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# INFLUENCE OF SALICYLIC ACID ELICITATION ON SECONDARY METABOLITES AND BIOMASS PRODUCTION IN *IN-VITRO* CULTURED *WITHANIA COAGULANS* (L.) DUNAL

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## Abstract

*Withania coagulans* is an important medicinal plant possessing several secondary metabolites collectively termed as withanolides. Salicylic acid is widely known to stimulate plant growth and production under different conditions. Therefore, the present study was conducted to determine how SA regulates secondary metabolites and biomass production in tissue culture raised seedlings of *W. coagulans*. Among all the four treatments, exogenously supplemented salicylic acid at 150 and 200 µM concentrations exhibited maximum potential to modulate growth and physiological processes of the seedlings compared to 50 and 100 µM treatments. Exogenous application of SA at 150 and 200 µM significantly improved photosynthetic pigment contents, increased biomass, enhanced secondary metabolite contents *viz*., phenol, proline, and anthocyanin and strengthen antioxidative defense system for scavenging reactive oxygen species. Findings of the present study illustrated that exogenous supplementation of salicylic acid-regulated the mRNA level of genes involved in secondary metabolite biosynthetic pathway thus enhancing the production of the secondary metabolite, photosynthetic efficiency, improved plant growth and increased plant biomass of tissue culture raised seedlings of *W. coagulans.*

***Keywords*:** Salicylic acid, *Withania coagulans*, Secondary metabolites, Biomass.

## Introduction

Various plants with medicinal potential have been reported to be useful to cure several health problems and diseases (Mukherjee *et al.,* 2006) thus playing an important role in the development of new herbal drugs. *Withania coagulans* Dunal which belongs to family Solanaceae is commonly known as punir bundh (Indian cheese maker) is one of the important medicinal plants whose health benefits are well documented in ancient Ayurvedic and naturopathic documents (Naz *et al*. 2009; Jain *et al*., 2012). In the ancient era, the plant was extensively used by tribal peoples for its milk coagulating properties and in the recent decades, it has been extensively used by modern communities to cure various health problems and diseases such as ulcer, dyspepsia, rheumatism, dropsy, consumption and sensible debility (Hemalatha *et al*., 2008). Additionally, it is also a natural source of withanolide (steroidal lactones) which is widely used as antitumor (Senthil *et al*., 2007), antimicrobial (Choudhary *et al*., 1995), and in the treatment of asthma, biliousness and nervous breakdown (Jain *et al*., 2009). The pharmaceutic properties of *W. Coagulans* plant is due to presence of a large number of steroidal alkaloids, fatty acids and lactones known as withanolides (Jain *et al*., 2012) which are synthesized via two independent pathways *viz.,* mevalonate pathway localized in cytosol and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastid where squalene is believed to be a metabolic intermediate for biosynthesis of diverse triterpenoids. The biosynthesis of these secondary metabolites has been reported to be stimulated under several biotic and abiotic stress conditions as well as with the application of phytohormones *perse.,* salicylic acid and jasmonic acid (Sivanandhan *et al*., 2013; Jacob *et al*., 2014).

Recently, *W. Coagulans* have been enlisted as "critically endangered plant" as the direct result of overexploitation by pharma industries causing destruction of its natural habitat, low seed germination, poor reproductive rate, and seed set, and frequent use as a fodder for animals has caused significant constraint in its reproduction and isolation of secondary bioactive metabolites (Jain *et al*., 2016; Thomas and Hoshino, 2016; Rathore and Kheni, 2017). Consequently, *In-vitro* technique has become imperative for rapid mass propagation using various explants such as shoot tips, leaf discs and inter-nodal parts for its conservation and mass production of bioactive medicinal compounds (Valizadeh and Valizadeh, 2011; Rathore *et al*., 2012).

