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ENHANCEMENT AND MASS PRODUCTION OF ANDROGRAPHOLIDE A MEDICINALLY IMPORTANT SECONDARY METABOLITE IN KALMEGH (*Andrographis paniculata*) THROUGH METHYL JASMONATE AND GIBBERELLIC ACID ELICITATION

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ABSTRACT
Andrographolide is one of the chief active ingredient secondary metabolite of *Andrographis paniculata* biosynthesized through both MVA and MEP/DXP pathways. It has multiple health promoting effects including therapeutic properties. In this study we have exogenously applied Methyl Jasmonate (MeJA) and Gibberelic acid (GA_3) as an elicitor, in variable concentration, to access the effect on andrographolide accumulation. The experiment generates an integrated data set by RT-PCR and HPLC analysis. The result indicated that the biosynthesis of andrographolide has enhanced and highest 193.66 ± 0.47 mg/g of dry weight after 12 hours in 50µM of sample treated with MeJA. On other hand higher andrographolide was reported 123.50 ± 0.35 mg/g of dry weight in 1000µM GA_3 treatment after 24 hours of observation. The relative expression levels of the GGPS gene in leaves showed different patterns of mRNA accumulation over the course of treatments. The expression of GGPS increased gradually within 48 hour reached the highest level 35.26 ± 0.24 at 12 hours when treated with MeJA, 21.41 ± 1.42 at 48 hours with the treatment of GA_3 fold difference in relation to control respectively. Increment in the diterpene content has shown its association with the expression level of GGPS genes in the andrographolide accumulation regulatory cascade. The results clearly showed that andrographolide elicitation process was coordinately regulated.

KEYWORDS: *Andrographis paniculata* , Andrographolide, elicitation, MeJA , GA_3

INTRODUCTION
Andrographis paniculata is an herbaceous plant in the family *Acanthaceae*, native to southern Asia and locally named the "King of Bitters" or creat or green chiretta holds exceptional demand globally and domestically. It is widely cultivated in Southern and Southeastern Asia, where it is used to treat infections and some diseases, often being used before antibiotics were created. The chief active ingredient confirming medicinal property is andrographolide, and numbers of active components are reported in this plant which mainly includes diterpene lactones, flavonoids and polyphenols (Li *et al.,* 2007). Andrographolide ($C_{20}H_{30}O_5$) is the major diterpenoid secondary metabolite in *A. paniculata* has been mainly attributed for therapeutic properties. The plant is reported to possess protective activity against various liver disorders. The primary medicinal constituents of *A. paniculata* are andrographolide and related compounds which are diterpenoids showing antipyretic, antimalarial, anti-inflammatory, immune stimulatory activities (Misra *et al.,* 1992). Plants are a rich source of valuable secondary metabolites and consistently synthesize and accumulate secondary metabolites during their life cycle. These secondary metabolites can be isolated and produced in large amount under *in vitro* conditions by applying biotechnological approaches. Secondary metabolites have been used by humans to treat infections, health disorders and illness since the early days. Recently, there has been a rapidly increasing interest in plant secondary metabolism to search for new plant-derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity.
Elicitation is a biotechnological strategy used to stimulate or enhance the production of high-value secondary metabolites (such as alkaloids, flavonoids, and terpenoids) in plant cell, tissue, and organ cultures. Methyl jasmonate (MeJA) and Gibberellic acid (GA_3) are the two prominent elicitor used in present investigation. MeJA is a comprehensively characterized phytohormone associated with regulation of plant defense responses and its role in enhancement of secondary metabolites production is well documented (Cheng *et al.,* 2009 and Shan *et al.,* 2009). MeJA has been thoroughly characterized phytohormone associated with regulation of plant defense responses and its role in enhancement of secondary metabolites production is well documented. It is involved in the elicitation of secondary metabolites in plants, such as ginsenosides (Hu *et al.,* 2008) paclitaxel

(Ketchum *et al.,* 1999) and terpenoid (Martin *et al*., 2002). GA_3 acts primarily as a plant growth regulator and biomass promoter in *Andrographis paniculata*. While GA_3 application enhances overall plant height, internodal length, leaf number, and total herbage yield, other targeted biotic or abiotic elicitors (such as yeast extract, silver nitrate, or fungal filtrates) are significantly more effective for hyper-accumulating andrographolide. The quantitative estimation of andrographolide was performed with regard to specificity, precision, accuracy and linearity by HPLC. The composition and concentration of chemical compounds in plants are affected by factors such as genetics, environmental growth conditions, and the harvest and post-harvest conditions. The type of solvent used for extraction and its concentration play an important role in amounts of bioactive compound extracted (Mannade *et al.,* 2026). *GGPP* synthase is crucially important for our understanding of compartmentalization of isoprenoid biosynthesis. It is likely that synthesis of GGPP in Arabidopsis is regulated and compartmentalized in different organelles where different kinds of GGPP synthases occur.

