



# Comparative analysis of biomass, ethrel elicitation, light induced differential MVA/MEP pathway gene expression and andrographolide production in adventitious root cultures of *Andrographis paniculata* (Burm. F.) Nees

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## Abstract

*Andrographis paniculata* (Burm. F.) Nees is a rich source of biologically active andrographolide and other diterpene lactone compounds. The in vitro adventitious root culture system is an improved alternative strategy for mass cultivation of the plants and enhanced production of secondary metabolites in general and diterpene lactones in particular. The present study was initially envisaged, to find out how two different andrographolide biosynthetic pathways (MVA and MEP) contribute towards the accumulation of andrographolide under the influence of light. In addition, the effect of different liquid culture techniques were evaluated for enhanced biomass and elicitation facilitated andrographolide production. The culturing of adventitious roots on a platform and by not immersing directly in the liquid medium was found best for improved biomass generation. With 1.23 g of initial inoculum were able to generate 4.45 fold (5.48 g) biomass in just 4 weeks by this modified liquid culturing technique. Further, the elicitation of adventitious roots with ethrel enhanced both biomass (up to fivefold) and andrographolide content (fourfold). The differential gene expression results indicated that, under the influence of light, the MEP pathway was found to dominate for the biosynthesis of andrographolide by the up regulation of the DXR, DXS, HDR, HDS genes as well as down stream GGPS gene expression. In absence of light, MVA pathway was observed as a major contributor for the production of andrographolide. The andrographolide content was enhanced upto 4.29 fold in light elicited adventitious roots compared to the control roots (grown in the absence of light). Our results implied that the light has not only significantly enhanced the MEP pathway gene expression but also inturn elevated the andrographolide accumulation in adventitious root cultures of *A. paniculate* indicating the role of elicitation and tissue specific gene expression.

## Key message

The liquid culture technique, ethrel elicitation promoted enhanced adventitious root biomass (5 fold) and andrographolide content (4 fold). Light has shown significant effect on andrographolide production (4.29 fold) by upregulating the expression of MEP pathway and downstream genes.

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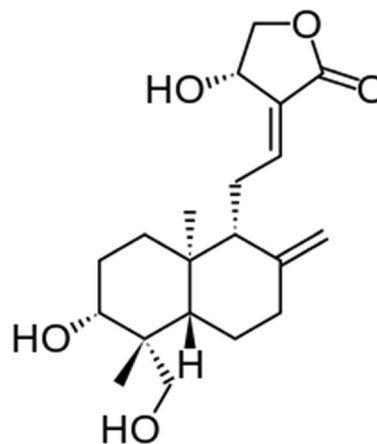
**Keywords** *Andrographis paniculata* · Andrographolide · Adventitious roots · Elicitation · Biomass · qRT-PCR

### Abbreviations

AD	Andrographolide
AR	Adventitious roots
ARC	Adventitious root cultures
DGE	Differential gene expression
GA <sub>3</sub>	Gibberellic acid
GI	Growth Indices
IAA	Indole acetic acid
IBA	Indole butyric acid
MS	Murashige and Skoog
MSO	MS basal medium without PGRs
NAA	Naphthalene acetic acid
PGRs	Plant growth regulators
DXR	Deoxy xylulose 5-phosphate reductase
DXS	Deoxy xylulose 5-phosphate synthase
HDR	4-Hydroxy 3-methyl 2-butenyl 4-diphosphate reductase
HDS	4-Hydroxy 3-methyl 2-butenyl 4-diphosphate synthase
GGPS	Geranylgeranyl pyrophosphate synthase

### Introduction

The plants are considered as the “Chemical Factory” where enormous number of primary and secondary metabolites are synthesized (Marchev et al. 2020; Kandar. 2021). The potential of plant derived bioactive compounds have been exploited in Indian traditional medicine from the ancient times. *Andrographis paniculata*, a medicinal herb is native to India and other tropical and subtropical Asian countries (Hossain et al. 2014; Neeraja et al. 2015; Gupta et al. 2019; Verma et al. 2019). *A. paniculata* (Burm.f.) Nees is considered as “bile of the earth” for its bitterest taste and small size up to a height of 30–110 cm (Subramanian et al. 2012; Hossain et al. 2021). It has been used to treat infectious diseases and respiratory tract infections and is referred to as the next big immune booster (Kumari et al. 2021). It is famous for its herbal formulations such as “Andrographis Plus” (Metagenics Inc. CA, USA), “Kan Jang” (Swedish Herbal Institute, N.H, USA), “Tribulus Complex” (Jarrow Formulas, LA, USA) and “Stimuliv” (Franco-Indian Pharmaceutical Pvt.Ltd, Mumbai, India). In recent years, the novel uses such as anti-helminthic (Banerjee et al. 2019), excito-repellency (Sukkanon et al. 2019) anti-viral (Li et al. 2020) and anti-snake venom (Nayak et al. 2020) properties of this plant have been assessed. The phytoconstituents and the traditional uses of the plant were recently reviewed in detail by Kumar et al (2021). The diterpene lactones and flavones are the contributors of medicinal properties of this



**Fig. 1** The chemical structure of the andrographolide

plant. Amongst these, andrographolide (Fig. 1) is the leading compound, in terms of bioactive properties and its abundance (Chao and Lin 2010). Due to the current “Demand and Supply” for *A. paniculata* plants, the National Medicinal Plant Board (NMPB) has extensively surveyed the herbal market of India in collaboration with The Indian Council of Forestry Research and Education (ICFRE), Dehradun and estimated the consolidated commercial demand of *A. paniculata* estimated to be 2000–5000 metric tonne (MT) Gupta et al. 2019. The over exploitation of the in situ natural resources for pharmaceutical needs pose a major threat to the plant’s ecological system and biodiversity at large (Devi et al. 2021). In addition, the current natural habitat of the plants is being contaminated with pesticides, herbicides and heavy metals derived from environmental impact and human interventions. The use of these contaminated plants can be harmful to human health (Hossain et al 2014). Thus, the natural production cannot meet the present market demands and quality control guidelines (Rahmat and Kang 2019). In the recent past, through the use of tissue/organ culture techniques and elicitation of in vitro cultures with signal molecules, it has been demonstrated that the amount of diterpene lactones and other bioactive compounds have increased to an accountable extent (Zaheer and Giri. 2015, 2017; Giri and Zaheer 2016; Pandey et al. 2017, 2022; Singh et al. 2018; Das and Bandyopadhyay 2020; Ganapumane and Nagella 2020; Devi et al. 2021; Sun et al. 2021, 2022; Ahmad et al 2022).

