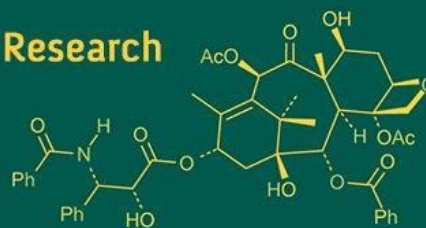


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Effect of salicylic acid on morphological and biochemical parameters of *Withania somnifera* L.

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Abstract

Withania somnifera (Ashwagandha), a medicinal plant of the Solanaceae family, is widely recognized for its therapeutic properties. This study explores the impact of salicylic acid (SA), a plant hormone known for its role in stress tolerance and growth regulation, on the morphological and biochemical traits of *W. somnifera*. Seeds were treated with SA concentrations of 0.25 mM, 0.5 mM, and 1 mM, and monitored over nine weeks.

Morphological parameters such as shoot and root length, leaf number, biomass, and leaf area were recorded. Biochemical estimations included chlorophyll, proline, and phenol content. Micromorphological features like stomatal and trichome indices were also analyzed.

The 0.5 mM SA treatment yielded the most significant improvements in growth and metabolite production. Plants treated with this concentration showed increased leaf number, shoot and root length, biomass, and leaf area. Enhanced chlorophyll content indicated improved photosynthetic efficiency, while elevated phenol and proline levels suggested better stress resilience and metabolic activity.

Microscopic analysis revealed higher stomatal and trichome indices, supporting the physiological benefits of SA application. These findings suggest that 0.5 mM SA is an optimal concentration for promoting growth and secondary metabolite synthesis in *W. somnifera*.

This study highlights the potential of SA as a sustainable growth enhancer and elicitor of bioactive compounds in medicinal plants, with implications for agricultural and pharmaceutical applications.

Keywords: *Withania somnifera*, salicylic acid, plant growth, secondary metabolites, chlorophyll, proline, phenol

Introduction

Withania somnifera (synonyms: ashvagandha, suranjan, winter cherry, Indian ginseng) is a xerophytic plant that nurtures abundantly in Africa, Sri Lanka, Pakistan and India. *Withania somnifera* is a small shrub which belongs to family Solanaceae. This plant acclaimed as medicinally essential due to the high therapeutic and nutraceutical potential. It possessed a plethora of medicinal uses and pharmacological application.



Fig 1: Morphological characteristic features of *W. somnifera*; A-Habit, B-Leaves, C-Fruit

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In Ayurvedic system of medicine, *W. somnifera* is regarded as an important medicinal herb (Singh *et al.* 2011). 'Ashwagandha' is commonly known as 'Indian ginseng', (Kulkarni and Dhiri 2008). Ashwagandha has attributed immense pharmacological research interest. According to the researchers, the plant is reported to have adaptogenic, anticancerous, immunomodulatory, antioxidative properties and in treating neurological disorders

(Schliebs *et al.* 1997; Dhuley 2001; Naidu *et al.* 2003). The extract of *Withania somnifera* was reported to prevent the skin carcinoma induced by DMBA (dimethyl benzantracene) and croton oil (Davis and Kuttan 2001). It was also reported to treat arthritis and neurological behavioural problems (Kulkarni and Ninan 1997). Ashwagandha is one of the most commonly used sedatives in India, similar to ginseng in China (Bown 1995). It has also been reported to have rejuvenating effect on the reproductive and nervous systems and improves the vitality and helps in early recovery after an illness (Bown 1995; Chevallier 1996).

Salicylic acid (SA) is a natural plant hormone that plays an important role in plant growth and development, as well as in the plant's response to biotic and abiotic stresses. SA is a defense-related plant hormone that plays a key role in resistance to different microbial pathogens, such as virus, bacteria, fungi, and oomycetes (Kunkel and Brooks,

2002; Vlot *et al.*, 2009) [29]. SA has been shown to regulate plant growth by promoting cell division, elongation, and differentiation, as well as by modulating the balance between growth and defense pathways. SA has also been shown to increase the activity of photosynthetic enzymes and to enhance plant tolerance to various stresses, such as drought, salt, and heat.

The mechanisms underlying the effects of SA on plant growth and stress tolerance are complex and involve the regulation of numerous genes and signaling pathways. SA interacts with other plant hormones, such as auxin, gibberellins, and cytokines, and modulates their biosynthesis, transport, and signaling. SA also regulates the expression of genes involved in the biosynthesis of secondary metabolites, such as flavonoids, alkaloids, and phenolics, which play important roles in plant defense against pests and diseases.

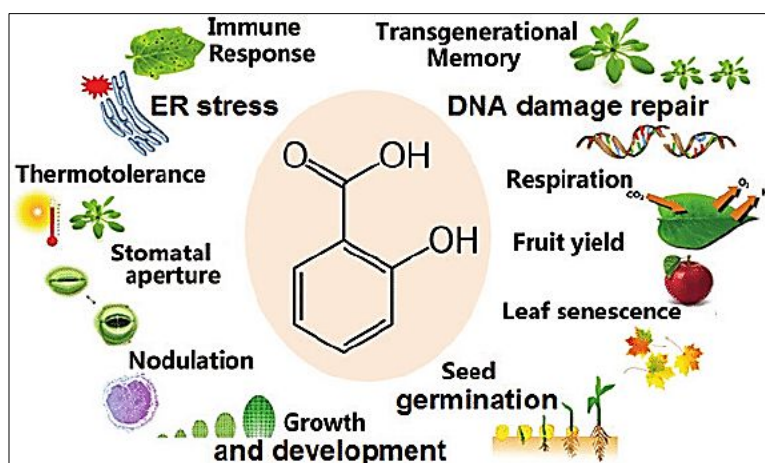


Fig 2: Biological function of salicylic acid in plants Adapted from Karolina Pajeroska-Mukhtar (2015)

The application of SA as a plant growth regulator has gained increasing attention in recent years, particularly in the field of horticulture and agriculture. SA has been shown to enhance the growth and yield of various crops, such as rice, wheat, soybean, and tomato, under both normal and stress conditions. SA has also been shown to improve the quality of fruits and vegetables by increasing their antioxidant content and delaying senescence. SA treatment increased the level of antioxidants in tomato plants which helped protect the plants from oxidative stress (Khan *et al.* 2019). It also increased the growth and biomass of tobacco plants (Raskin, I., & Skubatz, H. 1995). It can also enhance plant growth and stress tolerance by regulating various physiological and biochemical processes.

