein was estimated by the method of Bradford, 1976 [22]. Hundred mg of plant material was taken for analysis. Each sample was taken in test tubes containing 10 ml of 80% boiling ethanol in a water bath for one min. Then it was allowed to cool at room temperature. After that it was homogenized with the same ethanol using pestle and mortar. Volume of all the homogenates was made to equal level to avoid imbalance during centrifugation with the help of 80% ethanol.

After thorough grinding the homogenate was centrifuged using a Remi centrifuge at 5000 g for 10 min. The residue was re-extracted with 10 ml of 5% perchloric acid (PCA) with same speed and time. After discarding the supernatant, the residue was re-extracted with 5 ml of 1 N NaOH and dissolved it by keeping in warm water at a temperature of 50 ºC for 30 min. After 30 min it was centrifuged again at 5000 g for 10 min and the supernatant was stored as protein source. 0.3 ml of the extract was taken in triplicate to which 0.7 ml of double distilled water was added. Along with

this 5 ml of Coomassie brilliant blue G-250 dye was added and shaken well at room temperature by vortexing. Absorbance was measured at 595 nm in a spectrophotometer. Here the blank was made up of 1 ml distilled water along with 5 ml of dye. The protein content of the samples was determined against a standard calibration curve of bovine serum albumin (BSA, Sigma product, USA).

Estimation of Total Phenolics: The amount of total phenolics present in extracts of *M. spicata* species was determined (as gallic acid equivalent µg $ml^{-1}$of extract) using Folin-Ciocalteau reagent (FCR) as described by Karamian and Asadbegy, 2016 [23] with slight modification. Each sample (1 ml) was mixed with 2.5 ml of a 10-fold diluted FCR and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 min at room temperature before the absorbance was read at 760 nm spectrophotometrically. All the analyses were performed in triplicate. A gallic acid standard curve (R^2 = 0.93) was used to measure the phenolic content.

Estimation of Total Flavonoids: The total flavonoids content of each plant extract was estimated by the method described by Karamian and Asadbegy, 2016 [23] with slight modification. Based on this method, each sample (1.0 ml) was mixed with 4 ml of distilled water and subsequently with 0.30 ml of a $NaNO_2$ solution (10%). After 5min, 0.30 ml $AlCl_3$ solution (10%) was added followed by 2.0 ml of NaOH solution (1%) to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was then determined at 510nm versus the blank. A quercetin standard curve (R^2 = 0.96) was used to measure the total flavonoid content.

Extraction and Estimation of the Catalase and Peroxidase: Catalase activity was analysed following the modified method by Biswas and Choudhuri, 1978 [24]. Peroxidase activity was assayed following the method of Kar and Mishra, 1976 [25]. For assaying these enzymes, the blank was taken as zero-time control and the activity was expressed as (OD × TV) / (t × v), where OD is the difference of OD of blank and sample. TV is the total volume of filtrate, t is the time (min) of incubation with the substrate and v is the volume of filtrate taken for incubation.

Antioxidant Activities:

DPPH Radical Scavenging Assay: The antioxidant activity of methanol extract of leaves of the plant material was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical according to the method described by Karamian and Asadbegy, 2016 [23] with slight modifications. One ml of 0.1 mM DPPH solution in methanol was mixed with 1ml of various concentrations (3-21 µg ml^{-1}) of methanol extract of leaves. Quercetin was used as the reference standard. Mixer of 1 ml methanol and 1 ml DPPH solution was used as the control. The decrease in absorbance was measured at 517nm after 30 min in dark using UV-Vis spectrophotometer. The inhibition % was calculated using the following formula.

$$\text{DPPH scavenging activity (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Reducing Ability (FRAP Assay): The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain, 1996 [26]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C before use. Plant extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM Fe (II) g^{-1} dry mass and compared with that of ascorbic acid.