The objective of this work was to establish a simple and reliable technique for quantification of andrographolide and also to study the influences of MeJA and GA_3 on production of valuable andrographolide through elicitation by spraying on plant in *A. paniculata* and also to investigate expression of *GGPP* synthase.

MATERIALS AND METHODS

Plant growth condition and treatment

A well characterized genotype of *A. paniculata* (Indira megha) of Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India was grown under greenhouses condition with a 15-h light/9-h dark cycle and a temperature of 26±2°C. Relative humidity was maintained at 65-75% in whole growing periods. Thirty day old plantlet of was transplanted in the plastic tray containing soil: sand: FYM with the ration of 1:1:1. There are no chemical fertilizers, pesticide and plant protection measures were adopted during the investigation. Three different concentrations of MeJA (10µM, 30µM, 50µM and 100µM) and GA_3 (100µM, 300µM, 500µM and 1000µM) each were used for treatment after 40 days of transplantation. Sampling for expression analysis was done at 1, 12, 24 and 48 hours after foliar application. Andrographolide estimation was done just before the blooming of plants.

Crude extraction, estimation and analysis of andrographolide

Young leaves were collected from plants at before flowering time, but avoiding material containing flowers or damaged by insects or fungi. Leaves were then cut into small pieces to permit homogeneous sampling. Drying was carried out at an ambient temperature of 45ºC, followed by grinding. Two gram of fine power was extracted by reflux with 100 mL of 70% (v/v) ethanol, with 3 cycles in soxhlet system. The ethanolic extracts were filtered and concentrated in a rotary evaporator at 60°C. The extracts were stored at 4°C until used (Sharma *et al.*, 2009).

HPLC condition and standard preparation:

HPLC analysis was carried out on an Agilent Series 1100 chromatograph, equipped with a vacuum degasser, a quaternary pump and a diode array detection system, connected to a reversed-phase column (Diamonsil C18, particle size 5 µm, metachem (150 mm X 4.6 mm column, Agilent, USA). Data collection was performed using Chemstation software (Agilent). The temperature was fixed at 25°C. The injection volume was 20 µL and elution was monitored at 223 nm. Andrographolide (Sigma, USA) at different concentrations (22.0 mg, 11.0 mg, and 5.5 mg) was used for method validation and preparation of calibration curves. Crude extract was evaporated under reduced pressure until dryness and used for HPLC quantification (Sharma *et al.*, 2011).

Identification and Selection of Pathway based Gene

The functional analysis of genes of metabolic pathways responsible for andrographolide biosynthesis was studied by *in silico* as well as quantitative expression analysis. Differential expression analysis was performed under elicitor and inhibitor treatments in compared to control. *Geranylgeranyl diphosphate synthase* (*GGPS*) a pathway regulatory enzymes was selected for estimating the gene expression and association analysis with andrographolide accumulation. The putative sequence of these enzymes was selected from the database for designing the suitable primer pairs for detecting the expression pattern.

RNA isolation and qPCR analysis

Total RNA was extracted and quantified. A total of 3µg of total RNA was reverse transcribed for each samples. QPCR was carried out in 2 ml of the RT product, using Stratagene (M x 3000p) Real-time PCR system with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. Thermal cycling consisted of 5 min initial denaturation at 94°C, followed by 40 cycles of 60s at 94°C for denaturation, 30S at 60°C for annealing and 30s at 72°C for amplification. The MEP and MVA pathway gene-specific primers were identify through tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm against available corresponding refseqcDNA. The highest similar sequence was selected and hundreds of primers designed by using Primer 3 (Primer3_www.cgi v.0.2; http://frodo.wi.mit.edu/) from cDNA sequences. qPCR was performed with designed primers (http://www.ncbi.nlm.nih.gov/sutils/e-pcr/reverse.cgi). Finally the designed primers were tested by amplifying *A. paniculata* cDNA before going for expression analysis of the particular genes. The 150 -250bp predictive product size and 60ºC Tm of primers was considered for expression analysis. The adjustment of baseline and threshold was performed according to the manufacturer's instructions. The relative abundance of transcripts was normalized to the constitutive expression level of actin. The data were analyzed by using the comparative threshold cycle method (Livaka *et al.,* 2001).