The application of SA as a plant growth regulator can be done through different methods, such as foliar sprays, root drenches, and seed treatments. The optimal dosage and timing of SA application depend on the plant species, growth stage, and environmental conditions. Excessive or untimely application of SA can lead to negative effects, such as phytotoxicity and reduced yield. However, further research is needed to better understand the mechanisms underlying the effects of SA and to develop efficient and sustainable application strategies for different crops and environmental conditions.

Withania somnifera is an adaptogenic herb that is commonly used in traditional Ayurvedic medicine to help the body cope with stress. Salicylic acid, on the other hand, is a type

of plant hormone that plays a role in regulating plant growth and development, as well as in the plant's response to stress. There is limited research on the specific effect of salicylic acid on *Withania somnifera*. Some studies have investigated the effect of salicylic acid on other plants in the same family as Solanaceae, such as tomato and tobacco. So, based on these studies, it is possible that salicylic acid may have similar effect on *Withania somnifera* by increasing promoting growth. However, further research needed to determine the optimal dosage and application method of salicylic acid for *Withania somnifera*.

Material and Method

Seeds of *Withania somnifera* (L.) Dunal. Collected from shreeji beej company (Baroda) We took 108 *Withania somnifera* seeds and divided them into 4 parts. 27 seeds were put in Control, 27 seeds in 0.25mM, 0.5mM and 1mM concentration of SA respectively. After that seeds were soaked into each concentration for 3-4 hrs and put it for the germination. Prepare black plastic nursery bags with soil for the further growth of plant. Keep triplicate for each concentration. Observed the growth of the plants for 9 weeks and performed qualitative and quantitative features at a particular time interval.

Morphological characteristics like measurement of shoot and root length and number of leaves measured at the 5th, 7th and 9th weeks intervals. Fresh weight of each plantlet was also recorded at harvested time and put them for drying for

4-5 days at 80°C, and then take dry weight for the same. Biochemical estimation of chlorophyll, proline and phenol were estimated for all harvested plants (5th, 7th and 9th week's interval).

Micromorphological analysis of leaf samples were done by simple scratch method and peel was prepared and observe the internal structures under a compound microscope (Karl zeiss).

Chlorophyll Estimation done by Arnon *et al.* (1949) [4], Proline estimation done by Folin - Ciocalteu method (Journal of Science of Food and Agriculture 95(1),204-209,2015) follow for the estimation and proline estimation done by Acid Ninhydrin method (Bates, L.S., Waldern, R.P. and Tare, I.D., *et al.* 1973).

Result and Discussion: Plant growth is a complex phenomenon and determined by several endogenous and exogenous factors. Internal factor like hormones play a vital role in regulating growth and development. The role of SA in plant growth regulation was the first physiological

process to be reported (Dekock *et al.*, 1974). Thereafter, both exogenous and endogenous levels of SA have been shown to impart various effects (Raskin, 1992).

Effect of Salicylic acid on quantitative morphological characters

There are few morphological characteristics like number of leaves, root length, shoot length, biomass and leaf area of leaflets, considered to check for the growth of plant (Fig. 3, 4 and 5).

Number of leaves

Salicylic acid is used to coat seeds, causing the number of leaf to increase. Number of leaves increased as the weeks are increasing. 0.25 mM showed negative result than the control, but 0.5 mM and 1 mM showed positive result. Among these two 0.5 mM concentrations of SA showed best results. After 9 weeks 0.5mM showed around 26 number of leaves, which was double than the control (Table 1; Graph 1(A)).

Table 1: Effect of Salicylic acid on number of leaves

Treatment of Salicylic acid (mM)	Weeks						
	3	4	5	6	7	8	9
Control	2.7±0.42	4.4±0.6	5.8±0.73	7.3±0.65	8.9±0.84	11.9±0.76	13.7±5.22
0.25mM	2.3 ±0.58	3.05±0.63	4.4±0.68	6.3±0.85	8.1±0.783	10.5±0.72	12.7±4.85
0.5mM	3.5±0.76	4.7±0.56	7.04±8.66	12.5±2.78	17.7±1.33	22.4±1.52	25.9±9.96
1.0mM	3.8±0.55	6.4±0.90	8.7±0.81	13.4±3.15	18.7±1.12	21.6±0.99	23.9±9.08

Shoot length: Application of salicylates to plants increased the shoot growth in different plant species such as *Clitoria* where the biomass production is important as forage for animal feed (Martin-Mex and Larqué-Saavedra, 2001).

Similarly, when it was applied to ornamental plants the biomass always increased. Table 2 showed that 0.5 mM and 1mM showed more shoot length compared to lower concentrations. This result also represented in Graph 1 (B).

Table 2: Effect of Salicylic acid on shoot length (cm)

Treatment of salicylic acid (mM)	Weeks						
	3	4	5	6	7	8	9
Control	1.78±0.11	2.24±0.14	2.27±0.13	3.2±0.26	4.14±0.29	5.01±0.26	6.27±2.40
0.25mM	1.61±0.12	3.01±0.24	2.94±0.28	3.07±0.37	4.02±0.26	5.01±0.38	5.76±2.18
0.5mM	2.44±0.13	3.01±0.24	2.92±0.18	4.54±0.59	6.2±0.46	7.47±0.38	8.25±3.14
1.0mM	3.48±0.12	3.87±0.12	4.51±0.26	5.64±0.62	7.54±0.50	8.67±0.34	9.62±3.64

Root length: Increasing the total soil volume explored by the root of the plant is one of the best ways to improve yield since more water and nutrients may be taken up by the plants. A second important aspect is to increase rooting density which occurs because of increased initiation of secondary roots. In experiments carried out with salicylic acid both aspects (root length and density) were found to be affected. Length of the plant's roots is increasing over time, but this growth is affected by the concentration of SA used

to treat the plant. The plants treated with 0.25mM SA showed less root length compared to control. The plants treated with 0.5mM and 1mM showed more root length compared to control. Among these two 0.5mM concentration of SA showed more root length than the 1mM. The showed result of root length is represented in Table 3 which also showed that upto 7th week length of root increasing but after that root length is decreasing. Graphical representation of results shown in Graph 1 (C).

Table 3: Effect of Salicylic acid on Root length (cm)

Concentration of Salicylic acid (mM)	Weeks		
	5	7	9
Control	2.4	2.4	2.5
0.25mM	3	5.5	1.5
0.5mM	2.4	7.9	3.7
1.0mM	6.9	5.9	5.1

Biomass: Asger *et al.*, 2015 observed that the A plethora of research has shown the role of exogenous SA in enhancing photosynthesis and other physiological processes in various crops, Highest Fv/Fm ratio was observed by the seedlings treated with 150 and 200 µM. Identically, total biomass.