RESULTS AND DISCUSSION: In the present investigation, results clearly revealed that SA particularly at its specific concentration (200 µgml^{-1}), increased the shoot length, branch number per plant, leaf area, leaf number, and plant biomass, when compared with control plants **Fig. 1, 2** and **Table 1**.

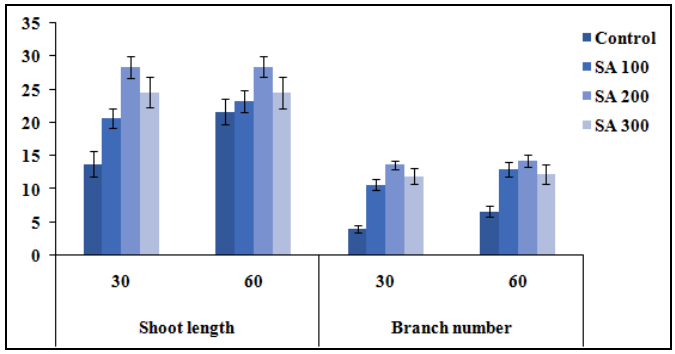

FIG. 1: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300µg ml^{-1}) OF SA ON SHOOT LENGTH (cm) AND NUMBER OF BRANCHES PER PLANT OF *M. SPICATA*. DATA WERE RECORDED AFTER 30 AND 60 DAYS AFTER TREATMENT

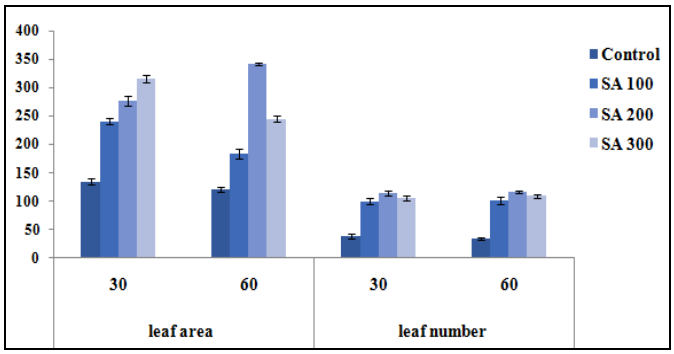

FIG. 2: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300µg ml^{-1}) OF SA ON TOTAL LEAF AREA PER PLANT (cm^2) AND NUMBER OF LEAVES PER PLANT OF *M. SPICATA* PLANTS. DATA WERE RECORDED AFTER 30 AND 60 DAYS AFTER TREATMENT

TABLE 1: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300 µg ml^{-1}) OF SA ON PLANT BIOMASS (g) OF *M. SPICATA*. DATA WERE RECORDED AFTER 60 DAYS OF TREATMENT

Treatments	Concentrations (µg ml^{-1})	Biomass per plant	
		Fresh weight (g)	Dry weight (g)
Control	0	45.04 ± 2.13	10.79 ± 0.86
SA	100	48.57 ± 1.80	12.26 ± 1.02
	200	63.73 ± 2.43	17.48 ± 1.36
	300	56.25 ± 2.23	14.56 ± 0.82

Total chlorophyll content was significantly higher in all the SA-treated plants over control **Table 2**. Maximum chlorophyll content was found in SA 200 µg ml^{-1} treated plant and it was approximately 21.7% higher than control at the observation period of 30 days after treatment whereas at 60 days after treatment it was almost 37.9% higher than control value. Similar trend was found in case of protein content.

TABLE 2: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300µg ml^{-1}) OF SA ON CHLOROPHYLL AND PROTEIN CONTENTS OF *M. SPICATA*. DATA WERE RECORDED AFTER 30 AND 60 DAYS AFTER TREATMENT

Treatments	Concentrations (µg ml^{-1})	Chlorophyll content (mg g^{-1} fr. wt.)		Protein content (mg g^{-1} fr. wt.)	
		30 days	60 days	30 days	60 days
Control	0	2.17 ± 0.12	1.82 ± 0.11	64.05 ± 2.55	48.76 ± 2.40
	100	2.26 ± 0.13	1.98 ± 0.13	79.26 ± 3.04	66.86 ± 3.23
SA	200	2.64 ± 0.14	2.51 ± 0.20	89.35 ± 4.52	83.70 ± 3.77
	300	2.47 ± 0.11	2.30 ± 0.14	83.23 ± 4.09	74.15 ± 3.05