The plant roots are considered, a unique site for the synthesis and storage of secondary metabolites. The adventitious root (AR) culture (ARC) is one of the promising plant tissue culture systems poised to increase the production of plant based bioactive compounds (Rahmat and Kang. 2019). The high rate of proliferation, tremendous accumulation

potential, stable production of the compounds defying genetic variation is unique to adventitious roots (Anjum et al. 2018). AR was induced in vitro from different plant parts by administering plant growth hormones in the media (Isah et al. 2018; Fathi et al. 2021; Kannan et al. 2021). The incubation of AR under different spectral lights can up regulate the secondary metabolite biosynthetic pathway genes and enhance the production of secondary metabolites (Ali et al. 2019). AR can be grown in the bioreactor to produce amounts of root biomass along with enriched bioactive molecules without producing harmful opine like compounds as observed in hairy roots (Baque et al. 2012). AR synthesize secondary metabolites into their intercellular spaces which can be extracted easily and can be grown with high growth rate even with low inoculum in a phytohormone amended medium (Murthy et al. 2021). Our laboratory is engaged in research initiatives on distribution of *A. paniculata* and related species, pharmacological study and yield enhancement of bioactive compounds, using biotechnological, genomic, proteomic and bioinformatics approaches (Neeraja et al. 2015; Zaheer and Giri 2015, 2017; Parlapally et al. 2016; Giri and Zaheer 2016; Bindu et al. 2017, 2020; Srinath et al. 2017, 2020, 2021; Shailaja et al. 2018, 2020, 2021). Besides the isolation and characterization of genes (HMGR, DXR and HDR) for diterpene lactone andrographolide biosynthesis, currently we are working on the elucidation and biomass production, synergy between external factors such as light/dark and tissue specific gene expression involving AR of *A. paniculata*.

In the present study, we established ARC system of *A. paniculata* from different explants and investigated the effects of different parameter on AR formation as well as andrographolide production. The different liquid culture techniques were established for the AR biomass production. Further, the current study aimed to explore the action of ethrel for the andrographolide accumulation via elicitation. The effect of light other than elicitors on the production of andrographolide and its biosynthetic routes mainly the MVA and MEP pathway genes was analysed.

## Materials and methods

### Collection of *A. paniculata* plant material

*Andrographis paniculata* seeds were collected from CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP) Research Centre, Hyderabad a regional Centre of CSIR-CIMAP, Head Quarters, Lucknow, India. The selected plots in the nethouse of Centre for Plant Molecular Biology (CPMB), Osmania University (OU), Hyderabad, India were cleaned, ploughed thoroughly and supplemented with manure (Soil and Vermicompost 1:1 ratio) prior to seed

sowing. The seeds were subsequently sown in the selected plots during March/April. The plots were watered twice in a day for efficient germination with additional supplement of nutrient solution, half strength Hoagland solution (Hoagland and Arnon 1938).

### Germination of *A. paniculata* seeds in vitro

The germination of *A. paniculata* seeds in vitro was carried out using two types of seeds i.e. immature, light yellow and mature brown seeds. The fresh green fruits/pods containing immature seeds were collected from the net house grown plants during the month of August and September. The pods/fruits were collected and brought to the lab and washed under running tap water. The washed pods were surface sterilized with 0.1% (w/v)  $\text{HgCl}_2$  for 5 min followed by repeated washing with autoclave sterilized distilled water in the laminar air flow. The surface sterilized pods were dissected using sterile scalpel blade to expose light yellow colour immature seeds and transferred aseptically to full strength Murashige and Skog (1962) medium devoid of plant growth regulator (MSO medium) solidified with 0.8% Agar, pH-5.8. The mature seeds were collected after the pods opened in the month of October/November and were surface sterilized with 0.1% (w/v)  $\text{HgCl}_2$  for 1 min following a similar protocol as described above. The seeds were then inoculated on to MSO as well as MS medium fortified with 2.0 mg/L gibberellic acid ( $\text{GA}_3$ ) (Sigma Aldrich, 99%, USA). The cultures were incubated in culture room conditions ( $25 \pm 2$  °C and relative humidity over 70%) under darkness. After germination, the seedlings were shifted to light and sub cultured every 4 weeks.

### Induction of adventitious root cultures in vitro

The in vitro germinated 30–40 days old seedlings were used for the adventitious root induction. The explants such as leaves/cotyledons, stem/hypocotyls and roots were excised from in vitro germinated seedlings and inoculated on to the full strength MS medium (solidified with 0.8% Agar; pH-5.8) supplemented with different concentrations (0.5, 1, 2, 3, 4 and 5 mg/L) of auxins. Auxins namely NAA, IAA and IBA (Sigma-Aldrich, USA) were used for the induction of adventitious roots. The cultures were incubated in the dark in culture room conditions. The adventitious roots induced on full strength MS + auxin medium were maintained by regular subculture in every 4 weeks interval on the same medium and incubated at  $25 \pm 2$  °C.

## Effect of light on adventitious root cultures and andrographolide production

The actively growing 3 week old healthy adventitious roots initiated under dark were aseptically transferred to freshly prepared MS + NAA (1 mg/L) medium to elucidate the effect of light on ARC for andrographolide accumulation. The cultures were then incubated under light with white fluorescent lights (1000 lx). The growth study was conducted for 4 weeks and compared with adventitious roots grown in the dark and light conditions. The adventitious roots induced on full strength MS + NAA (1 mg/L) medium were maintained by regular subculture every 4 weeks interval on the same medium and incubated at  $25 \pm 2$  °C. A small amount of adventitious root inoculum (0.93 mg) from 4 weeks old cultures was separated with the help of sterile forceps and inoculated on to MS + NAA (1 mg/L) medium. The cultures were incubated under dark and light conditions in the culture room.

### Fresh and dry weight (FW and DW) measurements for growth indices (GI)

The adventitious roots were harvested every week and weighed using an analytical balance (Mettler-Toledo India Private Limited) to determine the fresh weight (FW). The dry weight (DW) was obtained by drying the roots at 60 °C for 2–3 days and weighed using an analytical balance. The growth index (GI) was calculated as per the formula given below:

$$\text{Growth Index (GI)} = \text{Final FW} - \text{Initial FW (or) Final DW} - \text{Initial DW}$$

### Andrographolide extraction from different adventitious root cultures and HPLC analysis

To evaluate the andrographolide content in the adventitious roots developed from different explants (leaf, stem and root) were harvested after 4 weeks, dried in oven at 60 °C for 2 days and subjected to methanol extraction as per Zaheer and Giri (2017). The finely ground adventitious root powder (50 mg) was added with 5 mL of 100% methanol and incubated for 24 h at room temperature followed by sonication for 30 min for each sample. The sonicated samples were filtered through Whatmann No. 41 filter paper and then passed through Millex-GV Syringe Filter Unit, 0.22 µm, PVDF, 33 mm, gamma sterilized (Merck India). 200 µl of filtrate was used for HPLC analysis with 100% HPLC grade methanol as mobile phase using C18 column in Shimadzu HPLC machine. The flow rate of 1 mL/min, 20 µl of injection volume was used along with andrographolide standard (1 mg/mL) (Sigma-Aldrich, USA, 98%) detected by PDA detector

at the Department of Biochemistry, Osmania University, Hyderabad, India. Andrographolide was detected at 230 nm.