Table 4 showed the result of biomass of control and treated plants. As the root and shoot length are getting more in 0.5mM concentration of SA, biomass also getting more in same. This data is represented in graphical manner at Graph 1 (D).

Table 4: Effect of Salicylic acid on biomass of plants

Concentration of Salicylic acid (mM)	Weeks		
	5	7	9
Control	0.09	0.22	0.87
0.25mM	0.06	0.43	0.8
0.5mM	0.15	1.09	1.30
1.0mM	0.19	0.56	0.67

Leaf Area

Exogenous is well known to be involved in the regulation of various physiological processes such as the activity of photosystem II, plant biomass and leaf area thereby improving plant growth under adverse environmental conditions (Miura and supplementation of salicylic acid in nutrient media may help to improve *W. coagulans*,

maximum increase in leaf area was observed in SA 150 and 200 μ M treated seedlings compared to their respective control. To calculate the leaf area, three different sizes of leaves are considered for the study: large, medium and small. Again 0.5mM concentration showed a positive effect on the leaf area of all sized leaves. The result of it is represented in Table 5 and Graph 2 (A).

Table 5: Effect of Salicylic acid on Leaf area

Concentration of Salicylic acid (mM)	Weeks	Leaf Area (cm ²)		
		Large	Medium	Small
Control	5	5.66	0.61	0.003
	7	3.23	1.51	0.8
	9	11.17	7.04	1.88
0.25mM	5	1.5	1.034	0.006
	7	12.35	2.08	0.008
	9	4.1	0.44	0.01
0.5mM	5	2.56	0.43	0.0026
	7	14.99	4.42	0.41
	9	24.94	18.8	2.73
1.0mM	5	2.45	1.5	0.53
	7	28.78	2.8	0.61
	9	12.9	4.07	2.16

Effect of Salicylic acid on Chlorophyll

Several reports on the plant have confirmed the role of SA in improving photosynthesis in various crops (Clark *et al.*, 2004; Hasanuzzaman *et al.*, 2013) and these findings also corroborates the results of the present study. Nazar *et al.*, 2011 observed influence of salicylic acid elicitation on secondary metabolites and biomass production *in vitro* cultured *Withania coagulans*, they recorded highest chlorophyll amount in those seedlings which were treated

with higher concentration SA i.e. 100, 150 and 200 μ M. In our present study we also get same result in higher concentration of SA (0.5 mM) that's have more amount of chlorophyll a, chlorophyll b and total chlorophyll present. so that means the 0.5mM concentration prevents the improving photosynthesis in plant. Table 6 showed that 0.5mM concentration of SA showed maximum amount of chlorophyll a, chlorophyll b and total chlorophyll amount (Graph 2 (B)).

Table 6: Effect of Salicylic acid on Chlorophyll

Concentration of Salicylic acid (mM)	Weeks	Amount of chlorophyll (mg/g)		
		Chl a	Chl b	Total chl
Control	5	13.32	6.22	19.69
	7	10.08	5.4	15.47
	9	15.48	8.05	18.19
0.25mM	5	22.07	13.45	35.49
	7	14.98	7.97	17.99
	9	23.95	2.92	18.98
0.5mM	5	16.67	8.27	34.61
	7	13.76	14.47	16.74
	9	20.13	11.23	24.4
1mM	5	22.5	14.45	48.56
	7	21.04	12.29	25.99
	9	19.02	10	21.97

Effect of Salicylic acid on Phenol

Secondary metabolites are the substances generated biosynthetically from primary metabolites in plants as defense molecules which do not play any role in plant metabolic activity (Irchhaiya *et al.*, 2015). Salicylic acid has been well documented to play an important role in several secondary metabolite pathways including anthocyanin and

phenols (Ramakrishna and Ravishankar, 2011). Exogenous Influence of salicylic acid elicitation on secondary metabolites and biomass production in *in-vitro* cultured *Withania coagulans* (L.) Dunal application of SA at a varying concentration significantly increased secondary metabolites phenol contents in tissue cultured seedlings of *W. coagulans* compared to non-treated counterparts. A

maximum significant increase (50-200%) phenol contents was observed in those seedlings which were treated with SA at 150 and 200 μ M compared to control seedlings. However, seedlings treated with SA at 50 and 100 μ M also showed improved contents of secondary metabolites but the increase was inferior as compared to the effect of SA applied at higher concentrations (Wrolstand *et al.* 1982). Several studies have confirmed the involvement of SA in the stimulation of secondary metabolite synthesis in plants (Kiddle *et al.*, 1994; Ali *et al.*, 2007; Idrees *et al.*, 2013). In our present study the phenol

content in those seedlings which are treated with 0.5mM concentration of SA showed higher amount compare to various concentration of SA (Table 7, Graph 2 (C)). If the concentrations are increased than amount of phenol is going to be reduced. That is more benefited to these plant because of phenol is defense such as defense against pathogens and UV radiation, and regulation of plant growth and development. Thus, the right amount of phenol present in plant depends on the specific physiological and environmental context.

Table 7: Effect of Salicylic acid on Phenol

Concentration of Salicylic acid (mM)	Weeks	Amount of phenol(μ g)
Control	5	0.048
	7	0.15
	9	0.112
0.25mM	5	0.125
	7	0.224
	9	0.072
0.5mM	5	0.062
	7	0.115
	9	0.068
1mM	5	0.056
	7	0.047
	9	0.051

Effect of Salicylic Acid on Proline

The accumulation of osmolyte proline that adaptively regulates plants survival under different conditions. Proline is also known to potentially involved in the detoxification of reactive oxygen species (ROSs), protecting biological membranes by maintaining ion and osmotic homeostasis and stabilizes proteins/enzymes (Iqbal *et al.*, 2014). Exogenous Influence of salicylic acid elicitation on secondary metabolites and biomass production in *in-vitro* cultured *Withania coagulans* (L.) Dunal application of SA exogenous supplementation of salicylic acid at different concentration remarkably increased proline metabolism in all the treated seedlings compared to non-treated controls. However, control and seedlings treated with salicylic acid

(50 μ M) showed similar levels of proline whereas maximum increase (100- 200%) in the proline content was observed in the seedlings treated with 150 and 200 μ M of salicylic acid. Foliar application of SA (0.2 mM) significantly unregulated the activity of proline biosynthesis enzyme thereby increasing proline content and improved salt tolerance of *Lens esculenta* plants (Misra and Saxena, 2009). In our present study supplementation of salicylic acid at different concentration remarkably increased proline metabolism in all the treated seedlings but higher amount found in 1mM concentration of SA. Proline play important role in plant adaptability to environmental stresses and effects of reducing proline levels *Withania* on plant growth and development.