Statistical Analysis:
The statistical significance of variations in andrographolide was determined by one-way ANOVA analysis of data (significant at $P = 0.05$), was followed by multiple comparisons using Tukey's test for least-squared-difference (LSD at $P = 0.05$ level). Transcript levels as measured by qRT-PCR were correlated with andrographolide content. In addition, a composite total transcript index was computed by adding the relative expression values for each sample across the four time period. Data were recorded on 100 competitive plants for each accession for series of 8 metric traits: Days to 50% flowering, plant height (cm), LB Ratio (cm), leaf length (cm), leaf width (cm), Chlorophyll content (SPAD) Andrographiliode and Neoandrographolide content. The analysis was done by cluster formation was confirmed by Tocher's method. The relative contribution of each character towards genetic divergence was also worked out.

RESULTS AND DISCUSSION
Secondary metabolites are used by the plants during adverse conditions like environmental alteration and pathogen attack. Moreover, their production also depends on the developmental stage of the plant as well as the geographical and climatic conditions. Elicitor treatment was shown to affect the secondary metabolite biosynthesis in whole plant cultures with commercial potential (Kim *et al.,* 2004), (Namdeo 2007). The strategy behind selection of GA_3 is that, it increases signaling activity and expected increase the accumulation of andrographolide and chlorophyll. MeJA was selected because of its property of activating transcription of downstream target genes and its effect in increasing the phytoalexins, a diterpenoid.
Descriptive statistical analysis was performed to understand the variation and distribution of different agronomic and secondary metabolite related traits in *Andrographis paniculata* genotypes. The findings demonstrated substantial variation, suggesting ample genetic diversity for effective breeding and selection. The days to 50% flowering (DTF) ranged from 86 days to 130 days. Plant height (cm) varied widely, with the highest height recorded 98.45cm and the lowest 56.92 cm. The Leaf length ranges from 5.080 cm to 6.29 cm. Similarly; the Leaf breadth ranges from 1.73 cm to 2.15 cm. The length/breadth ratio falls between 2.93 cm to 3.05 cm Chlorophyll content (spad) ranged from 49.22 minimum to 64.50 maximum (Table 1). Comprehensive and precise phenotypic and genetic analysis were done by Hema *et al.,* 2017 which provide a brief idea for present investigation. In these agronomical analysis extent of variability, heritability and genetic advance for days to 50% flowering, plant Height (cm), leaf length (cm), chlorophyll content (spad), herbage fresh weight (g), herbage dry weight (g). Whereas, characters, leaf width (cm), petiole length (cm), stem diameter (cm), internodes length (cm), pod length (cm), no. of seeds/pod, andrographiloide content were determine in 134 accession include 2 checks collected from different district of the Chhattisgarh along with 2 check in Anand Kalmegh. The performance of *A. paniculata* genotypes was evaluated through field experimentation by Sharma *et al.,* 2009 in which they reported that morphological characterization (quantitative traits) of the *Kalmegh* genotypes including Plant height; Number of leaves; Number of branches; Leaf width; dry weight of whole plant; Seed yield of 15 genotypes collected from Chhattisgarh and there observation strongly support present investigation.
Effects on Andrographolide formation in the presence of MeJA and GA_3
Andrographolide accumulation was estimated in response to different concentrations of MeJA treatments at different time intervals. The andrographolide biosynthesis was significantly higher in the sample treated with MeJA compared to untreated control (Table 2). The accumulation of andrographolide was highest at 12 hours in most of the sample treated with MeJA in compared with 24 and 48 hours of treatment. Results denote more than fivefold increase in andrographolide accumulation in the sample treated with 50µM MeJA 193.66 ± 0.47 mg/g of dry weight after 12 hours of treatment (Fig.1). On other hand higher andrographolide was reported 123.50 ± 0.35 mg/g of dry weight in 1000µM GA_3 treatment after 24 hours of observation (Fig.2).
This observation demonstrates the stimulatory effects of MeJA on biosynthesis of andrographolide. As mentioned by *Jha et al.,* 2011 according to it after treatment, significant increase in andrographolide accumulation content was recorded. The highest level of andrographolide accumulation in GA3 treated plants was recorded at concentration of 5µM. The accumulation was more profound in case of JA treatment. 0.1mM JA treated plants has enhanced the andrographolide by 56.25% when compared to control. This approach is similar to studies that coupled biosynthetic gene expression and induce terpenoidindole alkaloid at the transcriptional level by switching on the coordinate expression of a set of biosynthesis genes under MeJA (Hirai *et al.,* 2004) and glucosinolate biosynthesis in *Arabidopsis thaliana* (Memelink *et al.,* 2001). Cho *et al*., 2006 optimized the production of the benzophenanthridine alkaloids in *Eschscholtzia californica* suspension cell cultures by adding 0.5 mg methyl jasmonate (MeJA) and get 10 times higher than in control cell cultures.

