Protoplasma (2018) 255:1387–1400
https://doi.org/10.1007/s00709-018-1211-7

ORIGINAL ARTICLE

# Anti-cancer labdane diterpenoids from adventitious roots of *Andrographis paniculata*: augmentation of production prospect endowed with pathway gene expression

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Received: 5 October 2017 / Accepted: 14 January 2018 / Published online: 20 March 2018


**Abstract**
Andrographolide (AD) is the time-honoured pharmacologically active constituent of the traditionally renowned medicinal plant—*Andrographis paniculata*. Advancements in the target-oriented drug discovery process have further unravelled the immense therapeutic credibility of another unique molecule—neoandrographolide (NAD). The escalated market demand of these anti-cancer diterpenes is increasingly facing unrelenting hurdles of demand and supply disparity, attributable to their limited yield. Callus and adventitious root cultures were generated to explore their biosynthetic potentials which first time revealed NAD production along with AD. Optimization of the types and concentrations of auxins along with media form and cultivation time led to the successful tuning towards establishing adventitious roots as a superior production alternative for both AD/NAD. Supplementation of IBA to the NAA + Kn-containing MS medium boosted the overall growth and AD/NAD synthesis in the adventitious roots. Compared to control leaves, the adventitious root exhibited about 2.61- and 8.8-fold higher contents of AD and NAD, respectively. The qRT-PCR involving nine key pathway genes was studied, which revealed upregulation of *GGPS1* and *HMGR1/2* genes and downregulation of *DXS1/2* and *HDR1/2* genes in the adventitious root as compared to that in the control leaves. Such observations highlight that in vitro cultures can serve as efficient production alternatives for AD/NAD as the cytosolic genes (*HMGR1/*2 of MVA pathway) are competent enough to take over from the plastidial genes (*DXS1/*2 and *HDR1/2* of MEP pathway), provided the accredited first branch-point regulatory gene (*GGPS*) expression and the culture requirements are optimally fulfilled.

**Keywords** *Andrographis paniculata* · Adventitious roots · Andrographolide · Biosynthetic pathway genes · Neoandrographolide · qRT-PCR

## Introduction

The ancient knowledge of the beneficial medicinal properties of plants have traversed a long way since time immemorial to attain the current thriving global platform where bioactive natural products or its derivatives have touched a new height in curing complex human illness (Atanasov et al. 2015; Jachak and Saklani 2007). Advancement of the drug discovery process based on modern molecular modelling, combinatorial chemistry and target-oriented pharmacokinetic analysis techniques have unravelled huge clinical potentials of numerous age-old bioactive phytomolecules to address several alarming health issues of modern days (Cordell and Colvard 2012). This narrative holds immense significance relating to a traditionally renowned medicinal plant—*Andrographis paniculata* (Burm. f.) Wall. ex. Nees, which has witnessed a creditable expansion of its therapeutic applications from its conventional relevance in Indian and Oriental medicines (Varma et al. 2009) to its diverse modern efficacious therapeutic leads owing to the presence of its unique bioactive secondary metabolites (Subramanian et al. 2012).

The India Herbal Pharmacopoeia and World Health Organization (WHO) monographs (Lim et al. 2012) have recognized the traditional therapeutic uses of this commonly

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Handling Editor: Peter Nick

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known "king of bitters" plant against diverse ailments, such as common cold, dysentery, fever, tonsillitis, diarrhoea, liver diseases, upper respiratory tract infections, inflammation, herpes and several other chronic and infectious disorders (Subramanian et al. 2012; Thakur et al. 2015; Varma et al. 2009). Andrographolide, a major *ent*-labdane diterpenoid (a bicyclic diterpenoid lactone) of the isoprenoid family of natural products, is believed to be the main bioactive ingredient of *A. paniculata* that predominantly fuelled most of its reported pharmacological activities (Hossain et al. 2014; Pholphana et al. 2004). Advancements in the target-oriented drug discovery process have further expanded the therapeutic applicability of this major constituent (i.e. andrographolide) towards complex disease conditions by demonstrating its incomparable and reputable efficacies relying on its antioxidant, immunostimulatory hepatoprotective, cardioprotective, hypoglycaemic, anti-cancer, anti-inflammatory, anti-rheumatoidal, anti-malaria, anti-leishmanial, anti-fertility, anti-obesity, anti-pyretic, anti-fungal and anti-bacterial attributes (Zaid et al. 2015; Sharma et al. 2017). This phytomolecule has also been acknowledged to possess remarkable anti-virus activity against a vast array of viruses including human papilloma pseudovirus, influenza A, hepatitis B/C, herpes simplex and human immunodeficiency virus (Wintachai et al. 2015). A recent report demonstrating the notable potential of andrographolide as an inhibitor of chikungunya infection by inhibiting replication of the virus without any noticeable cytotoxicity in a cell culture system has further substantiated the enormous future utility of this molecule pertaining to this specific as well as other virus-borne diseases that lack any specific treatment regime (Wintachai et al. 2015).