Salicylic acid (SA) is endogenously produced in the plants and an important plant growth regulator which playa key role in improving plant growth and defense against environmental cues. Salicylic acid plays a significant role in the regulation of plant growth and

developmental processes via modulating signaling networks associated with secondary metabolites biosynthesis pathway (Miura and Tada, 2014). SA is also involved in the regulation of several physiological processes such as stomatal regulation, germination, photosynthesis, ion uptake, production of proline and glycine betaine (Khan *et al*., 2003) thereby improving plant tolerance to several biotic and abiotic stress conditions (Karlidag *et al*., 2009). Apart from its role in plant growth, SA also stimulates regulation of stress-responsive genes and proteins to improve plant tolerance to major abiotic and biotic stresses (Khan *et al*., 2014; Nazar *et al*., 2015). Extensive studies have reported that exogenous application of SA to plants either by seed soaking, irrigation, foliar spray or adding in nutrient solution led to induction of plants secondary metabolite production (Khan *et al*., 2012, 2013; Anwar *et al*. 2013; Palma *et al*., 2013). SA is known to regulate plant functions in a dose-dependent manner, causing induction or inhibition of plant vital functions respectively. For example, 50 and 250 µM of SA has promoted and inhibited the growth of *M. chamomilla* plants (Kovacik *et al*., 2009). In another instance, 100 and 500 µM of SA promoted the photosynthesis and secondary metabolites production in *Vigna radiata*, however, inhibited growth was observed at 1.0 mM concentration (Nazar *et al*., 2011). Recently, several molecular studies have confirmed that SA can potentially regulate many aspects of plant growth, secondary metabolite productions and defense response thereby improving plant growth and productivity under adverse conditions (Jumali *et al*., 2011).

However, there is little or no information present regarding the effect of exogenous SA on growth, biomass, secondary metabolite production and induction of genes involved in the secondary metabolite biosynthesis pathway of tissue culture generated *W. coagulans* plants. Therefore, the objective of the present research was to evaluate the effect of exogenous SA in the improvement of plant growth and biomass, oxidative stress indicators and induction of genes involved in the secondary metabolite pathway.

## Material and Methods

### Plant Material and Growth Conditions

The seeds of *W. coagulans* were sown in tray containing sterilized sand for germination under dark condition at 25 ± 1 ºC for one week in Laboratory of Morphogenesis at Department of Botany, Institute of Science, Banaras Hindu University, Varanasi. The explants were collected from twenty-one days old seedlings which were surface sterilized using 4-6 drops of tween-20 in 100 ml distilled water for 10 min followed by re-washing in distilled water. Additionally, the explants were disinfected with 70% alcohol for 30s and 0.1% (w/v) mercuric chloride ($HgCl_2$) for 3 min followed by washing with double distilled water (5-6 times) under the aseptic condition of laminar air flow.

### Preparation of explants and salicylic acid treatment

The sterilized explants were cultured in liquid basal MS medium (Murashige and Skoog, 1962) supplemented with 3.0% (w/v) sucrose containing varying levels of SA (50, 100, 150 and 200 µM) replicated thrice with 10 explants in each replication. Cultures were maintained in a culture room at 25 ± 2 °C under 16-h photoperiod (40 mol $m^{-2}s^{-1}$) provided by white fluorescent lamp. Samples for various morphological, biochemical and molecular studies were collected on the 15$^{th}$ day after the completion of the treatment frozen immediately in liquid $N_2$, and kept at -80 °C for further analysis.

### Measurement of chlorophyll and carotenoid contents

The chlorophyll *a* and *b* (Chl), and carotenoid (car) contents were estimated by extracting 300 mg leaf sample with 80% v/v acetone. The mixture was then centrifuged at 7000 rpm for 15 min and the absorbance of aqueous supernatant was recorded at 663, 645, 480 and 510 nm (Porra *et al*., 1989) with a UV–visible spectrophotometer (Perkin Elmer, elico Ltd. China) and the content were estimated as per the equation given by Arnon (1949) and expressed as mg $g^{-1}$FW.