Table 8: Effect of Salicylic acid on Proline

Concentration of salicylic acid(mM)	Weeks	Amount of proline(μ g)
Control	5	0.037
	7	0.039
	9	0.01
0.25mM	5	0.038
	7	0.0155
	9	0.009
0.5mM	5	0.0445
	7	0.014
	9	0.003
1mM	5	0.1791
	7	0.1181
	9	0.0145

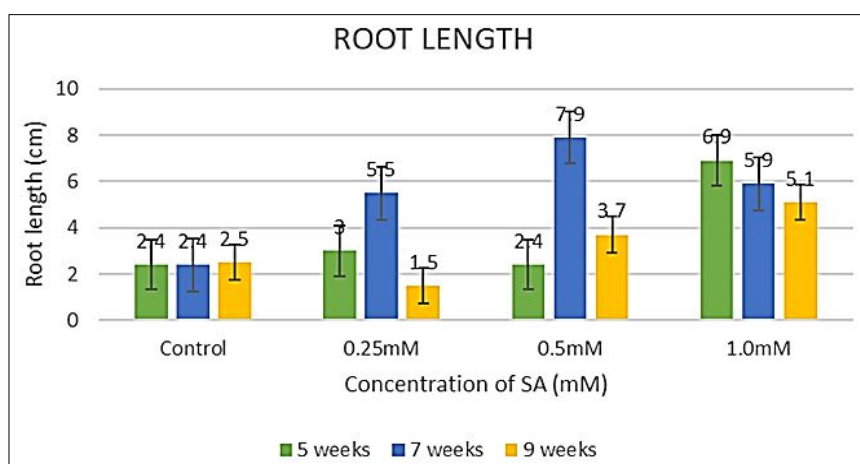
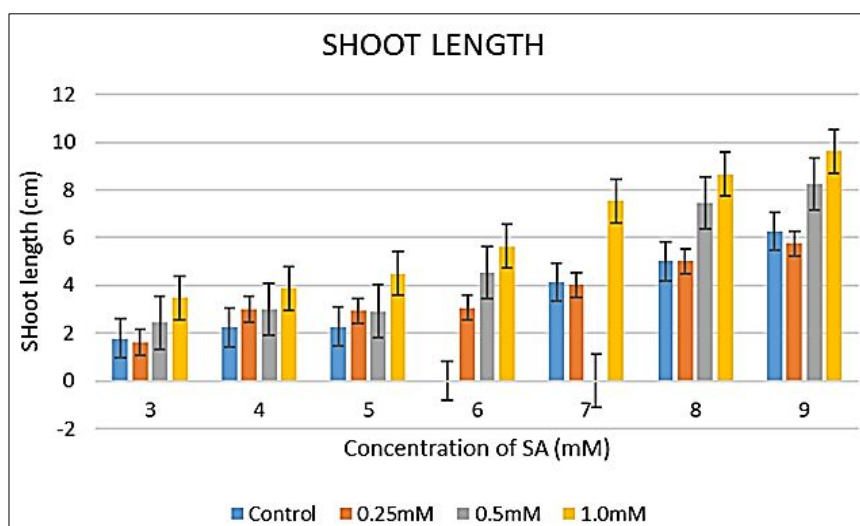
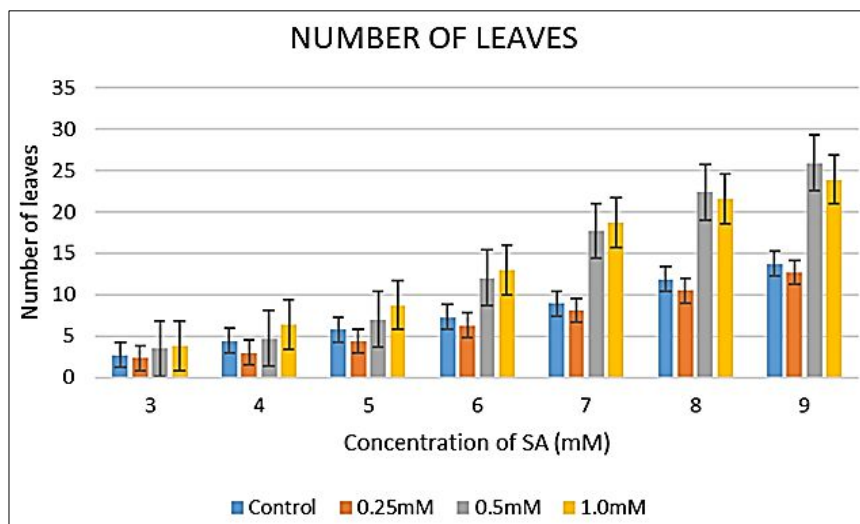
Effect of Salicylic acid on stomata and trichome

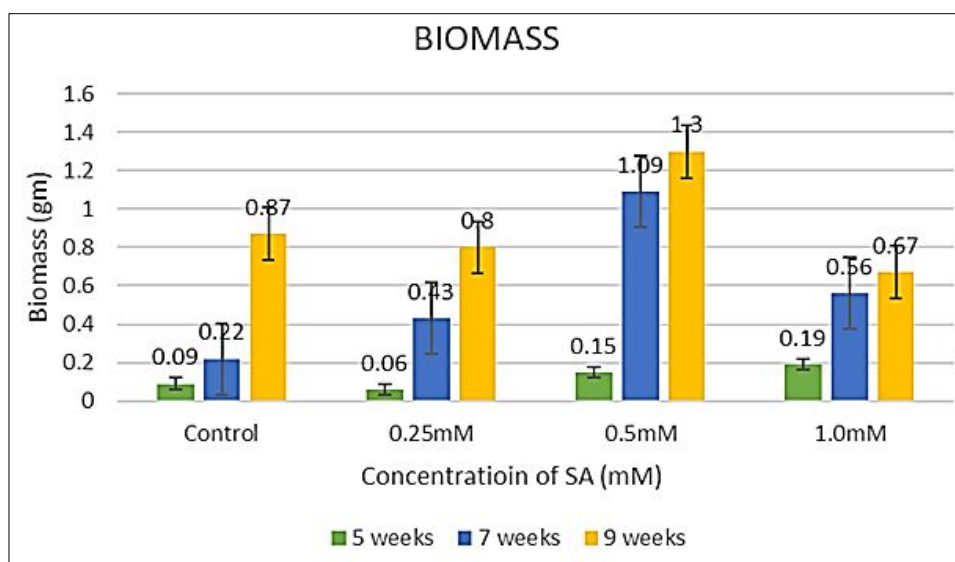
Foliar application of salicylic acid is an effective way to augment the adverse effects of salinity stress in plants. Foliar application of SA on *Withania somnifera* showed enhanced accumulation of the withanolides (Manali singh *et al.*, 2020). Anomocytic type of stomata present in the plant, and are observed in all concentration of SA (Fig. 6 and 7).