### Biomass enhancement of adventitious root cultures by different culture techniques in liquid media

The liquid cultures were generated for the enhancement of adventitious root biomass. An average of 1.23 g fresh inoculum from 3 weeks old adventitious root cultures was inoculated into 20 mL liquid MS + NAA (1 mg/L) medium in 150 mL Erlenmeyer flasks. The flasks were placed in the dark at  $25 \pm 2$  °C for 3 weeks in static condition. In order to increase the biomass of adventitious root cultures, a number of culture techniques were employed and compared for the growth of AR cultures in semi-solid medium (MS medium with 0.4% agar; pH-5.8). The actively growing adventitious roots from 3 week old liquid cultures were inoculated, into the liquid medium directly (Liquid adventitious roots-LAR), over blotting papers floating on liquid medium (Blotting paper adventitious roots-BAR) and over the cotton immersed in liquid medium (Cotton adventitious roots-CAR). The cultures were incubated in the dark at culture room conditions. The growth study was conducted for 4 weeks by taking the FW and DW every week and compared with solid adventitious root cultures (SAR). The harvested roots were oven dried, powdered and used for HPLC analysis. In addition, we checked the release of andrographolide in to the media by methanol extraction of the liquid media and HPLC analysis. The 5 mL liquid media was collected every week from each of the above three techniques, 5 mL of methanol was added

and left opened for 3–4 days for concentration. The liquid was further subjected to sonication, filtration and HPLC analysis as per standardised protocol mentioned earlier.

### Elicitation of adventitious roots with ethrel

Three week old actively growing adventitious roots were used for the elicitation experiment. The elicitor ethrel was first filter sterilized prior to the elicitation and added to the sterile solid MS + NAA (1 mg/L) medium in different concentrations (1, 10, 25, 50 and 100 µM concentrations). The adventitious root inoculum was inoculated onto elicitor media and incubated at culture room conditions. The root biomass was harvested every week and FW and DW was measured. The graph was plotted by taking FW and DW on Y-axis and time duration on X-axis. The air dried adventitious root samples were subjected to methanol extraction

**Table 1** The primer sequences for target transcripts of the genes

Target transcripts		Primer sequences (5' to 3')
DXS	F	CGTATCCGAGGATCGCAACT
	R	AAGGAACGCCGCTTGAGAT
DXR	F	TCCCTCTCGCTCACAAGCA
	R	GCCTTGAATGCACTGAAATATGG
GGPS	F	CCCTTCACTGTCCAAGTCCACTA
	R	AGCTGGCACTGGCAATCG
HDS	F	GGAGCACCGCCGACATAT
	R	TGCCATCATGGGTTGCATT
DXS	F	CCATTCCAAGTCCAGAAGATATGC
	R	GAAAGCCGGAAGATCTATCCAA
DXS	F	ACATGCCTTCTACCCAATTACAG
	R	GCACATTTGTGGGACGGTTT
HDR	F	TTGACCTAGAATGGCGATTTCTC
	R	GGTATCCGGCAAGGAAAAGCT
GGPS	F	GCCAAAGACTGTGTGGTTGGA
	R	CCACGACGACTTGCCATACA
GGPS	F	TGGATTTACCGACTTCCAGTACCT
	R	CGTGTGTGCCTTTATTGTGTG
DXR	F	GGCCGAGCGAATTATTG
	R	CGAGCCAAGCTTGCAAAGA
HDS	F	GGGAATGGTCGAGTCTGCAT
	R	TCAGCTACGAGAAGGCGATATG
DXR	F	GCCGTCGCTAGAGGAAATCA
	R	TGCAAACCTCGCTGCGTAGTC
HDR	F	GGGCTGTCCAGATTGCTTATG
	F	TCCATCTCTTCGAGCCTCTGA
HMGR	F	CATGGAAGTGATCGGCTTATCC
	R	GGCTTCGCACACCACTGATT
DXS	F	GACATTCGCTGCGGGTCTAG
	R	CCCGTCCATGATGAACCT
<i>Actin</i>	F	ACGATGTTACGGGCATTG
	R	GAGCCACCACCTTGATCTTCA
HMGS	F	TTCCATCGTACTCGCTTGAATCT
	R	CGCCTCACGACGAATCG

*DXS* 1-deoxy-D-xylulose-5-phosphate synthase, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *GGPS* geranylgeranyl pyrophosphate synthase, *HDS* 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, *HDR* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *HMGR* 3-hydroxy-3-methylglutaryl coenzyme A reductase, *HMGS* 3-hydroxy-3-methylglutaryl coenzyme A synthase F: Forward; R: Reverse

and HPLC analysis was performed. The 99% pure andrographolide procured from Sigma was used as standard. A graph was plotted by taking andrographolide content on Y-axis and time duration on X-axis. All the experiments were repeated three times to minimize the standard error and in each experiment a maximum of 6 replicates were

maintained. The statistical analysis one way ANOVA was performed by IBM SPSS Statistics version 25.0 software.

## Differential expression of MVA and MEP pathway genes in light influenced in vitro adventitious root cultures

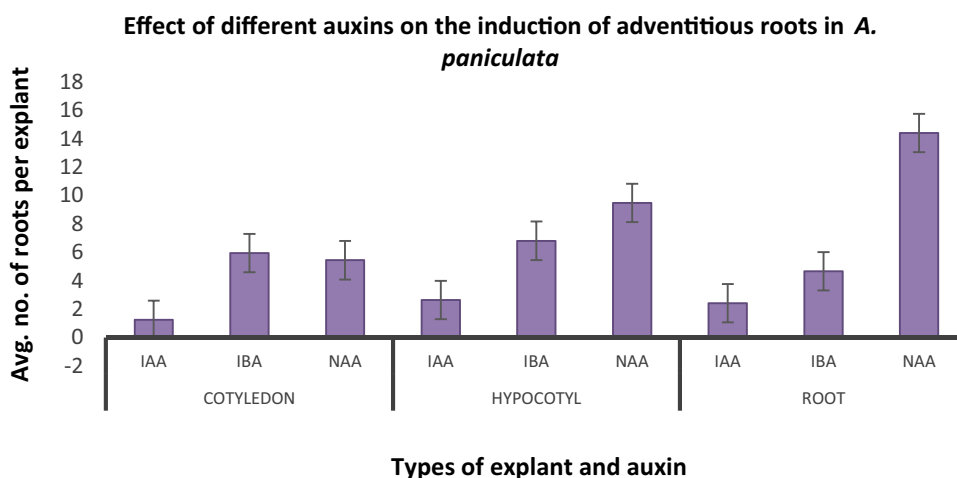
The total RNA from the AR tissue (incubated under light and dark) was extracted by trizol method and converted to cDNA using Takara Primescript first strand cDNA synthesis kit (DSS Takara, India) for qRT-PCR analysis. The primers for HMGR, HMGS, DXS, DXR, HDS, HDR and GGPS were procured from ReGene Biologics, Hyderabad, India. The primer sequences for target transcripts of the genes have been included as Table 1. The actin gene was used as internal control. The qRT-PCR was performed in QuantStudio™5 Real Time PCR instrument (Applied Biosystems from Thermo Fisher Scientifics, Singapore) using DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, USA) following manufacturer's instructions. The qRT-PCR program was as follows—95 °C—7 min; 95 °C—10 s; 40 cycles of 60 °C-30 s; 95 °C—15 s (absorption).