Table 1: Descriptive statistics showing traits difference between treated and untreated plants influencing Andrographolide content of *Andrographis paniculata*

Treatment (MeJA / GA_3)	Days to 50% flowering	Plant height (cm)	Leaf length (cm)	leaf breadth (cm)	L/B Ratio (cm)	Chlorophyll content (spad)
Control	85.33 ± 0.58	56.22 ± 1.15	5.10 ±0.07	1.74 ±0.02	2.93±0.06	49.17±0.07

(untreated)						
10µM MeJA	98.33 ± 0.58	63.24 ±0.90	5.19 ±0.02	1.96 ±0.04	2.65±0.05	55.71±0.25
30µM MeJA	104 ± 1.00	65.67 ±0.62	5.87 ±0.03	2.06 ±0.02	2.85±0.01	59.84±0.03
50µM MeJA	107.67± 1.53	82.71 ±0.62	6.17 ±0.02	2.10 ±0.01	2.94±0.02	63.51 ±0.40
100µM MeJA	119.33± 0.58	98.57 ±0.41	6.31 ±0.02	2.16 ±0.01	2.92±0.01	64.52±0.02
100µM GA_3	95 ± 1.00	72.75 ±0.54	5.23 ±0.05	1.91 ±0.02	2.74±0.04	53.75 ±0.03
300µM GA_3	101 ± 0.58	74.00 ±0.05	5.62 ±0.04	1.91 ±0.02	2.94±0.03	55.73 ±0.05
500µM GA_3	105.67± 0.58	82.46 ±0.10	5.76 ±0.04	1.96 ±0.03	2.94±0.03	56.13 ±0.05
1000µM GA_3	109 ± 1.00	88.65 ±0.26	5.93 ±0.05	1.95 ±0.01	3.04±0.03	56.87 ±0.02

Table 2: Enhancement of and rographolide content after MeJA and GA_3 treatment under different time and concentrations

Treatment (MeJA / GA_3)	**Average of the andrographolide content (mg/g DW)**			
	1 hours	**12 hours**	**24 hours**	**48hours**
Control (untreated)	39.68 ± 0.35	39.39 ± 0.56	38.95 ± 0.76	38.13 ± 0.09
10µM MeJA	65.70 ± 0.59	75.37 ± 1.16	47.02 ± 1.16	62.70 ± 0.14
30µM MeJA	88.35 ± 0.79	98.35 ± 0.79	62.24± 0.37	82.98 ± 1.16
50µM MeJA	173.66 ± 0.47	193.66 ± 0.47	122.44± 0.53	163.25 ± 0.56
100µM MeJA	123.97± 0.58	143.97± 0.23	89.31± 0.40	119.08 ± 0.37
100µM GA_3	55.68 ± 0.59	70.11 ± 0.08	69.55 ± 0.42	65.64 ± 0.37
300µM GA_3	65.59 ± 0.66	81.41 ± 0.38	105.21 ± 0.06	75.37 ± 0.16
500µM GA_3	88.41 ± 0.45	93.59 ± 0.43	103.60 ± 0.42	89.98 ± 0.05
1000µM GA_3	102.40 ± 0.23	113.37 ± 0.45	123.50 ± 0.35	112.33 ± 0.32