Stomatal index increasing as the number of weeks increased. 0.5mM and 1mM concentration of SA showed almost similar stomatal index and is 32.35 and 32.33 respectively. Multicellular, branched and unbrached type of trichome observed in plant on both the epidermises (Fig. 6 and 7). 1mM concentration of SA showed almost highest trichome index and is 12.5.

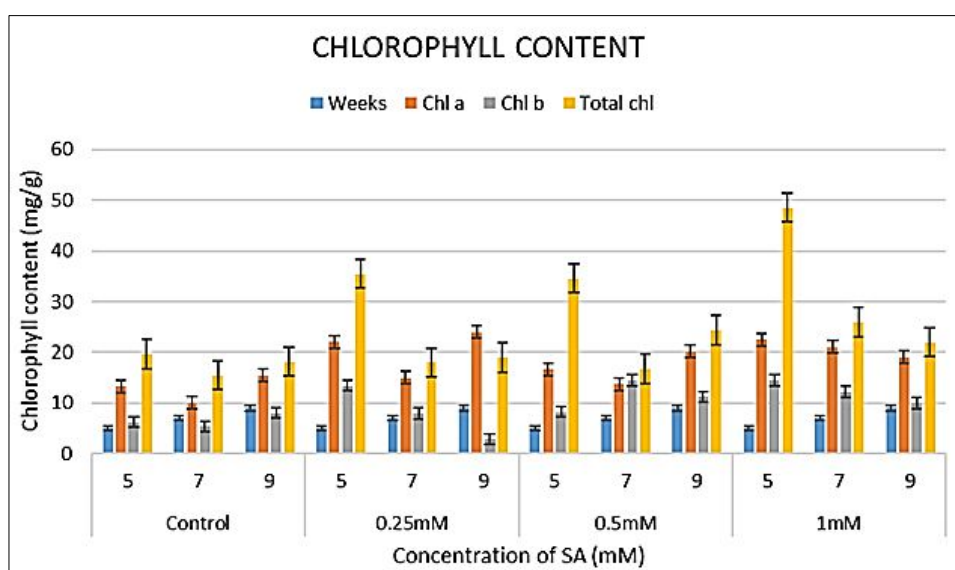
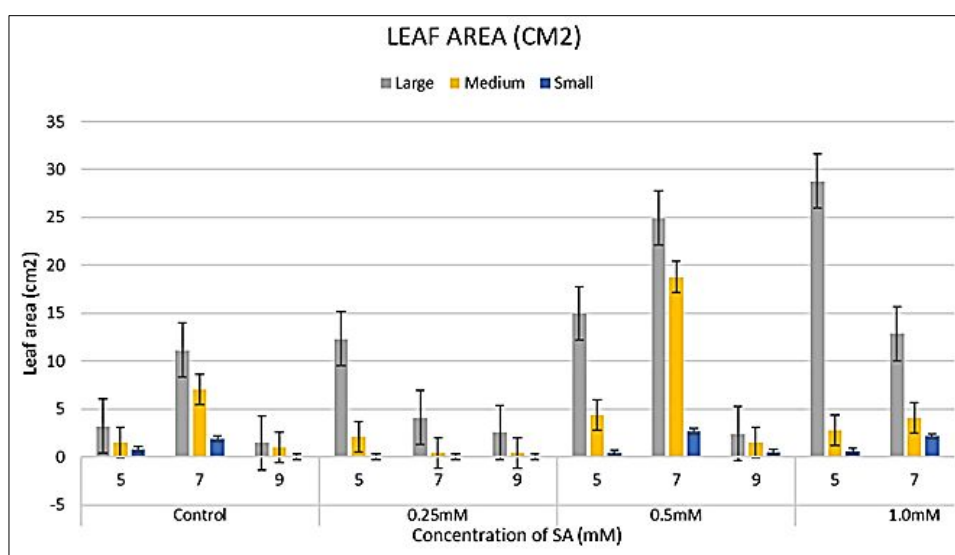
Table 9: Effect of Salicylic acid on Stomata index (%) and Trichome index (%)

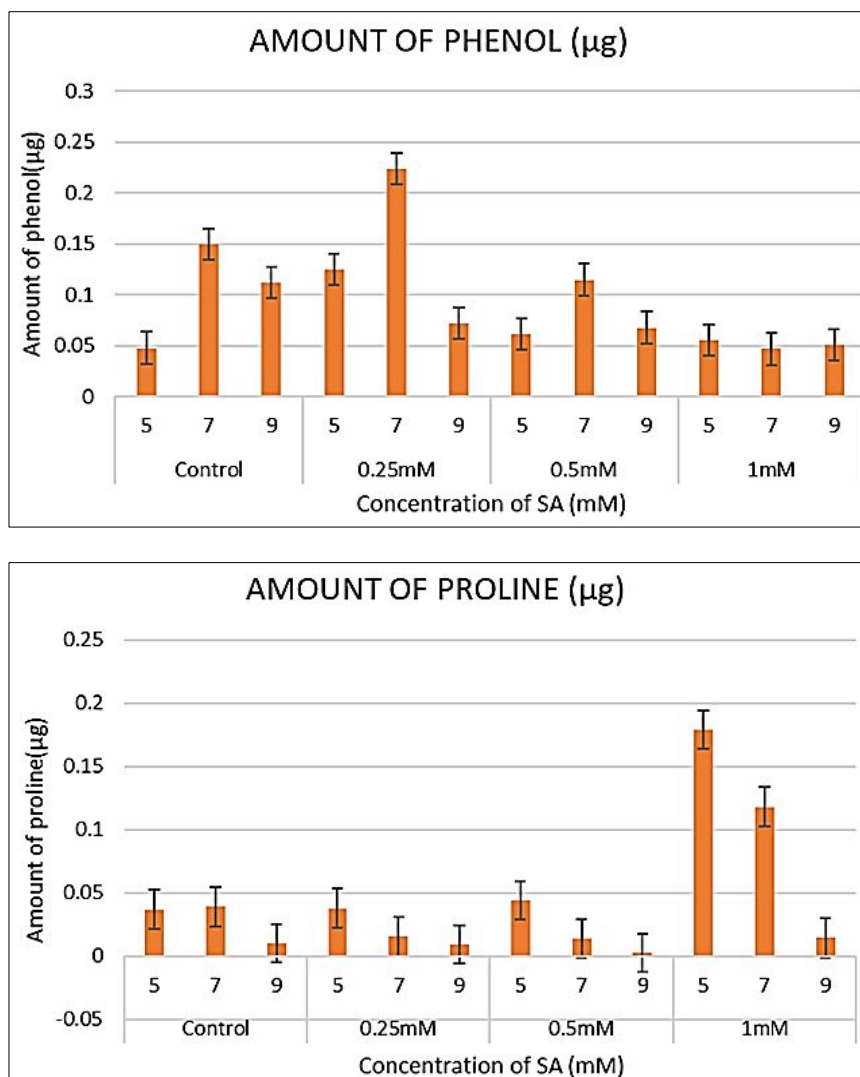
Concentration of Salicylic acid (mM)	Weeks	Stomata Index (%)	Trichome Index (%)
Control	7	30.7	11
	9	34.69	12.23
0.25mM	7	31.78	8.11
	9	32.47	9.02
0.5mM	7	31.55	9.53
	9	32.35	10.45
1mM	7	30.89	11.84
	9	32.33	12.5