## Results

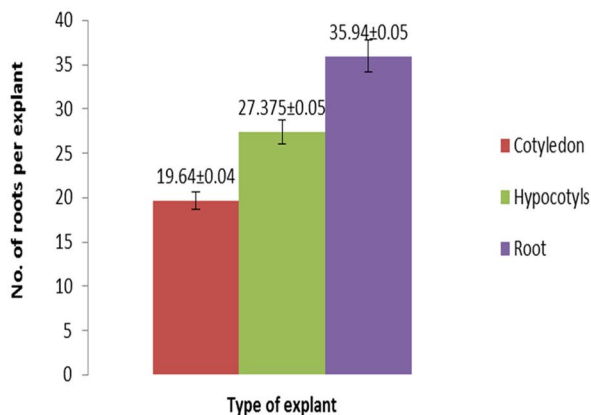
### Generation of in vitro cultures of *A. paniculata*

The immature seeds obtained from pods showed maximum germination percentage (up to  $75 \pm 0.029$ ) compared to mature seeds ( $21.2 \pm 0.036$ ). The average days taken for germination of immature seeds was 7–10 days and mature seed germination were 25–30 days. The number of roots ( $5.1 \pm 0.04$  from cotyledon,  $8.89 \pm 0.05$  from hypocotyl,  $14.3 \pm 0.02$  from root explant) was obtained in NAA amended MS medium. About  $6.02 \pm 0.035$  from cotyledon,  $6.56 \pm 0.042$  from hypocotyl,  $4.2 \pm 0.031$  from root explants were observed in MS + IBA medium. The cotyledon  $1.01 \pm 0.029$ ,  $2.12 \pm 0.039$  from hypocotyl,  $2.0 \pm 0.048$  from root explants were induced from MS + IAA medium (Fig. 2). A higher number of adventitious roots were induced from root explant [ $35.94 \pm 0.05$  roots per explant (RPE)] and hypocotyl explants ( $27.37 \pm 0.05$  RPE). The number of root induction ( $19.64 \pm 0.04$  RPE) was observed from cotyledon explants (Fig. 3a). There was no adventitious root induction observed on the MS medium without any PGR except callus formation and browning/blackening of the explant. HPLC analysis of adventitious roots induced from different explants (root, hypocotyl and cotyledon) revealed high amount of andrographolide content (1.58% DW) in the adventitious roots induced from the cotyledon explant and hypocotyl derived adventitious roots (1.22% DW). The

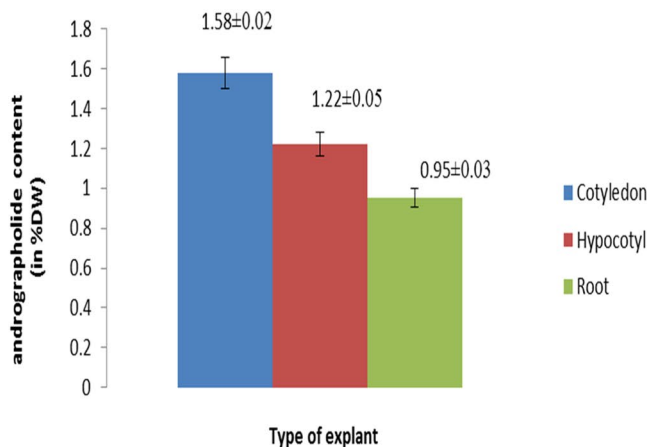
**Fig. 2** Graph showing the differential effect of auxins on the induction of adventitious roots from different explants



**a** Effect of explant on adventitious root induction in *A. paniculata*



**b** Effect of explant on andrographolide content in adventitious roots



**Fig. 3** **a** Graphical representation of effect of explant on the induction of adventitious roots in *A. paniculata*; **b** Graphical representation andrographolide content in adventitious roots induced from different explants such as cotyledon, hypocotyls and roots

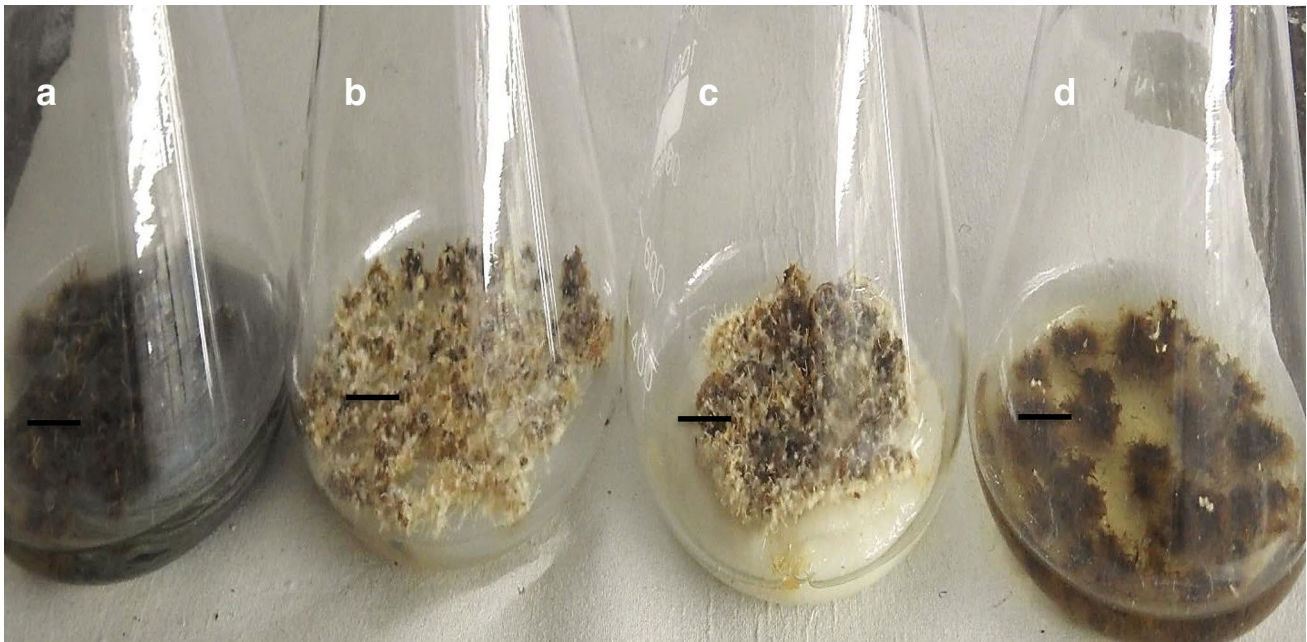
adventitious roots derived from root explants showed andrographolide content (0.95% DW) (Fig. 3b). The statistical one way ANOVA analysis shows the p value of <math><0.05</math> for all the experiments.