In addition to this molecule (andrographolide), the immense pharmacological qualifications of *A. paniculata* are also known to be contributed by another time-honoured molecule, known as neoandrographolide—a diterpene glucoside. Besides exhibiting strong free radical scavenging activity (Kamdem et al. 2002), this molecule is renowned for demonstrating stronger anti-inflammatory activity than andrographolide by inhibiting nitric oxide production both in vitro and ex vivo (Batkhuu et al. 2002). Moreover, neoandrographolide has also revealed natural chemosensitizing potential (Pfisterer et al. 2010) and viricidal activity against herpes simplex virus 1 (HSV-1) without significant cytotoxicity (Wiart et al. 2005), which has drawn significant research attention towards this molecule to unravel its hidden medicinal values. Notable anti-malarial activity of neoandrographolide and its hepatoprotective effects against carbon tetrachloride have also heightened the therapeutic credibility of this diterpene glucoside molecule towards the immense pharmacological qualifications of *A. paniculata* (Pholphana et al. 2013).

The unique and at the same time multifaceted pharmacological activity of the major bioactive constituent of *A. paniculata* has significantly escalated its market demand, which is increasingly facing the unrelenting hurdles of demand and supply disparity. On top of all the prior stated therapeutic advantages, notable anti-cancer merits of andrographolide and neoandrographolide and their semi-synthetic analogues against diverse forms of cancer cell lines (mainly breast cancer, colon cancer, ovarian cancer and prostate cancer) have gained escalating global attention over the years as evident in a patent review document (Aromdee 2014). Multiple issues, such as dependency on specific geographical/seasonal/environmental conditions in combination with the inescapable fluctuations in the levels of the active metabolite and anthropic pressure, are imposing the major hurdles (Atanasov et al. 2015; Pholphana et al. 2013; Zaheer and Giri 2015).

The prospect of exploiting the plant cell and tissue culture systems as stable and economically rewarding production substitutes to classical approaches (i.e. natural collection and chemical synthesis) has already been explored at several instances to address such impediments, which hold great promise for controlled production of innumerable purposeful secondary metabolites (Georgiev 2013). Amongst the different operational in vitro culture alternatives, adventitious root culture has gained enormous popularity over the years, not only because of its ability to overcome the cell culture-linked production instabilities, but also on account of its better amenability to large-scale bioreactor systems (Ahmad et al. 2015; Murthy et al. 2016). Adventitious root cultures have already proved highly proficient in serving as a steady commercial resource of contamination-free, authentic quality plant-based pharmaceutical compounds for their round-the-year supply involving diverse medicinal plants (Murthy et al. 2016; Naseem et al. 2015).

In case of *A. paniculata*, callus and suspension cultures have gained maximum priority to serve as alternative production source of andrographolide (Gandi et al. 2012; Sharma and Jha 2012; Sharmila et al. 2012; Vakil and Mendhulkar 2013; Vidyalakshmi and Ananthi 2013), whereas adventitious root cultures remained relatively obscured (Praveen et al. 2009; Sharma et al. 2013; Zaheer and Giri 2017) indicating the scope of its further explorations. In view of the well-established fact that the regulation and expression kinetics of key pathway enzymes actually govern the overall potential of any cell culture system, it was envisaged during the present course of study to emulate the molecular basis of andrographolide and neoandrographolide accumulation in the adventitious roots and calli of *A. paniculata*.