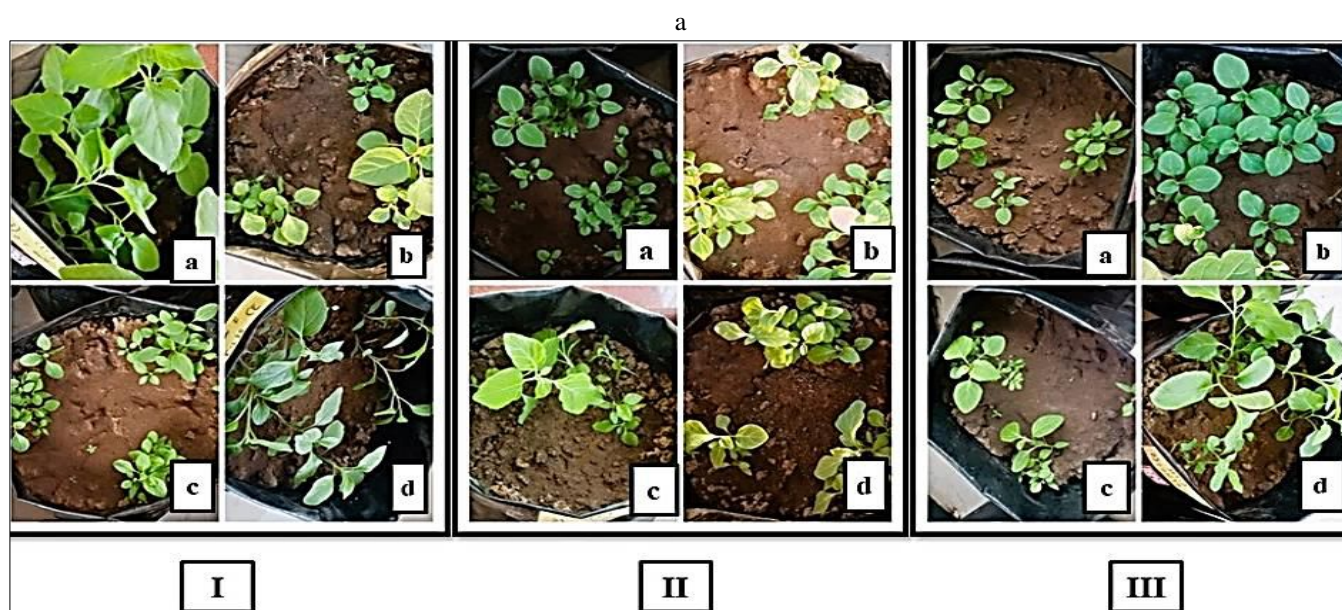


Graph 1: (A) Effect of Salicylic acid on No. of leaves; (B) Effect of Salicylic acid on Shoot length; (C) Effect of Salicylic acid on Root length; (D) Effect of Salicylic acid on Biomass



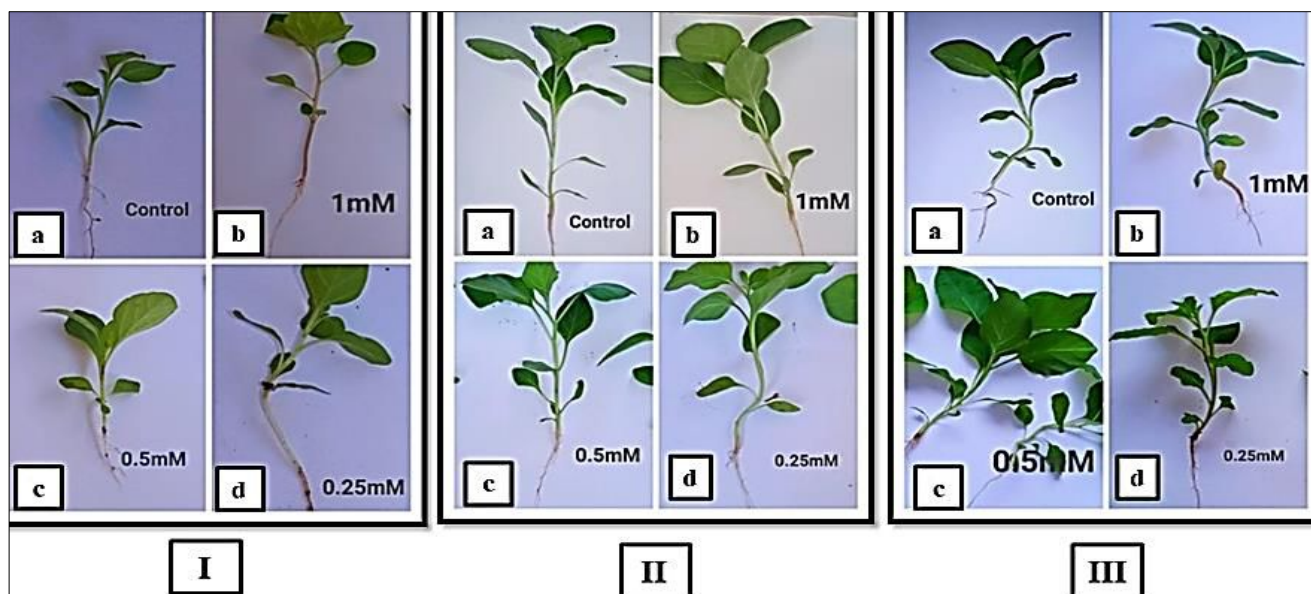


Graph 2: (A) Effect of Salicylic acid on leaf area; (B) Effect of Salicylic acid on Chlorophyll content; (C) Effect of Salicylic acid on Phenol amount; (D) Effect of Salicylic acid on Proline amount