### Determination of total phenol

Estimation of phenol was done following the standard protocol given by Imah and Khokhar (2002). Approx. 200 mg of leaf tissue was heated with 10 mL of 1.2 M HCl in 50% aqueous methanol for 2 hours at 90 ºC. 20 µL of sample (or standard) was taken and mixed with 1.58 ml water and 100 µL Folin- ciocalteu reagent was allowed to stand for 2 min, then mixed with 300 µL of 1.9 M sodium carbonate and incubated at 40 ºC for half hour. The absorbance was measured at 765 nm (Perkin Elmer, elico Ltd. China) and the concentration was expressed as mg $g^{-1}$ FW.

### Determination of anthocyanin content

The anthocyanin content was measured as per the method described by Wrolstand *et al*. (1982). Approx. 1 g of leaf extract was extracted in the solution of 0.05% HCl in methanol at 4 ºC for 12 h. The extract was then centrifuged at 10,000 rpm for 20 min and transferred to a fresh tube. 1 ml of supernatant was then mixed with 4 ml of 0.4 M potassium chloride and the absorbance of the resulting supernatant was measured at 510 and 700 nm. The content of anthocyanin in the leaf samples was calculated as per the standard equation given by Romero

*et al*. (2008) and expressed as mg $g^{-100}$ cyanidin-3-O-glucose equivalent.

**Estimation of proline content**

Estimation of proline content in leaves was done as described earlier (Bates *et al*., 1973). 500 mg leaves were crushed in 5 ml of 3% of sulfosalicylic acid and centrifuged at 12000×g for 10 min. The mixture was then centrifuged at 8000 rpm, resulting supernatant was then mixed with toluene (4 ml) and incubated at 100 °C for 1 hr. The absorbance of chromophore was recorded at 520 nm and the amount of proline in the samples was expressed as µg $g^{-1}$FW.

**Histochemical detection of superoxide anion radical**

The generation of superoxide radicals on leaves was detected by staining with nitro blue tetrazolium chloride (NBT) as described by Rao and Davis (1999) with some modifications. Leaves were incubated in 5 mM NBT solution for 8 h under yellow light at 25 ºC. After the appearance of blue (NBT staining) samples were kept in 70% ethanol to remove chlorophyll and picture was captured using computer attached Dewinter image microscope.

**Measurement of biomass, leaf area and fluorescence ratio**

The fresh and dry biomass (after drying at 80 °C) of Salicylic acid treated and untreated plant samples were taken after 15 days of treatment using digital balance(BL60S, Sartorious). Leaf area was measured using a Li-3000A portable area meter (LI-COR, NE, USA) from fifteen completely developed leaves and expressed as leaf area ($cm^2$). The ratio of fluorescence variable and fluorescence maximum (Fv/Fm) indicates damage caused to photosystem II which was measured by Hansatech Handypea (USA) meter on the fully expanded leaf after a 30-min dark adaptation.

**RNA isolation and cDNA preparation and Quantitative PCR analysis**

Total RNA from treated and non-treated samples were isolated using TRIZOL reagent (Invitrogen) following the manufacturer's recommendations. The quality of isolated RNAs were assessed on agarose gel electrophoresis (1.0%) and strength were assessed by measuring their absorbance ratio at 230/260/280 nm. The first stand of cDNA (1.0 µg of total RNA) were synthesized using iscript$^{TM}$ cDNA synthesis kit (Bio-Rad Laboratories, USA) following manufacturer's instructions. Semi-Quantitative PCR analysis (qRTPCR) were performed using genes involved in secondary metabolite pathway (Table 1) in thermocycler (BioRad Laboratories, USA) with the following steps-initial denaturation at 95 °C for 10 min superseded by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The intensities of qRT-PCR amplified genes were analysed in 2% agarose gel with Gel-DOC EZ imager (Bio-Rad) using Quantity One software (Bio-Rad).

**Statistical Analysis**

All the experiments were repeated thrice under the same condition and all the analysis were performed in three biological replicates. Error bars on the graph shows the standard error of mean value (±SE) and subjected to analysis of variance (ANOVA). The mean differences were compared using SPSS software (SPSS Inc., Version 20.0) and values at $P \leq 0.05$ were considered significant.

