Methyl jasmonate (MJ) and salicylic acid (SA) driven elicitation for enhanced production of curcuminoids from *in vitro* grown plants of Curcuma longa

Sandhya S., Swecha P., Bhuvaneswari Ch, Rao Kiranmayee and Giri Archana*
Centre for Biotechnology, Institute of Science and Technology, JNTUH, Kukatpally, Hyderabad – 85. Telangana State, INDIA
*archanagiriin@jntuh.ac.in

Abstract

Curcuma longa (turmeric) belonging to family Zingiberaceae is an important medicinal plant which has been used in the treatment of various ailments since time immemorial. C. longa is attributed with immense medicinal value owing to the presence of active constituents, the curcuminoids-a mixture of bisdemethoxy curcumin, demethoxy curcumin and curcumin (diferuloylmethane). The major pharmacological properties of these curcuminoids include antitumor, antioxidant, neuroprotection, antimicrobial, anticancer etc. Curcumin content of turmeric is greatly influenced by environmental factors and dearth of requisite high curcumin containing genotypes. Variations in the curcumin content at different agro climatic conditions, are major stumbling blocks in their commercial production. In this scenario, a promising new alternative sustainable system for plant secondary metabolite production would be plant cell culture based green chemical factories. One of the most effective biotechnological strategies for enhancing the production of secondary compounds from in vitro cultures is elicitation.

In the present study, the in vitro established plants of C. longa were elicited using methyl jasmonate (MJ) and salicylic acid (SA) for enhanced curcuminiod production. Based on the HPLC analysis, the content of curcuminoids was found to be best on the 54th day after culture. In the SA treated plants of C. longa, the accumulation of demethoxy curcumin (145.915µg.gDW^{-1}) and curcumin (145.506µg.gDW^{-1}) has been highest at concentrations of 200µM and 100µM respectively. Though, the accumulation of bisdemethoxy curcumin (90.114µg.gDW^{-1}) was much lower in comparison to the other two curcuminoids, the SA treatment was able to evoke 1.92 fold higher response than the MJ treated plants.

Keywords: *Curcuma longa*, Elicitation, Methyl jasmonate, Salicylic acid.

Introduction

Curcuma longa (turmeric) is a perennial plant from the Zingiberaceae family[25] distributed in various tropical regions of Asia. The *Curcuma* rhizome or root is the most useful part of the plant for culinary and medicinal purposes[29]. Phytochemical investigation of turmeric has revealed that it contains curcuminoids and volatile oils as the major components[20]. Curcuminoids are made up of three major phytochemicals – bisdemethoxy curcumin, demethoxy curcumin and curcumin responsible for various biological properties like antioxidant, antibacterial, hepatoprotective, antitumor, antiviral and anti-inflammatory activities[1,5,14,28].

Curcumin, as an anti-cancer agent is notable, as it can modulate tumour cell growth regulation through multiple cell signaling pathways[13]. Also, the antioxidant role of curcumin adds to the antitumor activity in keeping the DNA free from damage and peroxidation by free radicals[2,19].

Plant secondary metabolites extracted from various species are target specific and deemed to be safe for human consumption with negligible or no side effects. The yield of these secondary metabolites under normal conditions is very low and their chemical synthesis is highly uneconomical. Hence the possible approach for commercial production of therapeutically important secondary metabolites would be through use of plant tissue culture techniques *viz. in vitro* propagation, genetic transformation, suspension cultures, elicitation etc.

Elicitation is one of the most effective methods to improve secondary metabolite production in plant cell tissue and organ culture by using different stimuli - biotic, abiotic and signaling compounds[4,22]. Organ cultures have the advantage of tissue specific accumulation with high yield and hence organ cultures are preferred to avoid the major limitation of genetic instability and low product yield associated with undifferentiated cultures. The current study proposes the use of methyl jasmonate and salicylic acid for increasing the production of curcuminoids from *in vitro* grown plantlets of *C. longa*.