Literature survey revealed that terpene metabolites are produced from two 5C isoprenoid building blocks, dimethylallyl diphosphate (*DMAPP*) and isopentenyl diphosphate (*IPP*), that are biosynthesized via two independent biosynthetic pathways in plants: plastidial 2C-methyl-D-erythritol-4-phosphate (MEP) pathway and cytosolic mevalonic acid (MVA)

pathway (Garg et al. 2015; Fig. 1). The gene responsible for the first dedicated rate-limiting enzyme of the cytosolic MVA pathway had been identified to be hydroxymethylglutaryl-CoA reductase (*HMGR*), while 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) had been designated for the same function concerning the plastidial MEP pathway (Cordoba et al. 2009; Fig. 1). The active role of the 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*HDR*) gene as the penultimate regulatory gene of the MEP pathway has also gained substantial credit towards the IPP and DMAPP synthesis (Cordoba et al. 2009). Additionally, the decisive role of *GGPS* towards the biosynthesis of AD and NAD had also been established earlier, which chiefly catalyzes *DMAPP* and *IPP* to the major $C_{20}$ compound precursor—geranylgeranyl diphosphate (GGPP) (Srivastava and Akhila 2010; Sharma et al. 2015).

Previous report suggested a major role of the MEP pathway and a relatively minor role of the MVA pathway towards the supply of the 5C isoprenoid precursors for the biosynthesis of andrographolide in *A. paniculata* (Srivastava and Akhila 2010). However, ample evidences affirmed the cross talk between these two pathways for the biosynthesis of diterpenes (De-Eknamkul and Potduang 2003; Paetzold et al. 2010; Schramek et al. 2010; Srivastava and Akhila 2010). In the background of such revelations, the transcript expression pattern of the genes encoding enzymes of the diterpenoid biosynthetic pathway, such as *DXS1/DXS2* and *HDR1/HDR2* of the MEP pathway, *HMGR1/HMGR2* of the MVA pathway, and *GGPS1/GGPS2* and *IPP* of the central pathway, had been undertaken during the present investigation through qRT-PCR to comprehend the molecular basis of AD and NAD accumulation in the adventitious roots and calli of *A. paniculata* (Fig. 1).

## Materials and methods

### Plant material

*Andrographis paniculata* (CIM-Megha) plants, grown at the Institute's field, were used as source of explants for the in vitro culture establishment.

### Chemicals

The standards andrographolide (AD) and neo-andrographolide (NAD) and plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kn), α-naphthaleneacetic acid (NAA), benzylaminopurine (BAP) and indole-3-butryic acid (IBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents were procured from Merck (Mumbai, India).

### In vitro establishment, callus induction and adventitious root formation

The nodal explants from the field-grown plants of *A. paniculata* (CIM-Megha) were utilized for the in vitro establishment of sterile cultures, which were carried out