Both the catalase and peroxidase activities were found increased value in all the SA-treated plants over control at 30 and 60 days after treatment **Table 3.** Maximum catalase activity was found in SA 200 µg ml^{-1} treated plant at 30 days after treatment and it was approximately 23.3% higher than control value. In case of peroxidase activity at same developmental stage it was almost 66.6% higher than control value.

Secondary metabolites like phenol and flavonoid were significantly increased in SA-treated plants particularly at 200 µg ml^{-1} during later stage of development *i.e.*, 60 days after treatment **Table 4**.

Total phenol content at this stage in SA 200 µg ml^{-1} treated plant was almost 54.5% higher than control and total flavonoid content was approximately 89.5% higher than control value.

TABLE 3: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300 µg ml^{-1}) OF SA ON CATALASE AND PEROXIDASE ACTIVITIES (UNIT min^{-1}g^{-1}fr.wt.) OF *M. SPICATA*. DATA WERE RECORDED AFTER 30 AND 60 DAYS OF TREATMENT

Treatments	Concentrations (µg ml^{-1})	Catalase (Unit min^{-1} g^{-1} fr. wt.)		Peroxidase (Unit min^{-1} g^{-1} fr. wt.)	
		30 days	60 days	30 days	60 days
Control	0	0.60 ± 0.04	0.43 ± 0.03	0.36 ± 0.02	0.24 ± 0.01
	100	0.71 ± 0.03	0.64 ± 0.03	0.43 ± 0.02	0.33 ± 0.02
SA	200	0.74 ± 0.04	0.70 ± 0.04	0.60 ± 0.04	0.54 ± 0.03
	300	0.61 ± 0.03	0.47 ± 0.02	0.38 ± 0.02	0.27 ± 0.02

TABLE 4: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300 µg ml^{-1}) OF SA ON TOTAL PHENOL CONTENT (TPC) AND TOTAL FLAVONOID CONTENT (TFC) OF *M. SPICATA*. DATA WERE RECORDED AFTER 30 AND 60 DAYS OF TREATMENT

Treatments	Concentrations (µg ml^{-1})	TPC (µg mg^{-1} gallic acid equivalent)		TFC (µg mg^{-1} quercetin equivalent)	
		30 day	60 day	30 day	60 day
Control	0	41.52 ± 2.90	49.30 ± 3.74	5.76 ± 0.38	6.38 ± 0.32
SA	100	52.07 ± 1.56	55.89 ± 1.84	8.25 ± 0.61	11.74 ± 0.85
	200	72.51 ± 3.92	76.19 ± 3.12	6.84 ± 0.58	12.09 ± 0.72
	300	55.01 ± 2.32	55.23 ± 2.65	5.21 ± 0.36	7.85 ± 0.52

DPPH free radical scavenging activity showed better result in all the SA-treated plants over control. Among all the treatments of SA 200 µgml^{-1} concentration showed maximum percentage of free radical scavenging activity and it was almost 54.17% more over control value. FRAP activity also showed similar type of result and SA 200 µg ml^{-1} concentration showed maximum FRAP activity and it was almost 38.39% more over control value **Table 5**.