Material and Methods

***In vitro* plant establishment:** *C. longa* plant material was collected from CIMAP, Hyderabad. Rhizome buds were used as explants for initiation of aseptic cultures. Initially, the explants were washed thoroughly under running tap water for 30 min followed by washing with mild detergent. Later, the buds were treated with 0.2% (w/v) bavistin for 5 min followed by surface sterilization with 0.1% (w/v) mercuric chloride for 3 min. The explants were then thoroughly washed with sterile distilled water (8-10 times) and inoculated onto MS liquid media supplemented with

various combinations and concentrations of phytohormones (BAP, TDZ, Kn, NAA, IAA, IBA) and incubated under standard culture conditions (25 ± 2˚C temperature under a 16/8 h light/dark regime with 40 – 50 mol $m^{-2}s^{-1}$ light provided by the cool fluorescent tube light).

Growth kinetics: *In vitro* established plants of *C. longa* were further evaluated for biomass increase on MS liquid media fortified with different concentrations of sucrose (3, 6, 9% w/v). The growth of *C. longa* plants in MS liquid media was studied for a period of 60 days by taking a uniform shoot mass of 2g as initial inoculum. The increase in biomass was estimated at a regular interval of 5 days for a period of 60 days and expressed as gram fresh weight (gFW). Subsequently, the plantlets were used for extraction of secondary metabolites after excess moisture removal.

Elicitor treatment: In the present study, methyl jasmonate (MJ) and salicylic acid (SA) were used as elicitors for enhancing the production of curcuminoids from *in vitro* cultures of *C. longa*. The stock solution of elicitors was prepared at a standard concentration of 1mM in 50% ethanol and filter sterilized using 0.22µm membrane filter before its addition to the medium. MJ and SA from the stocks were added to MS liquid media at various concentrations *viz.* 50, 100, 150, 200 and 250µM and 50, 100, 200µM respectively.

Quantification of curcuminoids: The production of curcuminoids in *C. longa* was quantified at regular intervals along with the growth of *in vitro* grown plants. Elicitors were added to the media after 60 days of culture. After elicitor augmentation, the cultures are analyzed for biomass as well as curcuminoid contents at regular intervals of 24 hours for 4 days. Quantification of curcuminoids in the elicited and control plants was carried out by HPLC according to the protocol given by Kewu et al[10] in 2015. The extract samples were filtered using 0.22µm filter before being analyzed. HPLC analysis of curcuminoids was done using Schimadzu HPLC system, with UV-VIS detector equipped with C18 column. The mobile phase consisted of acetonitrile and water with 5% acetic acid with an absorbance maxima of 425nm. The injection volume was 20µl with a flow rate of 1ml. min^{-1}.

Results and Discussion

Aseptic cultures of *C. longa* were established from the rhizome bud explants on MS media supplemented with 2.5mg.L^{-1} BAP and 1.5mg.L^{-1} NAA (Fig.1). The response of rhizome buds in plant initiation was comparable to the other *Zingiberaceae sps*[11,24]. The importance of BAP and NAA in establishment of *C. longa in vitro* cultures in the present study was in accordance with the earlier reports[15,27]. The growth of *in vitro* plants was evaluated on MS media supplemented with different concentrations of sucrose (3, 6 and 9%) to study the biomass increase.

MS media supplemented with 6% (w/v) sucrose displayed highest biomass accumulation (17.32gFW) on the 55th day of incubation i.e. mid stationary phase (Fig. 2). The positive effect of sucrose at a higher concentration of 6% towards micropropagation was reported earlier in *Curcuma sps*.[8] The role of carbon source sucrose 6% (w/v) at higher concentration in enhancing the formation of aerial shoots and roots was studied[7] which is Important in the establishment of *in vitro* cultures from rhizome explants. Sucrose at a concentration of 9% (w/v) was found to be growth inhibitory. Cultures were harvested at regular intervals of 5days for a period of 60 days, after which the cultures reached decline phase.