## Results and Discussion

**Effect of salicylic acid on chlorophyll and carotenoid contents**

The present study revealed how exogenous supplementation of salicylic acid in nutrient media may help to improve *W. coagulans* growth and secondary metabolites production by increasing photosynthetic pigments, biomass, and antioxidant defense. In the present study, exogenous supplementation of SA at different concentrations significantly improved chlorophyll and carotenoid contents of *W. coagulans* seedlings compared to non-treated seedlings (Fig. 2a and 2b). Both chlorophyll and carotenoid contents increased upon exogenous application of SA. However, the contents did not significantly increase by SA (50 µM) supplementations. Highest chlorophyll and carotenoid contents about 20-50% was recorded in those seedlings which were treated with higher concentration SA i.e. 100, 150 and 200 µM (Fig. 2a and 2b). Dose-dependent effect of salicylic acid on several plant functions have been previously reported by many researchers where both stimulatory and inhibitory effect are possible with low and high concentrations of SA (Nazar *et al*., 2011). 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.

**Effect of salicylic acid on anthocyanin and phenol contents**

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). In the present study, exogenous

application of SA at a varying concentration significantly increased secondary metabolites *viz*., anthocyanin and phenol contents in tissue cultured seedlings of *W. Coagulans* compared to non-treated counterparts (Fig. 2c and 2d). A maximum significant increase (50-200%) in anthocyanin and phenol contents was observed in those seedlings which were treated with SA at 150 and 200 µM compared to control seedlings. However, seedlings treated with SA at 50 and 100 µM also showed improved contents of secondary metabolites but the increase was inferior as compared to the effect of SA applied at higher concentrations. 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). Elicitation in anthocyanin and subsequent increase in phenol content have been reported in SA-supplemented *C. chinense* (Rodas-Junco *et al*., 2013). In another instance, exogenous application of SA at 100 and 150 µM led to the induction of secondary metabolites such as coumarins, sterols and phenols in *S. Glauca* (Awate and Gaikwad, 2014). These findings are in accordance with the results of the present study.

**Effect of salicylic acid on proline content**

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). Researchers have well reported that exogenous application of salicylic acid can stimulate proline biosynthesis under adverse condition (Khan *et al*., 2013). In the present research, exogenous supplementation of salicylic acid at different concentration remarkably increased proline metabolism in all the treated seedlings compared to non-treated controls (Fig. 2e). However, control and seedlings treated with salicylic acid (50 µM) showed similar levels of proline whereas maximum increase (100-200%) in the proline content was observed in the seedlings treated with 150 and 200µM of salicylic acid (Fig. 2e). Foliar application of SA (0.2 mM) significantly upregulated the activity of proline biosynthesis enzyme thereby increasing proline content and improved salt tolerance of *Lens esculenta* plants (Misra and Saxena, 2009). Similar results have also been reported by Khan *et al.* (2013) where they have confirmed that exogenous application of SA (0.5 mM) alleviated the toxic effect of heat stress by inducing proline biosynthesis in wheat plants, which also corroborates the result of the present study.

**Effect of salicylic acid on photosystem II, biomass and leaf area**

SA 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 Tada, 2014). A plethora of researches has shown the role of exogenous SA in enhancing photosynthesis and other physiological processes in various crops (Asger *et al*., 2015). Similarly, in the present study, exogenous supplementation of SA significantly improved the activity of photosystem II by 40-60% in all the treated plants compared to control (Fig. 2f). Highest Fv/Fm ratio was observed by the seedlings treated with 150 and 200 µM. Identically, total biomass (Fig. 2g) and leaf area (Fig. 2h) were also collaterally increased in all the treated plants from 12.5-50% and 5.4-37.5%. Maximum increase in the total biomass and leaf area was observed in SA 150 and 200 µM treated seedlings compared to their respective control (Fig. 1). Studies have revealed that exogenous supplementation of SA positively regulate plant physiological functions to improve their growth and survival under adverse conditions. SA-mediated improved growth of *V. vinifera* and wheat has been advocated as a result of SA induced accumulation of plant biomass and increased activity of photosystem II (Li *et al*., 2013).