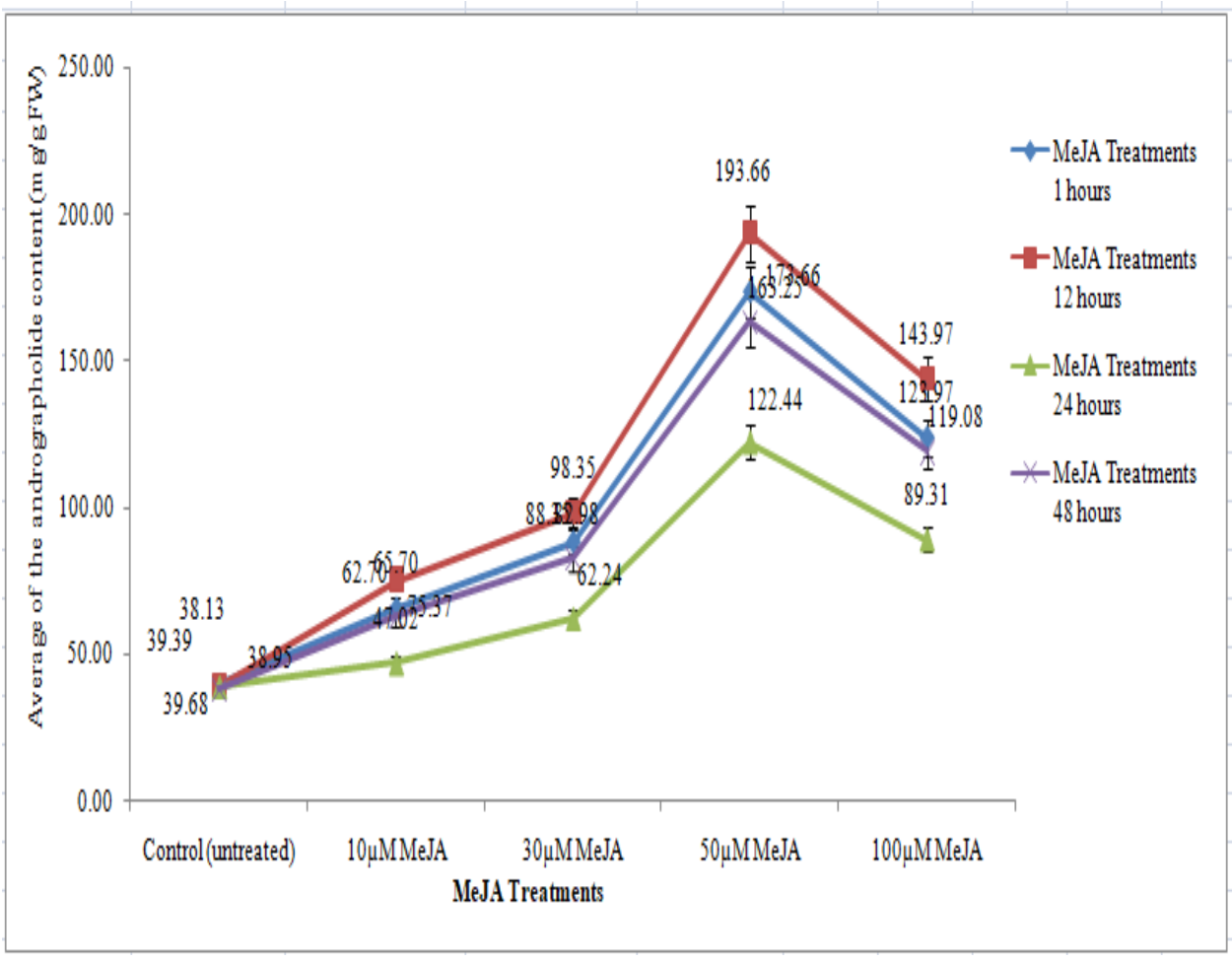

Fig. 1: Fold increment of andrographolide content under MeJA elicitation in different time and concentrations regime.