Fig. 1: *In vitro* grown *plants of C. longa* on MS medium supplemented with 6% (w/v) sucrose

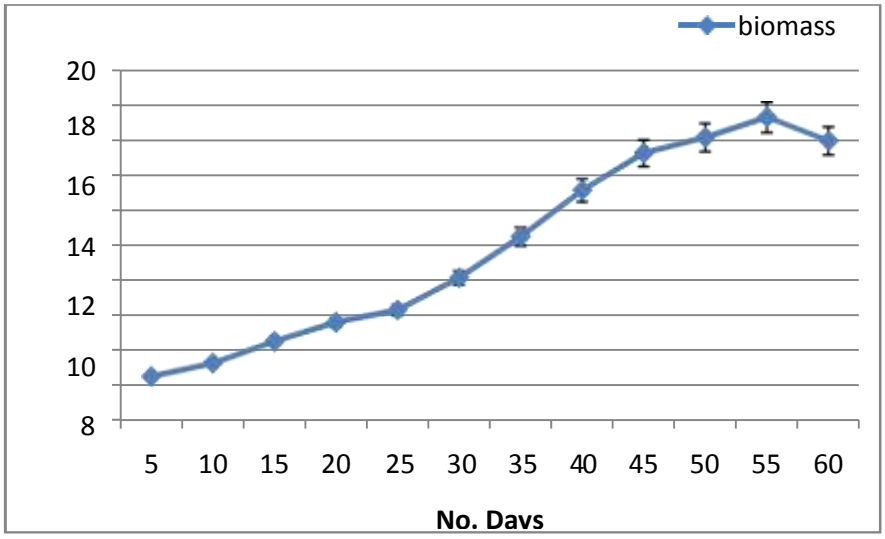

Fig. 2: Biomass increase in *C. longa in vitro* cultures on MS liquid media with 6% (w/v) sucrose

The curcuminoid content in the *in vitro* cultures of *C. longa* was analysed along with the increase in biomass. Based on the HPLC analysis, the content of curcuminoids was found to be best on the 55th day after culture, which is following the similar pattern as the growth. The curcuminoids i.e. bisdemethoxy curcumin, demethoxy curcumin and curcumin, were found to be 18.694 µg.gFW^{-1}, 9.81 µg. gFW^{-1} and 7.52 µg.gFW^{-1} respectively. Among the curcuminoids, the content of bisdemethoxy curcumin was found to be the maximum in comparison to the other two components. The results in this study were in contrast to the earlier findings[6,30] wherein curcumin was the major component under *in vitro* conditions. The variation in the obtained results could be dependent on the plant material source and geographical and climatic conditions.

Secondary metabolite production can be induced in various plant species under *in vitro* conditions by elicitor treatment with abiotic[4], biotic[9] and signaling molecules[4]. Phytochemical analysis of *in vitro* grown plants at different stages has revealed maximum curcuminoid accumulation on the 55th day of inoculation. Hence, the elicitors were added to the *in vitro* grown whole plants of *C. longa* on the 54th day of incubation in order to enhance the curcuminoid content.

Methyl jasmonate: MJ was added to 54 day old *C. longa in vitro* plants at varying concentrations (50 to 250µM). The effect of MJ for enhanced curcuminoid accumulation was analyzed for a period of 4days at 24h interval. Bisdemethoxy curcumin production was maximum (46.189µg.gDW^{-1}) at 250µM concentration after 24h treatment (Fig. 3). There was a 4.25 fold increase in the bisdemethoxy curcumin content in comparison to the untreated plants while the lowest production (4.241µg.gDW^{-1}) was observed at 250µM concentration of MJ after 96h.

Demethoxy curcumin production was highest (72.561µg.gDW^{-1}) after 24h treatment time with 200µM MJ concentration. The production was enhanced greatly (8.08 fold) when compared to the control (8.981µg.gDW^{-1}) (Fig. 4). Lowest amount (5.909µg.gDW^{-1}) of demethoxy curcumin was shown after 96h with 250µM concentration which was similar to the accumulation pattern of bisdemethoxy curcumin.