### Biomass enhancement of adventitious roots in vitro

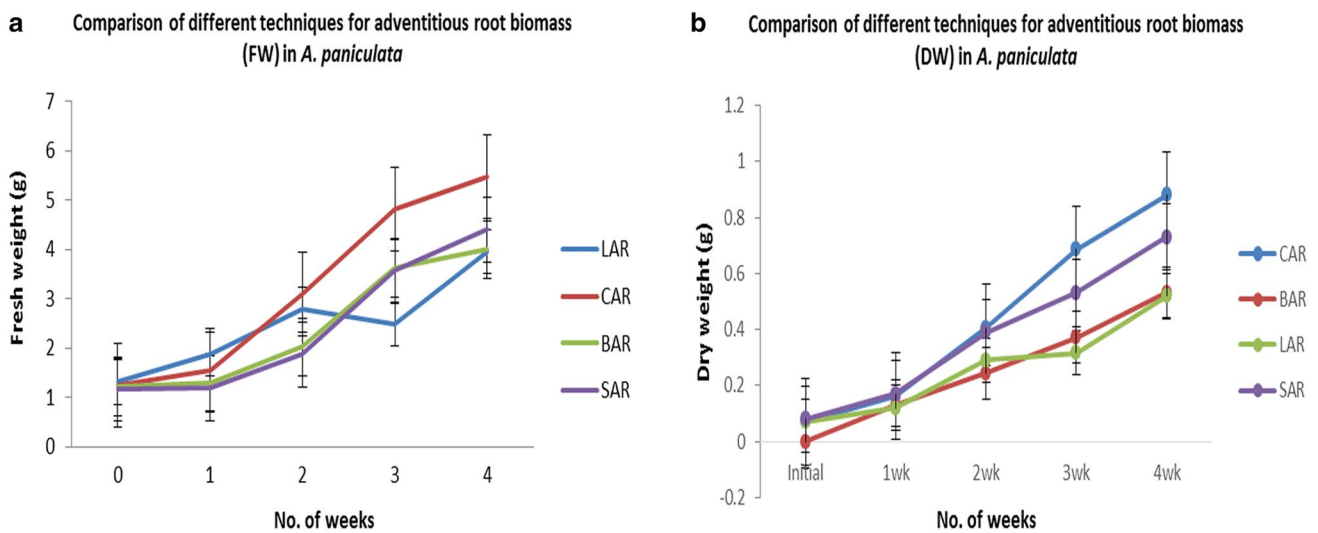
The adventitious roots placed on blotting paper were bunchy, small and short in length without any additional branched structure (Fig. 4a). The adventitious roots grown on solid medium were dispersed and long (Fig. 4b). The adventitious roots induced on cotton paper were short and bunchy (Fig. 4c). The adventitious roots directly immersed in liquid medium were small and branched (Fig. 4d). The highest amount [5.48 g (FW)] of biomass per explant was generated when the adventitious roots were cultured on CAR. The adventitious roots cultured on SAR produced 4.4 g (FW)

of BPE whereas adventitious roots grown on BAR generated 4.0 g (FW) BPE after 4 weeks of culture. The adventitious roots that directly immersed in the liquid medium (LAR) generated biomass (3.0 g FW) BPE. The growth study of adventitious root cultures by different techniques was compared by plotting a graph with FW (Fig. 5a) and DW (Fig. 5b). The chromatogram for the standards and that of the different samples through HPLC analysis is depicted in Figure S1a–d.

The HPLC analysis of the adventitious roots developed using the different culture techniques revealed high amount of andrographolide (7.952% DW), from the SAR cultures and BAR (5.799% DW) and CAR (5.377% DW) cultures. The andrographolide content (4.57% DW) was observed in LAR cultures (Fig. 6a). The HPLC analysis of liquid culture medium revealed that over the culture time, andrographolide was released by the adventitious root cultures in to the surrounding medium (Fig. 6b). The release of andrographolide



**Fig. 4** **a** Adventitious roots placed on blotting paper which is floating on liquid medium; **b** adventitious roots on solid medium; **c** adventitious roots placed on cotton immersed on liquid medium; **d** adventitious inoculated in liquid medium (Bar = 1.0 cm)

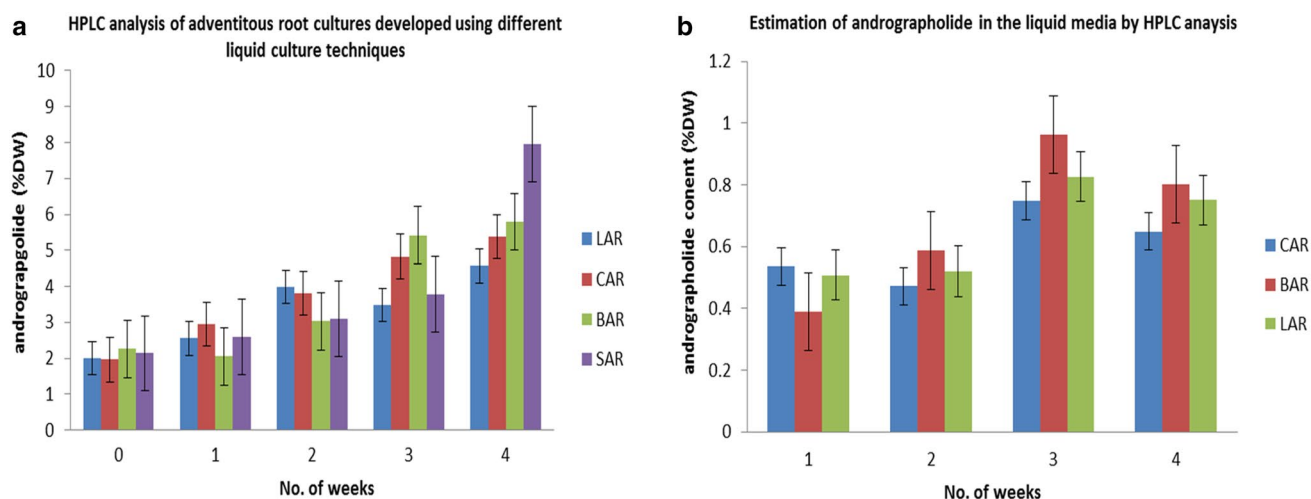


**Fig. 5** **a** Biomass (fresh weight) comparison of adventitious roots developed using different liquid culture techniques; **b** Biomass (dry weight) comparison of adventitious roots developed using different liquid culture techniques