**Effect of salicylic acid superoxide anion generation**

The generation and scavenging of reactive oxygen species such as hydrogen peroxide, superoxide radical and hydroxyl radical are an important process of aerobic metabolism. At the basal level, these ROSs form an integral component of various signal transduction pathways thereby regulating/modulating plant functions to varied conditions (Gill and Tuteja, 2010). However, when their level is increased beyond the threshold value, can result in the generation of oxidative stress which may severely arrest plant growth, block the functioning of vital biomolecules causing cell death ultimately affecting plant growth and development (Anjum *et al*., 2012). Keeping the above facts in mind, accumulation of superoxide anion in the leaves of SA treated and non-treated *W. Coagulans* seedlings were analyzed by staining them NBT (Fig. 3). Exogenous supplementation of SA resulted in a significant increase in superoxide anion generation in all the treated seedlings. Maximum superoxide generation was observed in those seedlings which were treated with a higher level of salicylic acid i.e. 150 and 200 µM compared to 50 and 100 µM treated seedlings as well as control seedlings. Exogenous supplementation of SA has been reported to play compelling roles in the

stimulation of antioxidant defense system to have stringent control over ROS (Khan *et al*., 2014) and increased level of superoxide anion radical observed in present study may have been due to modulation of SA dependent ROS signalling to stimulate an appropriate growth response (Kang *et al*., 2013).

**Effect of salicylic acid on modulation of mRNA level**

Salicylic acid-mediated differential modulation of the transcript level of the genes involved in secondary metabolite pathway and other defense signaling pathways have been extensively advocated to regulate antioxidant metabolism which results in the development of systemic acquired resistance involved in the regulation of the complex process of plant growth and development (Csiszar *et al*., 2014). In the present study eight genes involved in secondary metabolite synthesis pathway *viz.,* 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase1*(HMGR1),*1-deoxy-D-xylulose-5-phosphate synthase 1*(DXS1),*Farnesyl diphosphate synthase *(FPPS1),* Squalene synthase *(SQS),* Selenocysteine methyltransferase *(SMT),* Sinapate: UDP-glucose glucosyltransferase *(SGT),* obtusifoliol-14α-demethylase *(ODM)* and Ribosomal Protein S9 *(RPS9)* were quantified using reverse transcriptase PCR in SA treated and non-treated plants (Table 1). The mRNA levels of *DXS1, FPPS1, SQS, SMT, SGT,* and *ODM* genes exhibited concentration-dependent increase in both SA 150 and 200 µM treated seedlings compared to control, SA 50 and 100 µM treated seedlings (Fig. 4a and 4b). However, other genes *viz., FPPS1* and *RPS9* responded similarly with respect to control i.e. the mRNA level were minimum as observed in control seedlings without any supplementation of SA. Several studies have reported that exogenous supplementation of SA significantly modulate mRNA levels of several genes involve in defense and secondary metabolite biosynthesis pathway, thus strengthening plant's growth and innate immunity under biotic and abiotic stress (Nazar *et al*., 2015).

## Conclusion

On the basis of above results, along with the results present in available literature, we, therefore, conclude that exogenous supplementation of salicylic acid was effective in stimulation of secondary metabolites production, which could be partially attributed by the increase in photosynthetic pigment contents and ROS scavenging capacity as well as improved plant growth and biomass. Although several reports have indicated the exogenous application of salicylic acid is able to enhance secondary metabolite production by modulating the expression of genes involved in the biosynthetic pathway, however to date research conducted to enhance secondary metabolites production upon exogenous application of SA is scarce. Therefore, further studies are required to elucidate the signaling pathways and molecular mechanisms underlying the role of SA in the stimulation of secondary metabolite productions in plants. Detailed analysis of the biochemical and molecular mechanisms of SA along with elucidation of signaling pathways would be helpful in engineering *W. coagulans* plants with improving withanolide contents.

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**Compliance with ethical standards**

Conflict of interest: The authors declare no conflicts of interest.