Maximum production of curcumin (25.628µg.gDW^{-1}) was observed at 72h with 200µM MJ concentration (Fig. 5). MJ treated plants have shown better production of curcumin content after 72h in comparison to 96h treated plants at similar concentrations of MJ.

Methyl jasmonate on the whole has a positive effect on enhanced production of curcuminoids with the best response for demethoxy curcumin followed by bisdemethoxy curcumin from the whole plants of *C. longa*. These findings have also indicated that higher concentrations of MJ have resulted in higher accumulation of demethoxy curcumin – the more stable curcuminoid equipped with antitumour properties[19]. Jasmonates play a key role during signal transduction process acting on the receptor-elicitor complex, consequently, leading to the triggering of enzymes responsible for the synthesis of secondary metabolites involved in the plant defense mechanism[18].

Methyl jasmonate induced accumulation of secondary metabolites in various plant systems has been reported e.g. triterpenoid saponins in *Bupleurum falcatum*[5], ginsenoside in *Pinax ginseng*[21], soyasaponins from *Glycyrrhiza glabra*[11] and asiaticoside from *Centella asiatica*[9].

Salicylic acid: Salicylic acid was added to 54 day old *in vitro* grown whole plants of *C. longa* at varying concentrations ranging from 50 to 200µM. The influence of SA on curcuminoid accumulation was analyzed at a regular interval of 24h for a period of 4 days (24 to 96h). A maximum of 90.114µg.gDW^{-1} bisdemethoxy curcumin at 200µM SA concentration was observed after 48h treatment (Fig. 6) which was 8.5fold higher in comparison to the untreated plants.

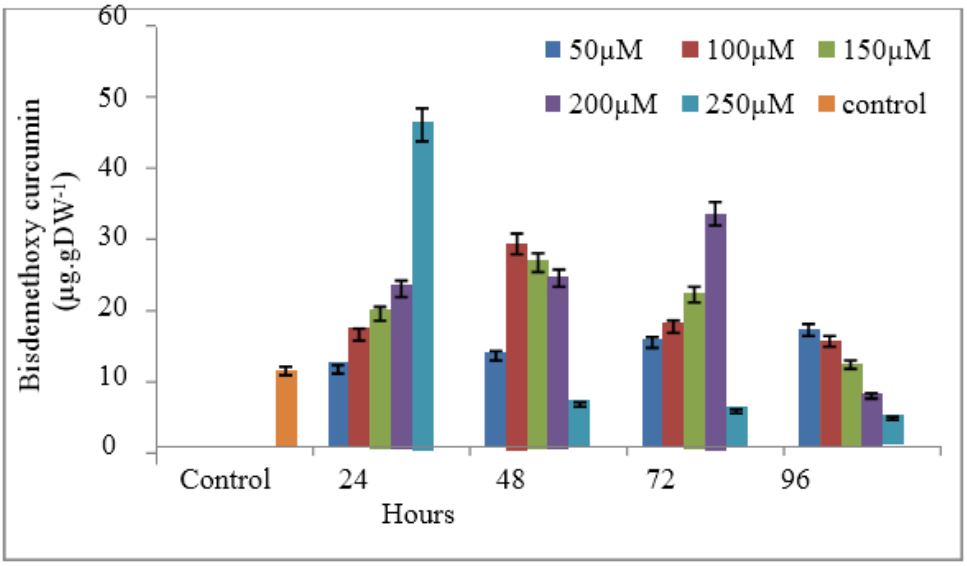

Fig. 3: Quantification of Bisdemethoxy curcumin in MJ treated plants of *C. longa*