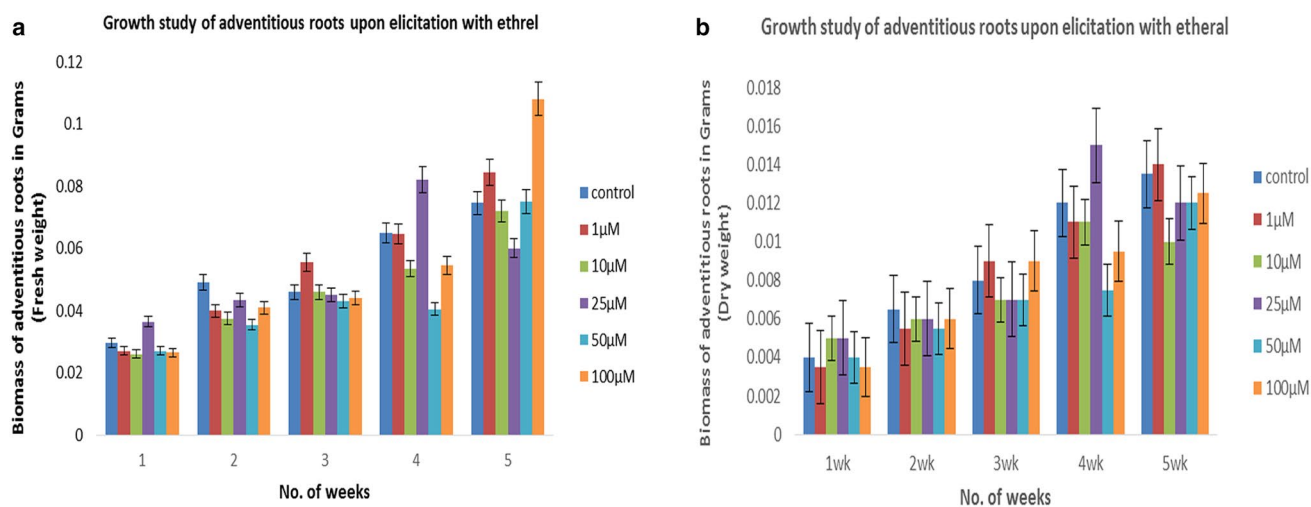
is more in the 3rd week. The adventitious roots cultured in BAR released more andrographolide (0.9% DW) followed by LAR (0.8% DW) and CAR (0.7% DW). The andrographolide from SAR medium could not be extracted and observed no peak in the HPLC chromatogram.

### Elicitation of adventitious roots with ethrel

Ethrel elicitation of adventitious roots enhanced the biomass accumulation as well as andrographolide content. The highest biomass accumulation (0.108 g FW) was observed in the 5th week in 100  $\mu$ M ethrel concentration (Fig. 7a). The highest dry weight (0.015 g DW) accumulation was observed in 25  $\mu$ M in the 4th week (Fig. 7b). 1 and 25  $\mu$ M ethrel concentrations also enhanced the adventitious root biomass



**Fig. 6** **a** Estimation of andrographolide content in adventitious roots developed from different liquid culture techniques. **b** Estimation of andrographolide from the liquid medium of different culture techniques (CAR, BAR and LAR)



**Fig. 7** Growth study of adventitious roots for 5 weeks upon elicitation. **a** Fresh weight comparison **b** Dry weight comparison

by (0.082 g FW) in the 5th week and 0.0845 g FW in the 4th week. A fivefold increase of andrographolide content (1.12% DW) was observed in 50  $\mu$ M ethrel elicited adventitious roots followed by fourfold increase (0.86% DW) in 100  $\mu$ M ethrel elicited roots (Fig. 8). The statistical analysis of the experiments revealed the significance of all the experiments was  $\leq 0.05$ .

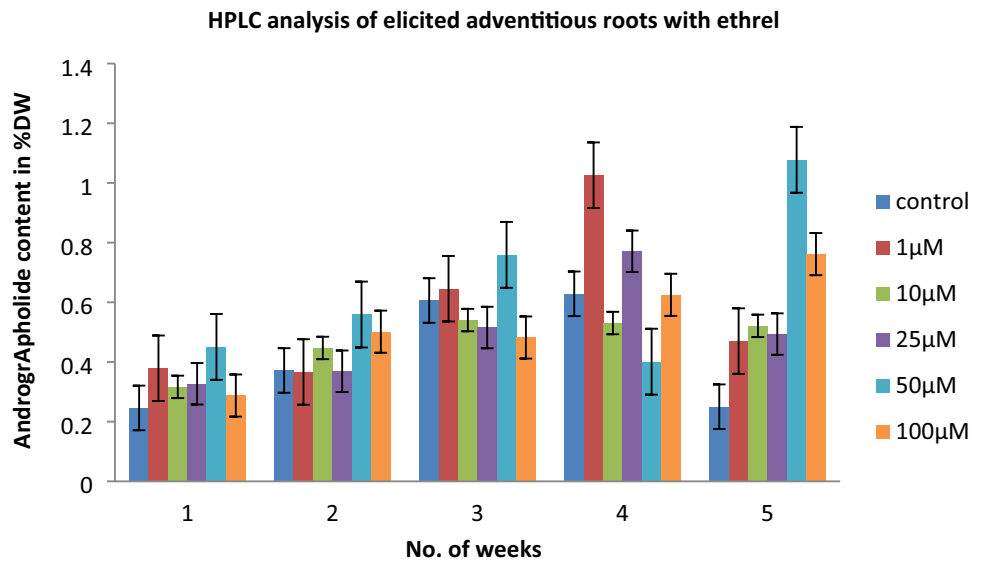
### Effect of light on andrographolide production and differential expression of its biosynthetic pathway genes in *A. paniculata*

The adventitious roots incubated under white light have shown morphological changes and andrographolide

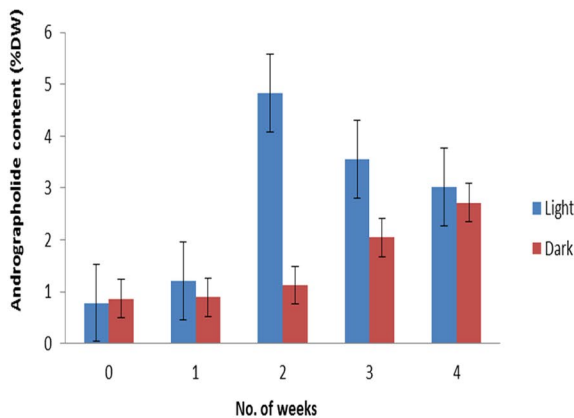
content. The dark induced AR was found to be white, long, dispersed and turned to green under light incubation over the time. The HPLC analysis revealed high andrographolide content was observed at second week (4.291-fold) in the light incubated AR (Fig. 9a). The differential gene expression analysis carried out using RT-PCR revealed that the MEP pathway genes, DXS, DXR, HDS and HDS were gradually up regulated (up to 5.5 fold) in the light incubated AR until 4th week. In the dark incubated AR, only MVA pathway genes (HMGR, HMGS) were highly expressed (up to 3.5 fold). It is observed that downstream pathway gene GGPS expression is similar in both light and dark conditions (Fig. 9b).