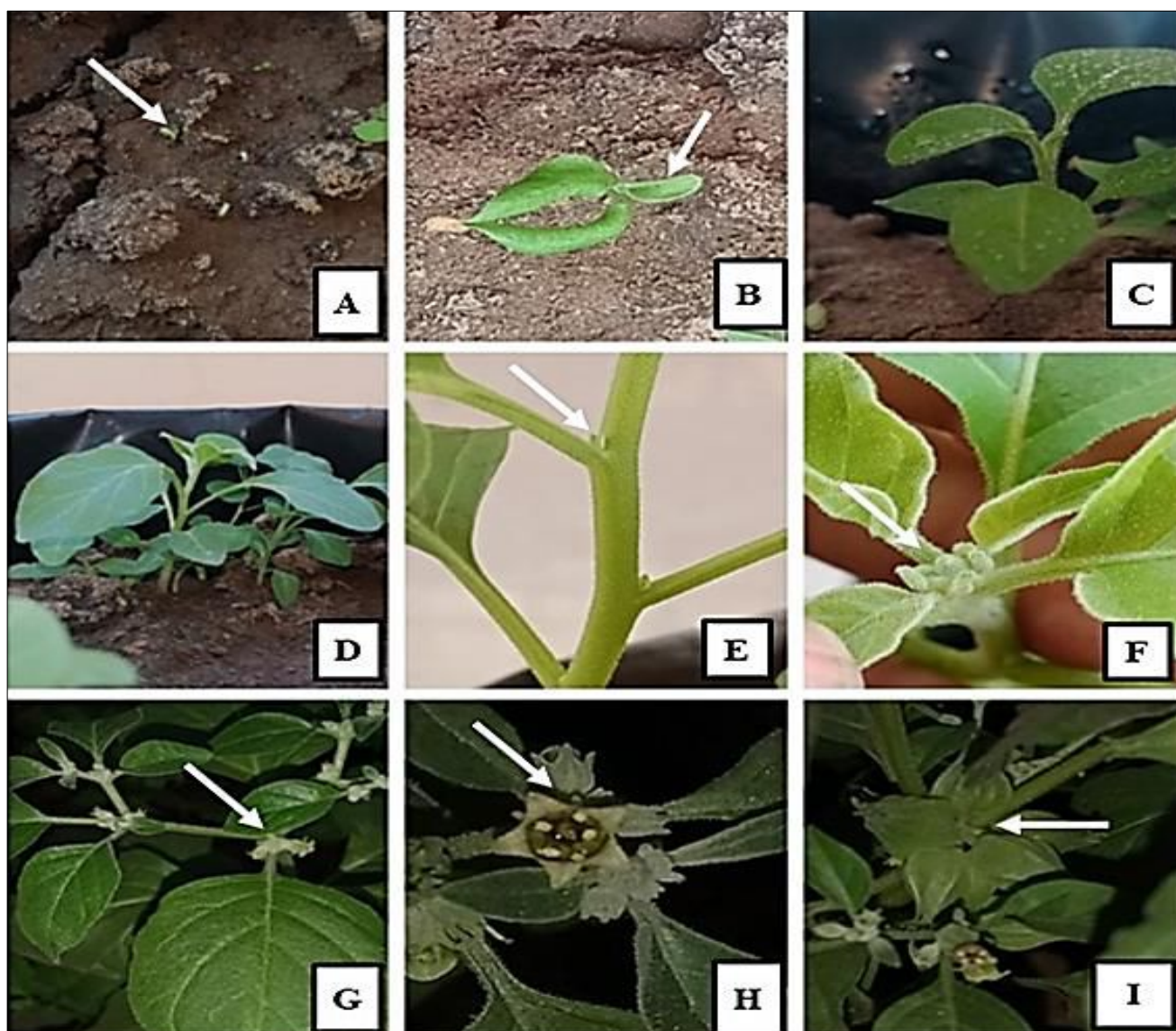
(I) 1st harvest (5th week), (II) 2nd harvest (7th week), (III) 3rd harvest (9th week). a - control condition; b - 0.25 mM concentration; c - 0.5 mM concentration; d - 1 mM concentration.

Fig 3: Effect of SA on seeds of *Withania somnifera* during growth.



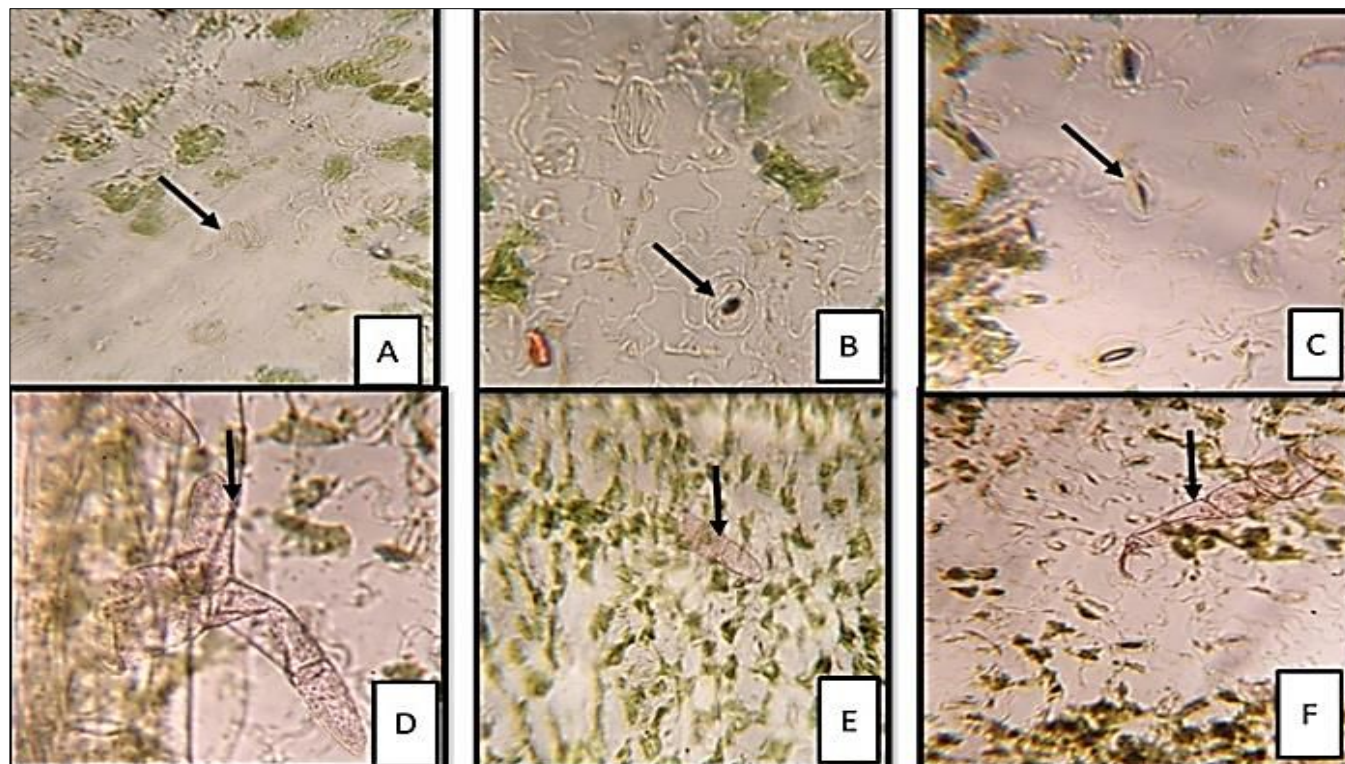
a - control condition; b - 1 mM concentration; c - 0.5 mM concentration; d - 0.25 mM concentration (I) 1st harvest (5th week), (II) 2nd harvest (7th week), (III) 3rd harvest (9th week)