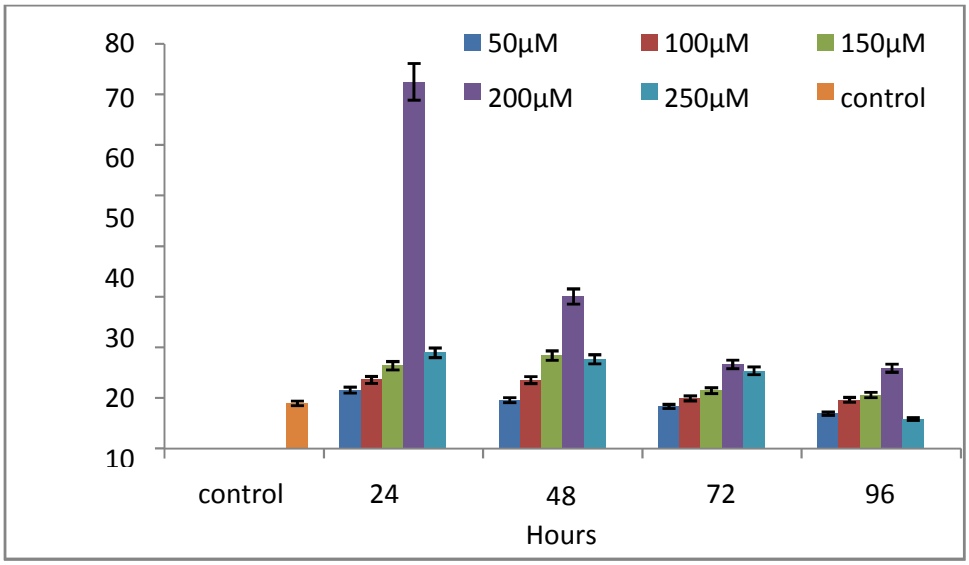

Fig. 4: Quantification of Demethoxy curcumin in MJ treated plants of *C. longa*

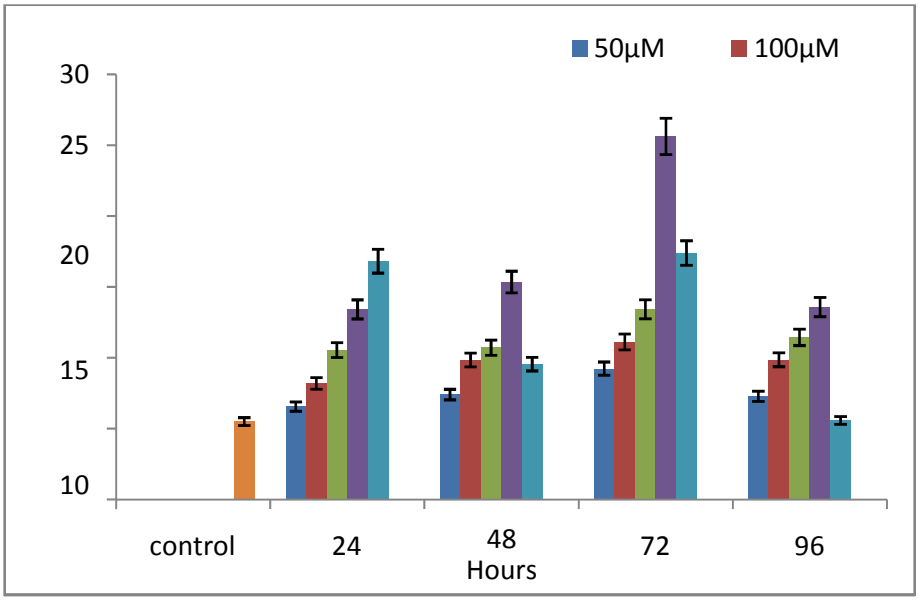

Fig. 5: Quantification of Curcumin in MJ treated plants of *C. longa*

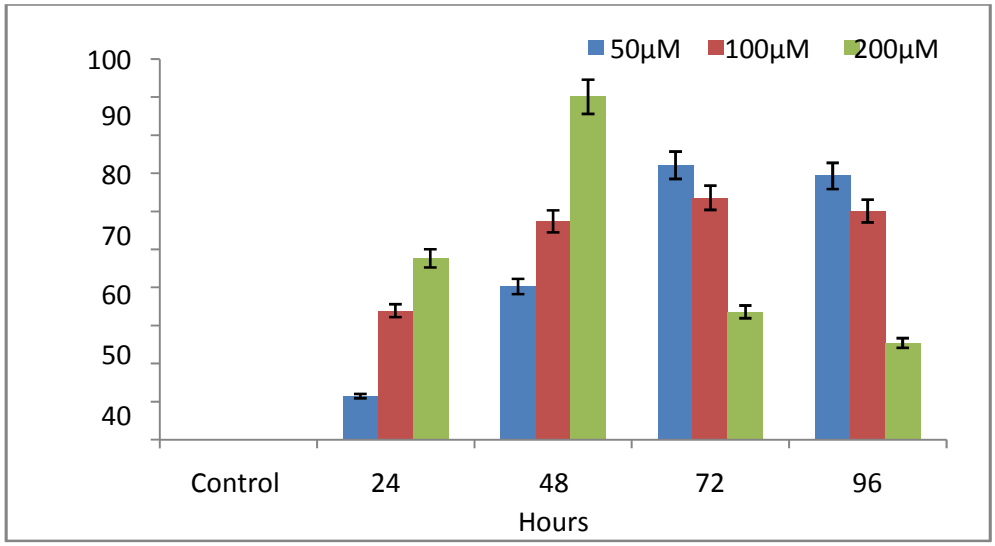

Fig. 6: Quantification of bisdemethoxy curcumin in SA treated plants of *C. longa*