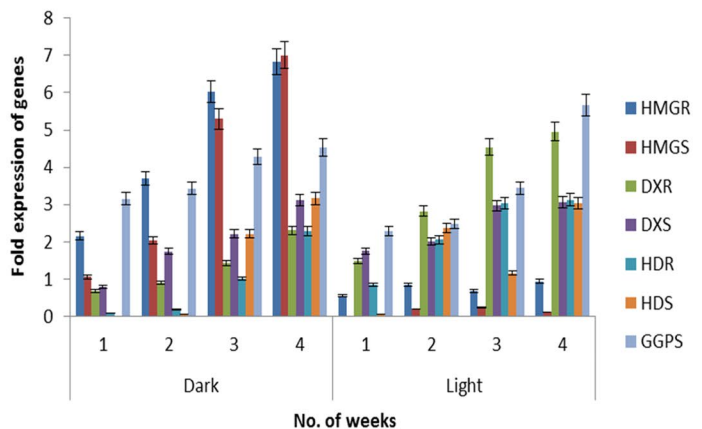
**Fig. 8** Graph showing the andrographolide content in adventitious roots elicited with different concentrations of ethrel up to 5 weeks



**a** Effect of light on andrographolide production in adventitious root culture of *A. paniculata*



**b** Differential expression of andrographolide biosynthetic pathway gene under light and dark



**Fig. 9** **a** Differences in andrographolide content among light and dark induced adventitious root cultures; **b** Differential expression of MVA (HMGR, HMGS), MEP (DXS, DXR, HDS, HDR) and downstream

(GGPS) pathway genes of light and dark incubated ARC for andrographolide production

## Discussion

In the present study, we have developed an efficient protocol for the induction of in vitro adventitious root cultures (ARC) system of *A. paniculata* to enhance the production of andrographolide (AD) as well as AR biomass. ARC system is proved as the best alternate way for the production of various secondary metabolites in different medicinal plants such as *Panax quinquefolius*, *Astragalus membranaceus*, *Oplopanax elatus*, *Morinda coreia*, *Panax ginseng* (Lee et al. 2018; Jin et al. 2019; Han et al. 2019; Kannan et al. 2021) and other industrial important compounds (da Silva et al. 2021; Fan et al. 2021; Rodrigues et al. 2021).

The germination frequency of seeds sown in the net house was observed to be less compared to in vitro. The immature *A. paniculata* seeds has shown high germination frequency compared to mature seeds. The type of seed used for germination has an impact on breaking seed dormancy. It is easy to overcome seed dormancy and achieve high germination frequency using immature seeds within short span of time when conducting in vitro experiments (Lekamge et al. 2020; Dinarti et al. 2021). The different explants displayed varied capacity of AR induction although the adventitious roots were successfully induced from all the explants such as cotyledons, hypocotyls and roots. From our study, root explant generated a higher number of adventitious roots than hypocotyls and cotyledons/leaf explants for the production

of biomass. The adventitious roots induced from different explants of *A. paniculata* were subjected to HPLC analysis to study the effect of explants on andrographolide content. We found a higher andrographolide content in the AR induced from cotyledon explants followed by stem and root. There by, it is suggested to use cotyledon/leaf derived AR for andrographolide production. The earlier studies also revealed a similar correlation between biomass and production of secondary metabolites in ARs induced from different explants of *Polygonum multiflorum* (Ho et al. 2019). A similar type of study was also conducted in *Periploca sepium* Bunge (Zhang et al. 2011) and *Camellia japonica* (Jang et al. 2019) to find out the efficiency of different explant for the induction of ARs and production of periplocin. Induction and submerged cultivation of *Valeriana jatamansi* adventitious root cultures for production of valerenic acids and its derivatives was reported (Gehlot et al. 2022).

The auxins such as NAA, IAA and IBA are used alone and/or in combination with low concentration of cytokines promoted AR induction. From our study, it is also observed that NAA is a preferred auxin for the induction of AR than other auxins IAA and IBA. The implications of different auxins on induction of ARs from our study is supported by earlier studies by Simao et al. (2015) in *Passiflora pohlii* Mast., Deepthi and Satheshkumar (2017) in *Ophiorrhiza mungos*, Manokari and Shekhawat (2016) in *Couroupita guianensis* Aubl and Kannan et al. (2021) in *Morinda coreia*.

Further, different culture techniques were evaluated for their efficacy towards enhancement of biomass. With the initial inoculum weight of 1.23 g a 4.45 fold increase (5.48 gm) in biomass was observed after 4 weeks of culturing by CAR. This is more (1.04 fold) than the previous report by Zaheer and Giri (2017) in 4 weeks of culturing on solid medium. Zaheer and Giri reported 0.085 g of fresh weight from 0.02 g of initial inoculum in 4 weeks and regular sub-culturing up to 20 weeks could generate substantial amounts of biomass (1200 fold). The different culture techniques in the present study promoted the enhancement of AR biomass as well as AD content. Additionally, growing the AR in the liquid media facilitated the release of AD in to the liquid medium. Cai et al (2012) in their review explained in detail the benefits of product release in to medium. Cannabionoid metabolism was intensified under the influence of light in *Cannabis sativa* L. (Danziger and Bernstein 2021). According to Cai et al (2012) the efficiency of product isolation and purification will be increased with release of the product in to the medium. The optimization of liquid AR cultures for the production of gentiopicroside in *Gentiana kurroo* Royle was achieved by Alphonse and Thiagarajan (2021). The combination of elicitation and different culture techniques were implemented in *Tripterygium wilfordii* Hook and *Rubia tinctorum* L. to improve the triptolide, wilforgine, wilforine and anthraquinone production as well as product