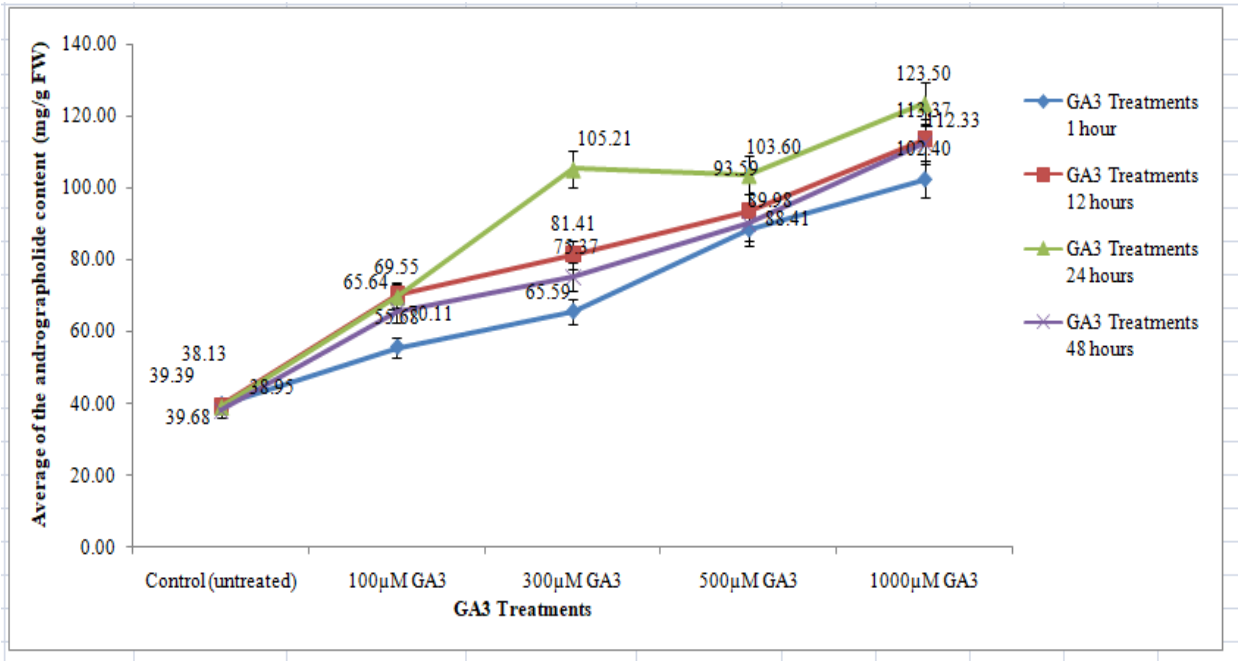

Fig. 2: Fold increment of andrographolide content under GA_3 elicitation in different time and concentrations regime.

Elicitor mediated changes in the expression of genes involved in andrographolide biosynthesis

The expressions of major regulatory genes involved in andrographolide biosynthesis were quantified. To avoid bias, real-time RT–PCR is typically referenced to a housekeeping gene as the internal control gene. Ideally, the conditions of the experiment should not influence the expression of this internal control gene. Actin was used as internal control gene. The inhabited genes *ggps* were first undergone to semi quantity PCR analysis. The result of semi-quantitative PCR exhibited down-regulation and amplicon were hardly visible in MeJA and GA3 treated plants. The genes were expressed well in untreated control suggesting extreme down-regulation of the genes in response to MeJA and GA3 treatments. The actin gene expression was used as internal control that showed no difference in amplicons.

GGPS expression under MeJA and GA_3 induction

The relative expression levels of the GGPS gene in leaves showed different patterns of mRNA accumulation over the course of treatments. The expression of GGPS increased gradually within 48 hour reached the highest level 35.26 ± 0.24 at 12 hours when treated with MeJA, 21.41 ± 1.42 at 48 hours with the treatment of GA_3 fold difference in relation to control respectively. The transcripts for both GA_3 and MeJA treated plant had increased rapidly by the first time point measured and maintained levels well above those found in non-treated control plant. The 100µM concentration GA_3 induced GGPS expression at the maximum level after 48 hours (Fig. 3 A & B). In the other hand MeJA has also influenced the expression level of GGPS. The maximum expression of GGPS was recorded by treating 50µM MeJA at 12 hours after treatment. Treatment with MeJA produced greater effects than GA_3 and the timing of GGPS transcript changes also appeared to be somewhat different when comparing GA_3 treatment with MeJA. The study indicated that the GA_3 and MeJA has substantially influenced the GGPS activity and shows that GA_3 and MeJA has induced the GGPS activity and regulates the production of Andrographolide accumulation in *A. paniculata* (Table 3). Differential expression of genes acting in the andrographolide biosynthesis pathway was examined over various concentration and time regime. This approach is similar to studies the coupled biosynthetic gene expression and induce terpenoidindole alkaloid at the transcriptional level by switching on the coordinate expression of a set of biosynthesis genes under MeJA (Hirai *et al.,* 2005). Similarly MeJA increased CPT production 11 times in *C. acuminata*cells (Song *et al.,* 1996). MeJA elicitation was characterized in mRNA levels consistent with results reported in *T. cuspidate* (Jennewein 2004) and *Taxuschinensis* (Li *et al*., 2012). Sharma *et al*. 2013 worked on the exogenous application of JA on the biosynthesis of andrographolide and relating to *hmgr* expression. *GGPP* synthase is crucially important for our understanding of compartmentalization of isoprenoid biosynthesis. *GGPS* presumably generates GGPP by sequential coupling of three *IPP* molecules to DMAPP (Kasahara *et al*., 2002). The intermediate enzymes activity (*IPP* and *GGPS*) indicated that the regulation of andrographolide the MEP pathway and downstream metabolic pathways are coordinately regulated through the regulation of the expression levels of key enzymes involved. *GGPS* generates geranylgeranyl diphosphate (GGPP) for diterpenes most probably andrographolide in *A. paniculata*. *GGPS* activity was recently described (Cervantes *et al*., 2006). The analyzed genes MeJA treatment showed higher relative expression in compare to control induced andrographolide biosynthesis support the fact of up regulation of metabolic pathway leading to enhanced secondary metabolite production