TABLE 5: EFFECT OF DIFFERENT CONCENTRATIONS (0, 100, 200 AND 300µg ml^{-1}) OF SA ON FREE RADICAL SCAVENGING ASSAY (DPPH AND FRAP) OF *M. SPICATA*. DATA WERE RECORDED AFTER 60 DAYS OF TREATMENT

Treatments	Conc. (µg ml^{-1})	% DPPH Scavenging (at 100 µg ml^{-1})	FRAP {µM of ferrous equivalent Fe (II)} (d.w.)
Control	0	38.53 ± 1.33	330.00 ± 12.04
SA	100	46.66 ± 2.48	445.49 ± 24.60
	200	59.40 ± 3.08	456.62 ± 21.95
	300	42.37 ± 2.62	385.03 ± 17.95

In this study, increased growth attributes with concomitant increase of some biochemical parameters in SA treated plant samples are well established from repeated experiment. The use of plant growth regulators particularly salicylic acid on various plants to improve vegetative growth, metabolism and essential oil productivity were documented earlier by different workers [27, 28, 29, 30]. In our present study the beneficial role of SA on some growth parameters the physiological data like shoot length, leaf number, leaf area, plant biomass and branch number per plant of mint corroborated the previous findings. Jakhar and Sheokand, 2015 [31] also reported the increase of leaf biomass and shoot dry weight of soybean plant when treated with SA.

In 2007, Arfan *et al.,* [30] opined that the SA-induced promotion of growth in wheat plant was due to the increase of photosynthetic tissue as there was a positive correlation between photosynthetic tissue and leaf area. It may be speculated that in our study, production of a higher number of oil glands containing economically important mint essential oil in treated samples might be due to greater leaf area per plant. Our results are in agreement with some previous reports suggesting that SA can augment the essential oil content by stimulating vegetative growth [32].

SA-induced increase or decreases of photosynthetic pigments (chlorophylls and carotenoids) were reported to be species and cultivar-dependent [33, 34, 35]. In our experiment, total chlorophyll content was significantly higher than the control plants and our observation seems to be identical with that of Kareem *et al.,* 2017 [33]. Treatment induced significantly enhanced vegetative growth (shoot length, leaf number, leaf area, leaf biomass and branch number per plant) in this present work might be due to the enhanced photosynthetic rate resulted from the higher stomatal conductance and/or higher chlorophyll content.

In this work total soluble protein content in SA-treated plants particularly at 200 µg ml^{-1} concentration was found significantly higher after 30 days of treatment and this augmentation might be due to the increase of Nitrate reductase enzyme as suggested earlier [36].

Activities like catalase and peroxidase were higher in treated samples which indicate the good health status of plants. However, the enzyme activities were higher in both the control and SA-treated plants during early stage (30 days after treatment) than the later one (60 days after treatment). Thus, our results regarding SA-induced increased catalase and peroxidase activity corroborate those of

previous reports [37, 38]. During 60 days stage SA-treated plants showed significantly higher value of two secondary metabolites like total phenol content (TPC) and total flavonoid content (TFC). And promotive influence of SA on secondary metabolites at specific concentrations is available from some previous reports [39, 40, 41]. The SA-treated plants also showed more antioxidant activity measured in terms of DPPH free radical scavenging activity as well as FRAP activity. This higher free radical scavenging activity of the plants may be due to the presence of high level of phenol and flavonoids. Some earlier workers also reported such positive correlation between antioxidant activity and phenol content [40, 42, 43].

CONCLUSION: Thus, by virtue of increasing the biomass of leaves by SA treatment along with its enhancement of metabolite potential, as evidenced from some reliable biochemical parameters, it can be concluded that SA is potential enough for exhibiting better field performance which consequently resulted in coveted higher oil productivity of the experimental *Mentha* species. Further research work is in progress to pinpoint the exact mechanism for SA regulated augmented oil productivity of *Mentha.*

ACKNOWLEDGEMENT: The authors are indebted to the Centre for Advanced Study, Department of Botany, Burdwan University for rendering necessary facilities for carrying out the work, and the first author is thankful to Department of Science and Technology, Government of India for providing the INSPIRE Fellowship.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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How to cite this article:
Kundu M, Halder S and Bhattacharjee A: Salicylic acid-induced modulation of growth and metabolism of a medicinal plant *Mentha spicata* L. Int J Pharm Sci & Res 2018; 9(12): 5294-00. doi: 10.13040/IJPSR.0975-8232.9(12).5294-00.