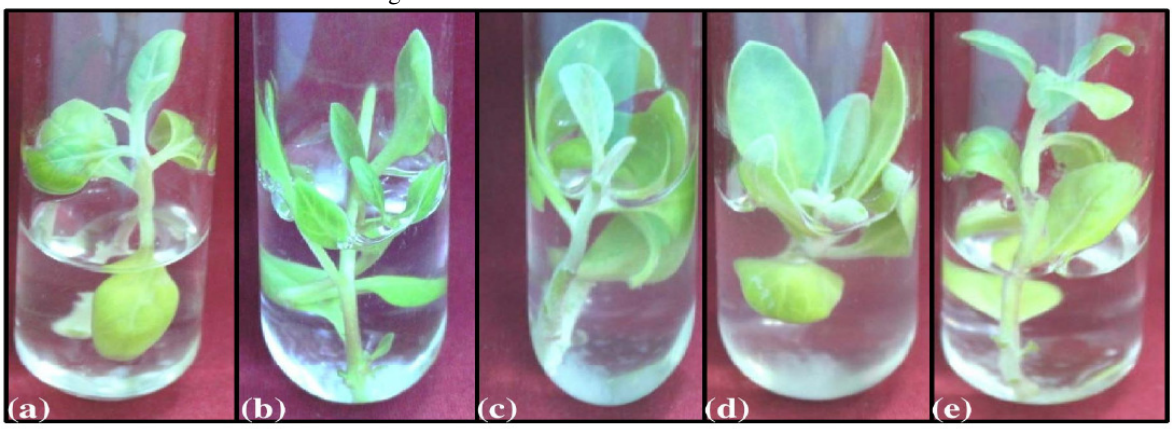


**Fig. 1 :** Improve growth and biomass in tissue cultured *W. coagulans* seedlings treated with different levels of salicylic acid. (a) Control, (b) SA 50 µM, (c) SA 100 µM, (d) SA 150 µM, and (e) SA 200 µM