Fig 4: Seedlings of *Withania somnifera* at different concentrations during growth



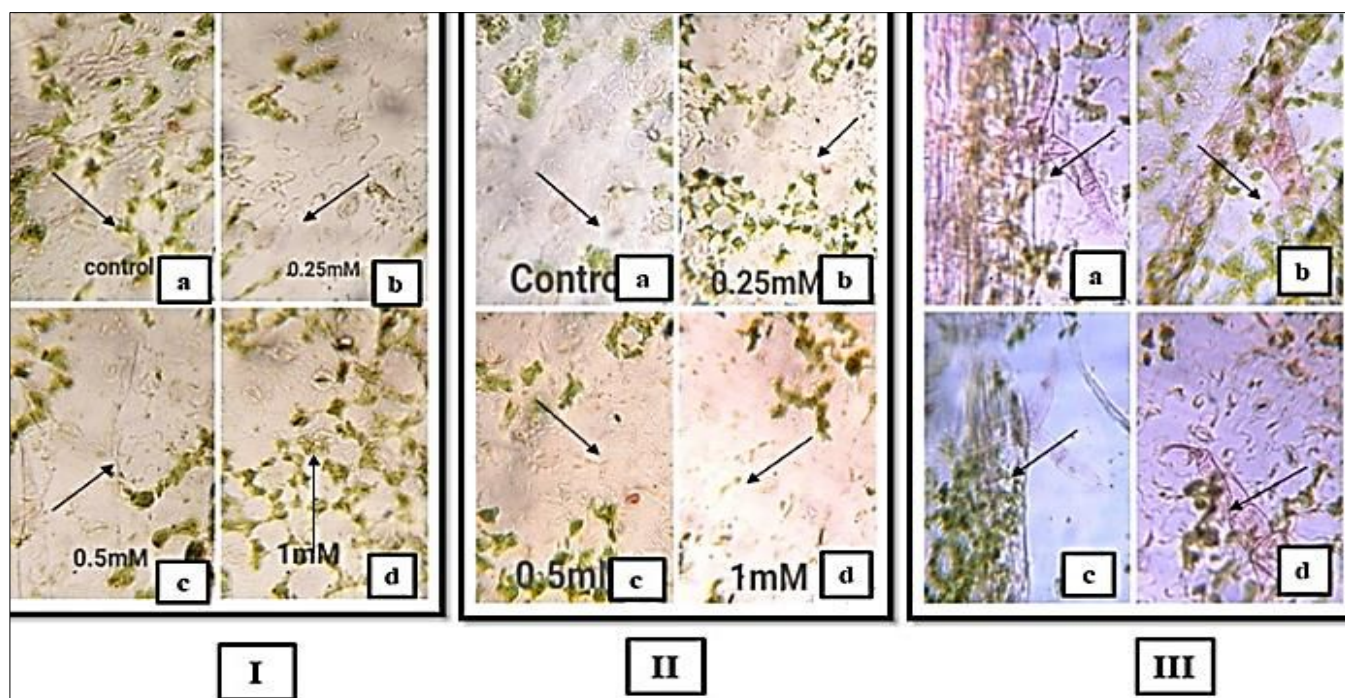
A - sprouting of seedlings (arrow), B - cotyledons of seeds (arrow), C & D - developed seedling showing leaf development, E - small scaly leaves (arrow), F & G - initiation of flower bud (arrow), H - flower (arrow), I - fruit (arrow)

Fig 5: Effect of SA on *Withania somnifera* as a growth regulator



(A) anomocytic stomata (arrow), (B) and (C) open and closed stomata, (D), (E), and (F) multicellular, branched, and unbranched types of trichomes

Fig 6: Types of stomata and trichomes



(I) 7th week; (II) 9th week; (III) different types of trichomes. a - control condition; b - 0.25 mM concentration; c - 0.5 mM concentration; d - 1 mM concentration.

Fig 7: Effect of SA on stomata and trichomes of *Withania somnifera* under different concentrations.

Conclusion

Salicylic acid is a great plant hormone which performs a good defense reaction in plants. In the present study, based on the result, we can conclude that exogenous supplements of salicylic acid on seeds were effective in stimulation of morphological characters and secondary metabolites like chlorophyll, proline and phenol etc. production.

Salicylic acid influence leaf chloroplast structure and chlorophyll amount. It also affects the proline, leads to adjustments in osmotic pressure and improves the root system. The major role of proline as a scavenger and improving growth character.

0.5mM concentration of salicylic acid shows interesting results, which stimulate least effect on plant growth and production of secondary metabolites also in high amounts.

Number of leaves, root and shoot length, biomass, leaf area like morphological characters and chlorophyll, proline and phenol like secondary metabolites are increased at 0.5mM concentration of salicylic acid. So, can conclude that 0.5mM if salicylic acid is more suitable for the growth of the *Withania somnifera*.

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