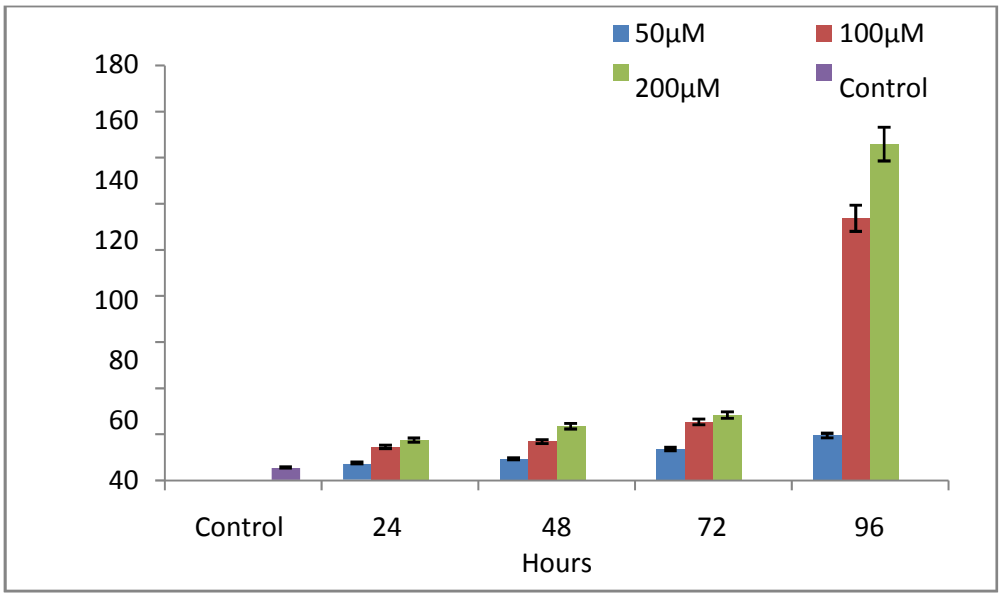

Fig. 7: Quantification of demethoxy curcumin in SA treated plants of *C. longa*

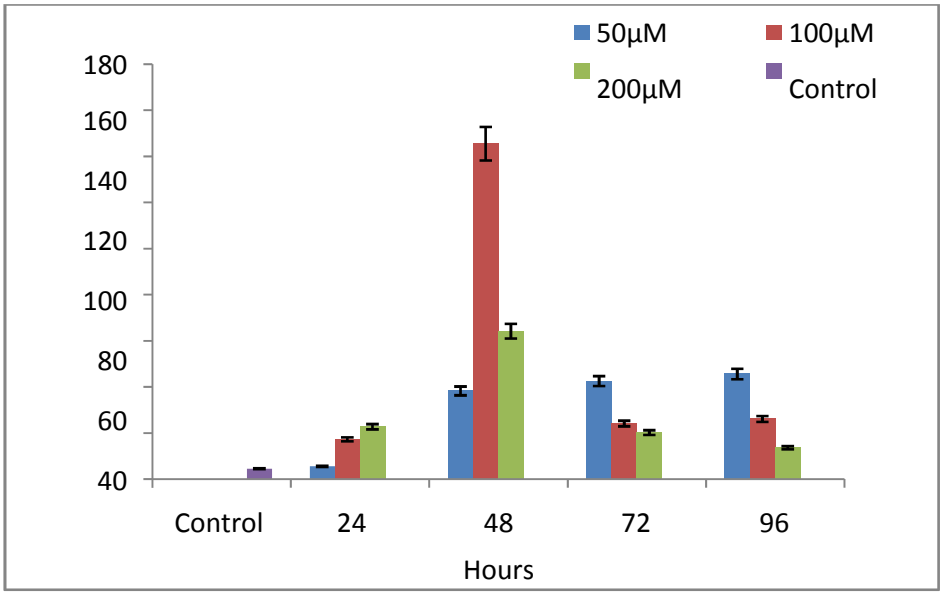

Fig. 8: Quantification of curcumin in SA treated whole plants of *C. longa*

The production was least at 200µM SA concentration with 25.41µg.gDW^{-1} after 96h of treatment. In case of demethoxy curcumin, the production was found to be highest (145.915µg.gDW^{-1}) after 96h treatment and concentration of 200µM SA, which is 25.5 fold higher than the content found in control plants (Fig. 7). Whereas for curcumin, the maximum production (145.506µg.gDW^{-1}) was recorded after a 48h treatment period at 100µM salicylic acid concentration (Fig. 8). The production was found to be 32.18fold higher in comparison to the control plants.

In the SA treated plants of *C. longa*, the accumulation of demethoxy curcumin (145.915µg.gDW^{-1}) and curcumin (145.506µg.gDW^{-1}) has been the highest at concentrations of 200µM and 100µM respectively. Though, the accumulation of bisdemethoxy curcumin (90.114µg.gDW^{-1}) was much lower in comparison to the other two curcuminoids, the SA treatment was able to evoke 1.92 fold higher response than the MJ treated plants. Salicylic acid is a well known inducer of plant defense metabolites by inducing the SAR (systematic acquired resistance) during the plant-pathogen interaction. However, SA induces gene expression related to the biosynthesis of a particular class of secondary metabolites. The elicitation studies in another *Zingiberaceae sps*, *Curcuma zedoaria* using SA have resulted in 2.31 fold higher accumulation of curcumin[21]. Recent evidences have suggested that it involves an extensive cross talk between signaling processes by the participation of methyl jasmonate and salicylic acid for enhanced secondary metabolite production[23].

Conclusion

Curcuminoids produced by *C. longa* have a wide range of applications in the pharmaceutical industry, however, these molecules in plants are produced in trace amounts. Production of these secondary metabolites has been improved using the elicitation process, by treating the plants with MJ and SA. SA has proved to be a better elicitor in comparison to MJ treated plants with higher accumulation of all the curcuminoids (bisdemethoxy curcumin, demethoxy curcumin and curcumin). The results obtained in the present study may pave the way for scale-up of curcumin production under economically viable conditions.

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