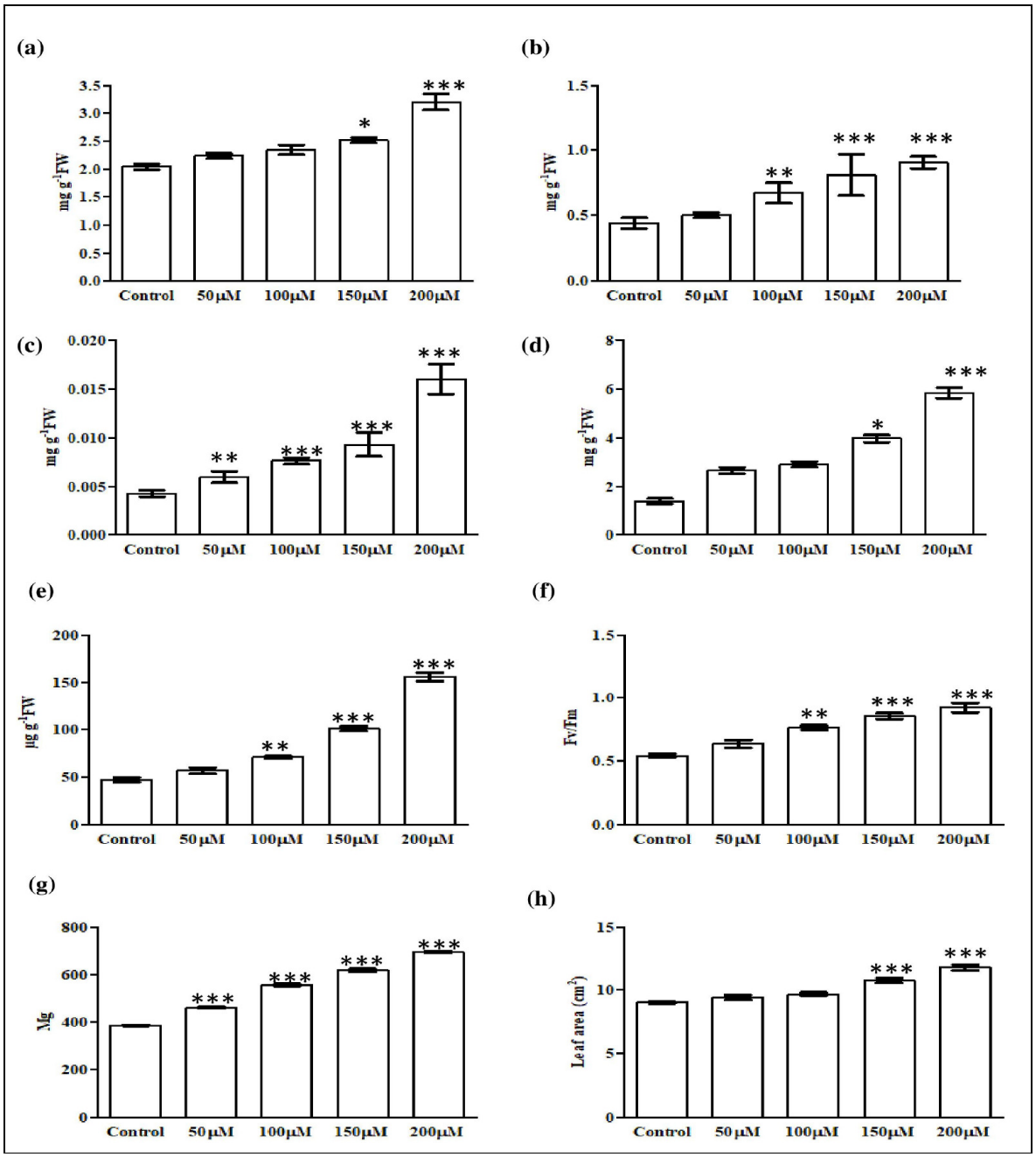


**Fig. 2 :** Effect of different levels of exogenous salicylic acid (SA) on (a) chlorophyll and (b) carotenoid(c) anthocyanin (d) phenol (e) proline (f) Fv/Fm (g) total biomass and (h) leaf area in tissue cultured *W. coagulans* seedlings treated with different levels of salicylic acid *viz*., Control, SA 50 µM, SA 100 µM, SA 150 µM, and SA 200 µM. Mean (±SE) was calculated from three replicates for each treatment. Bars with distinct asterisks are significantly different at $P \leq 0.05$.

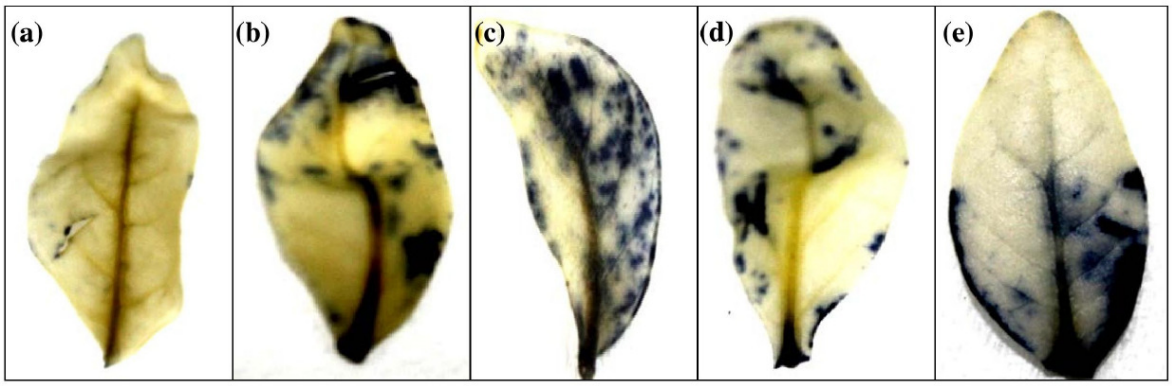


**Fig. 3 :** Effect of different levels of salicylic acid (SA) on (a) In-situ accumulation of superoxide ($O_2^{\bullet-}$) anion radicals by NBT staining in tissue cultured *W. coagulans* seedlings treated with different levels of salicylic acid. (a) Control, (b) SA 50 µM, (c) SA 100 µM, (d) SA 150 µM, and (e) SA 200 µM