Table 3: Fold change expression of GGPS genes after MeJA and GA_3 treatments

Treatment (MeJA / GA_3)	Fold difference in relation to control			
	1 hours	12 hours	24 hours	48hours
Control (untreated)	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10µM MeJA	0.01 ± 0.01	12.38 ± 1.22	0.06 ± 0.41	0.64 ± 0.10
30µM MeJA	0.03 ± 0.21	26.35 ± 3.81	1.15± 0.46	0.08 ± 0.06
50µM MeJA	0.01 ± 0.01	35.26 ± 0.24	0.69 ± 0.61	0.06 ± 0.05
100µM MeJA	0.07± 0.03	14.12± 0.18	0.04± 0.02	0.27 ± 0.05
100µM GA_3	1.01 ± 0.02	1.22 ± 0.29	1.10 ± 0.03	21.41 ± 1.42
300µM GA_3	1.21 ± 0.22	3.81 ± 1.93	1.06 ± 0.04	5.46 ± 1.45
500µM GA_3	1.01 ± 0.02	0.24 ± 0.21	1.61 ± 0.81	3.51 ± 1.81
1000µM GA_3	1.00 ± 0.01	0.05 ± 0.01	1.02 ± 0.01	1.78 ± 0.83

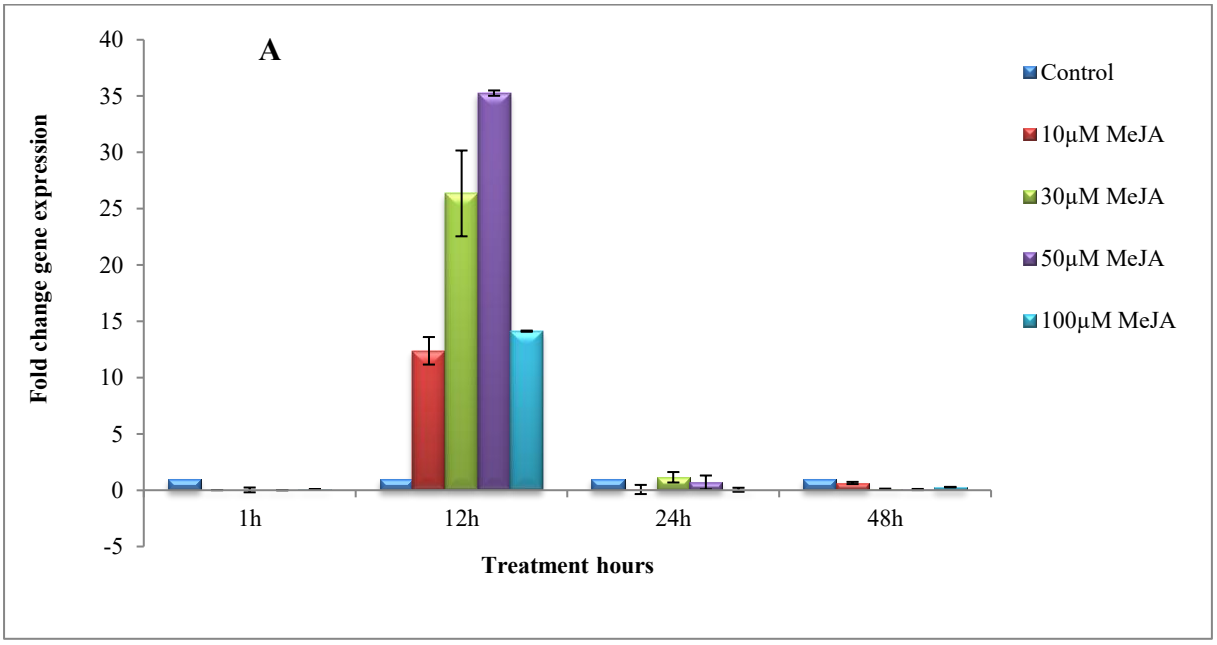

Fig. 3: (A) Changes in steady-state transcript levels of GGPS genes in *A. paniculata* following MeJA treatments.