recovery (Miao et al. 2017; Perassolo et al. 2017). Furthermore, The adventitious roots grown in different culture techniques in liquid medium in the present study displayed different growth patterns and structures. It was observed that initially LAR and CAR has more andrographolide production than BAR and SAR but at the end of 4 weeks high amount of andrographolide was observed in BAR and SAR. The growth techniques such as CAR and BAR which accumulated higher biomass compared to LAR indicated that aeration and support are the crucial aspects for the efficient growth and AD production. An orthogonal test established by Wang and Qi (2010) also proved the importance of aeration for the enhancement of AR biomass in *Pseudostellaria heterophylla*. Ruta et al (2020) proved the temporary immersion system for the in vitro plant tissue culture was best to enhance biomass as well as secondary metabolite production in *Lycium barbarum* L. The present work describes a novel way of liquid media culture of ARCs, which could be extrapolated for small scale to large scale bioreactor experiments. The bioreactor cultivation of adventitious roots in Malaysian ginseng (*Eurycoma longifolia* Jack) and soybean (*Glycine max*) in small scale bioreactor resulted in the highest biomass and excellent secondary metabolites production of total phenolics, flavonoids and coumestrol (Cui et al. 2019; Lee et al. 2020). There are many other studies in which industrially high demand secondary metabolites were produced using ARCs in large scale. The biomass and production of flavonoid was achieved in Balloon-type Bubble-bioreactor using ARCs of *Gynura procumbens* (L.). Merr (Manuhara et al. 2019; Faizah et al. 2018). The improvement of bio-active saponin accumulation in bioreactor with ARCs of *Panax vietnamensis* was achieved by Linh et al. (2019). But the agitation and aeration in the bioreactor could adversely affect viability of the cultures and influence the biomass production (Jang et al. 2016). Hence, in our study, we have developed a improved, modified liquid culture technique where the AR inoculum will be placed on cotton or blotting papers immersed in the liquid medium. Here, the inoculum is devoid of anaerobic conditions and absorbs the nutrients from liquid medium. Hence, the present techniques could be a suitable alternative to overcome shear stress caused by agitation. In addition, enhanced biomass was observed to be equal to the amount of biomass produced using solid cultures. The BAR and LAR liquid culture systems could be used as alternative to SAR where the maintenance is easy.

Furthermore, the effect of elicitor for the production of biomass and AD was carried out using in vitro ARC. In the present investigation, elicitation with ethrel enhanced the biomass as well as AD in ARC of *A. paniculata*. The growth study of elicited AR for 5 weeks revealed no growth inhibition until 3 weeks. The highest fresh weight was observed at the 5th week in 100  $\mu$ M followed by 1 and 25  $\mu$ M in the 4th week. The growth study of ethrel elicited adventitious roots

shows there is no negative effect of elicitor on the adventitious root growth unlike other elicitors. Moreover, there was high growth observed in 25  $\mu\text{M}$  (4th week; 1.25-fold more than the control) and in 100  $\mu\text{M}$  (5th week; 1.5-fold more than the control). In the earlier study, the quantities of aloe emodin and chrysophanol were enhanced 5 and fourfold respectively by applying ethrel (500  $\mu\text{M}$ ) elicitation (Lee et al. 2013). Unlike Bulgako et al. (2002) study in *Rubia cordifolia*, our study enhanced both biomass as well as AD content. Ethrel in 10–100  $\mu\text{M}$  used in the Bulgako study not involved in the enhancement of anthraquinones. Ethrel elicitation of hairy root cultures of *Prunella vulgaris* and black carrot resulted in a 1.66 fold increase of rosmarinic acid and 82% more anthocyanin and 20% more hydroxycinnamic acid (Ru et al. 2016; Barba-Espin et al. 2020). Ethrel treatment of cell suspension cultures of *Vitis vinifera* enhanced the anthocyanin content up to 2.3 fold and phytoalexin up to 10.8 nmol.mg<sup>-1</sup> DW (Faurie et al. 2009; Saw et al. 2012). Ethrel treated roots of black carrot and *Lemna paucicostata* has shown highest anthocyanin concentration (1.17 more) and  $\gamma$ -aminobutyric acid ( $5.014 \pm 1.372$  mg/L) and ferulic acid ( $0.640 \pm 0.071$  mg/L) than untreated roots, with no negative effect on root fresh weight and dry weight (Barba-Espin et al. 2017; Kim et al. 2020). In general, elicitation with chemical elicitors in high concentrations negatively affects the growth of the cultures though enhances the secondary metabolite production. But here, ethrel promoted biomass unlike other chemical elicitors even in high concentrations along with AD production. The elicitation with ethrel could overcome the problem of growth inhibition which is a common in the chemical elicitation experiments across species (Bae et al. 2006; Ru et al. 2016).

The effect of light on the expression of AD biosynthetic pathway genes and AD production was elucidated. The incubation of ARC under dark and light brought significant morphological differences, growth patterns, differential expression of andrographolide biosynthetic pathway genes and andrographolide. In the dark ARC, MVA pathway genes played a crucial role in the production of AD whereas in the light cultures MEP pathway taken over the biosynthesis of AD. The genes responsible for important rate limiting and related enzymes were up regulated in the light cultures. In both dark and light conditions it was observed that rate limiting enzymes (HMGR and DXR) of the respective pathways (MVA and MEP) were highly expressed. The HDR and HDS genes were less expressed than other genes. In addition, the gene encoding downstream enzyme GGPS was also up regulated compared to dark cultures. The up regulation of MEP pathway genes and GGPS gene was correlated with enhanced AD production by HPLC analysis of the ARCs. As mentioned by Escobar-Bravo et al. (2017), light plays a crucial role in plant growth and metabolism. Several studies also proved the direct correlation between

light and secondary metabolites production. The duration of photoperiod and effect of different spectral lights in line with elicitation increased steviol glycosides, phenolics, and flavonoid contents in the ARCs of *Stevia rebaudiana* (Idrees et al. 2018; Alvarado-Orea et al. 2020) and *Ajuga bracteosa* (Ali et al. 2019). The biomass and hypericins (used in depression treatment and photodynamic therapy) accumulation was enhanced in *Hypericum perforatum* by the treatment of red light and darkness (Sobhani Najafabadi et al. 2019). Effect of darkness treatment on the morphology, hormone status and gene expression of developing adventitious root in apple rootstock has also been studied (Li et al. 2022). Here in our study, incubation under light changed the AR morphology where the roots became thick, turned to green unlike thin, short dark AR.

## Conclusion

In order to overcome the bottlenecks to meet the market demands of secondary metabolites, plant tissue and organ culture is the best alternative approach for mass production of biomass and subsequent production of secondary metabolites in vitro. In the present study, we have evaluated different parameters to obtain more number of ARs productions, biomass enhancement and production of andrographolide using different liquid culture techniques. Our results suggests one has to be selective in choosing the explant and auxin, for the efficient induction of ARC and follow different culture conditions/techniques according to the requirements as well as elicitation techniques for the effective biomass and AD production. Cotyledon explants were more suitable to generate high AD producing adventitious root cultures where as NAA in 1 mg/L is ideal for the adventitious root induction. Growing the adventitious roots in modified liquid culturing techniques (CAR/BAR) is favors the high biomass proliferation than growing the adventitious roots directly in liquid medium. Using ethrel as an elicitor not only enhanced the AD content but also minimized the growth inhibition which is a common problem in elicitation even in higher concentrations. The influence of light on the expression of andrographolide biosynthetic pathway genes and shift change from MVA to MEP pathway with regard to andrographolide biosynthesis was identified. The light factor plays a crucial role in regulating the biosynthesis of andrographolide that is partitioned between matching parallel MVA and MEP pathways.

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**Data availability** All data generated or analysed during this study are included in this published article.

**Code availability** N/A.

**Material availability** N/A.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** N/A.

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