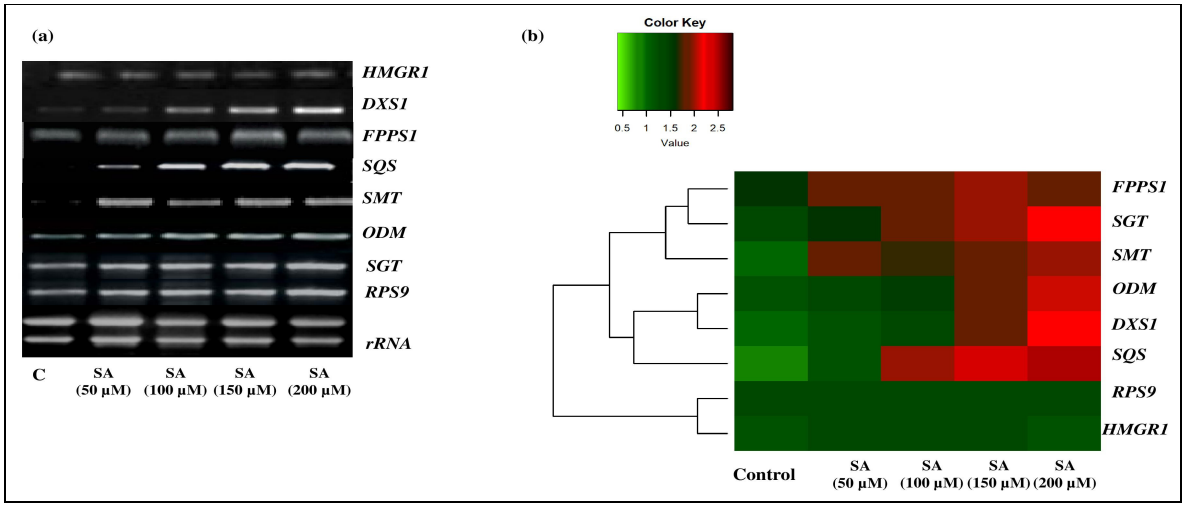


**Fig. 4 :** Effect of different levels of salicylic acid (SA) on (a) mRNA level of biosynthetic genes analyzed with semi-quantitative RT-PCR (b) Heat Map and clustering analysis of biosynthetic genes SA treated and non-treated *W. coagulans* seedlings. Color corresponds to the expression level of transcripts with low, intermediate and high expression represented by Dark green, red and dark red color respectively.

**Table 1:** Gene sequences used for quantification of mRNA levels by qRT-PCR

| S. No | Gene | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|---|---|---|---|
| 1. | HMGR1 | ACATAGCCGTCGTGGACCGTGT | TGATACAAAGCCACCTTAGATGGTTGC |
| 2. | DXS1 | CAATTTTCAATGTATCAAAGACTGGAGG | TTGGGAGCATTGAAGACATAAGAAG |
| 3. | FPPS1 | TACTGATGATGCTAGTGAATGGTCGAA | CACCAGCCGAGGGAAGATGTTTC |
| 4. | SQS | CTCGGTCAAGGCAGTCCAATGTCTC | CACCATACACATCTGCCATAGTCCGA |
| 5. | SMT | GCCTCGCGGGCAATTTCCACACATC | TTAGCCTCAAATCCCTGAATGGTGG |
| 6. | ODM | ATATATGGATCCATGGATCTCGCCGACATCCC | ATCGTCGGCGGCGGCCGCTTCTTG |
| 7. | SGT | ACNCAYTGYGGNTGGAAC | GTTCCANCCYCAYTGNGT |
| 8. | RPS9 | AGGAGGCGGTGTTTCAGGTC | TGTCAGGAAGCCAGCGTTTC |
| 9. | rRNA | AATTGTTGGTCTTCAACGAGGAA | AAAGGGCAGGGACGTAGTCAA |

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