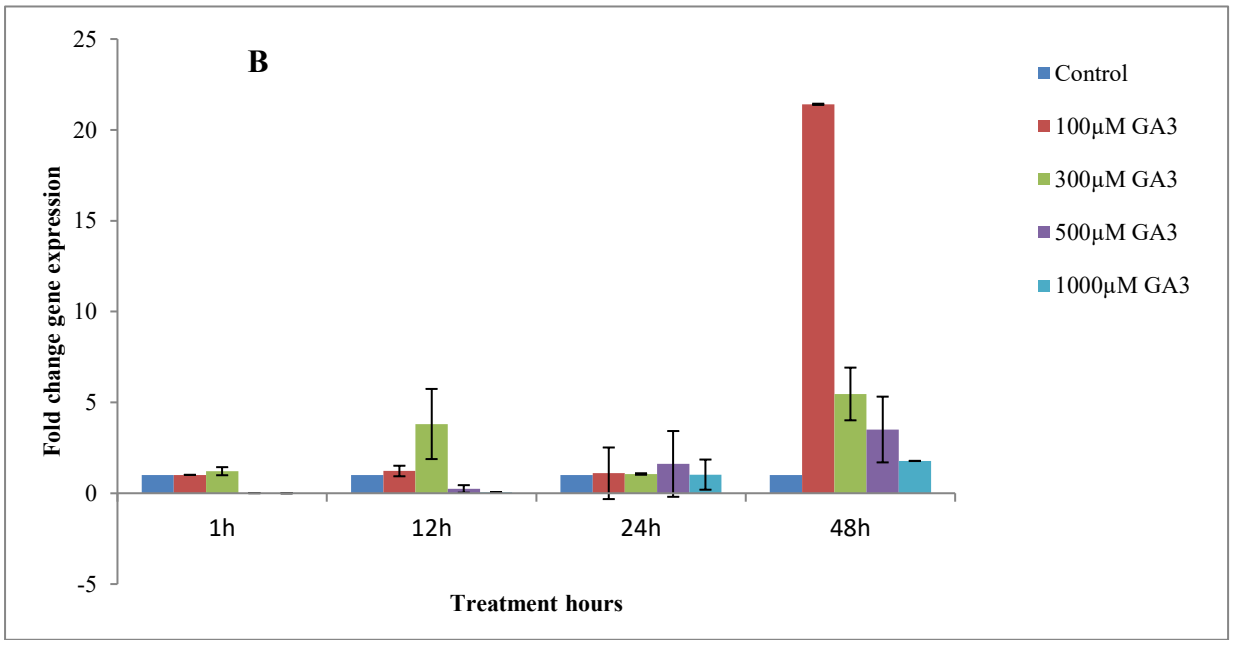

Fig. 3: (B) Changes in steady-state transcript levels of GGPS genes in *A. paniculata* following GA_3 treatments.

CONCLUSION

The present study establishes a simple and reliable technique for quantification of andrographolide and also to study the influences of MeJA on production of valuable andrographolide through elicitation by spraying on plant in *A. paniculata*. The strategy behind selection of GA_3 is that, it increases signaling activity and expected increase the accumulation of andrographolide. MeJA was selected because of its property of activating transcription of downstream target genes and its effect in increasing the phytoalexins, a diterpenoid. Results denote more than fivefold increase in andrographolide accumulation in the sample treated with 50µM MeJA 193.66 ± 0.47 mg/g of dry weight after 12 hours of treatment. On other hand higher andrographolide was reported 123.50 ± 0.35 mg/g of dry weight in 1000µM GA_3 treatment after 24 hours of observation. The expression of GGPS increased gradually within 48 hour reached the highest level 35.26 ± 0.24 at 12 hours when treated with MeJA, 21.41 ± 1.42 at 48 hours with the treatment of GA_3 fold difference in relation to control respectively. As current experiment clearly indicates that after elicitation of MeJA and GA_3 andrographolide content of plant increases rapidly, due to enhancement of expression level of GGPP gene and ultimately influencing diterpenoid synthesizing MVA and MEP pathway.

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