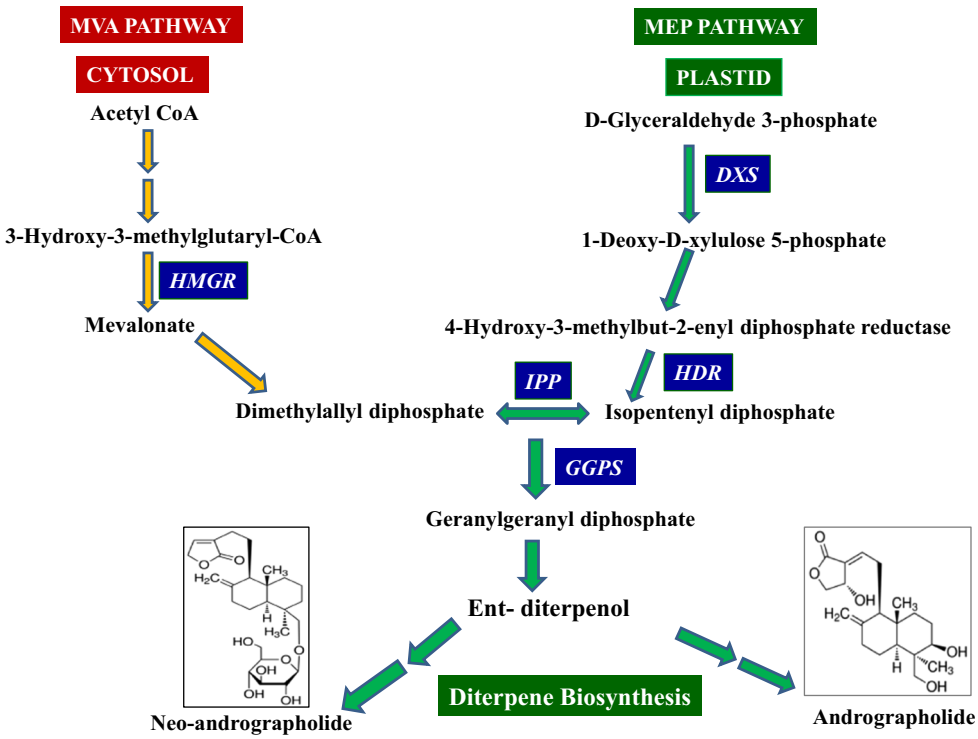


**Fig. 1** Schematic representation of the biosynthetic pathway of andrographolide in *A. paniculata*. The pathway genes selected for the qRT-PCR are shown in the boxes: deoxy-D-xylulose synthase (*DXS*), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*HDR*), hydroxymethylglutaryl-CoA reductase (*HMGR*), geranylgeranyl pyrophosphate (*GGPS*) and isopentenyl pyrophosphate synthase (*IPP*)

following previously reported protocol (Pandey et al. 2015). In brief, the explants were surface sterilized with 0.1% (*w*/*v*) $HgCl_2$ for 2 min and rinsed with autoclaved distilled water for four to five times and then cultured on MS (Murashige and Skoog 1962) medium containing 3% (*w*/*v*) sucrose, 0.8% (*w*/*v*) agar (HiMedia, India) and four different concentrations of BAP (0.5 to 2 mg/l). The pH of the media was adjusted to 5.88 prior to autoclaving at 121 °C and 15 lb pressure for 20 min, and the cultures were maintained at 25 ± 2 °C.

The leaf explants from these in vitro grown plants were inoculated on MS semi-solid medium containing the aforementioned concentration of sucrose and agar, supplemented with either NAA or 2,4-D (1.0 to 3.0 mg/l), along with a fixed concentration of Kn (0.25 mg/l) for the induction of callus cultures. The best-performing and fast-growing callus cultures were subsequently transferred to half-strength liquid MS medium supplemented with three different concentrations of NAA (1.0 to 3.0 mg/l) + a fixed concentration of Kn (0.25 mg/l). The established adventitious roots were afterwards multiplied in the best-responding media formulation consisting of half-strength liquid MS medium with 3 mg/l NAA + 0. 5 mg/l Kn designated as "N" along with/without the addition of three different concentrations of IBA (0.5 to 1.5 mg/l) designated as "P" for multiplication of the adventitious roots. The growth indices (GI) of the calli and both the adventitious roots cultures ("N" and "P") were determined on the basis of dry weight (DW) at the intervals of 4 and 8 weeks of cultivation by adopting our earlier published formula (Pandey et al. 2015).

### Extraction and HPLC analysis

The adventitious roots and the calli, cultivated at two different time intervals (4 and 8 weeks) in the best-responding media (N and P), were harvested, weighed, dried, powdered and extracted (~ 2 g each) with methanol (MeOH × four times). Likewise, the leaves from the normal field-grown plant (2 months) were also dried, powdered and extracted with MeOH. All the extracts were concentrated in vacuo and utilized for the quantitative analysis of AD and NAD. The quantification of these two targeted metabolites was performed through reversed-phase HPLC using a $C_{18}$ column (250 × 4.6 mm, Waters) with mobile phase consisting of solvent A as acetonitrile and solvent B as water (90:10 *v*/*v*) in isocratic elution mode with a flow rate of 0.8 ml/min. All the extracts were redissolved in 1 ml MeOH (HPLC grade) and filtered through a syringe filter (0.22 μm, Millipore) before injection. The injection volume was 15 μl, and detector wavelength was 210 nm. The stock solution of the standards AD and NAD (1 mg/ml) were prepared in HPLC grade methanol and identified in the samples by comparing its UV absorbance and retention time.

### RNA isolation and qRT-PCR

The expression analysis of nine triterpenoid pathway genes viz. deoxy-D-xylulose synthase (*DXS*1 and *DXS*2), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*HDR1* and *HDR2*), hydroxymethylglutaryl-CoA reductase (*HMGR*1 and *HMGR*2), geranylgeranyl pyrophosphate (*GGPS*1 and *GGPS*2) and isopentenyl pyrophosphate synthase (*IPP*) was investigated by quantitative real-time PCR in the callus and the adventitious root cultures of *A. paniculata.* The RNA was isolated from the calli and the adventitious root cultures upon their cultivation for 4 and 8 weeks in the best-responding media ("N" and "P") and purified following the reported protocol (Misra et al. 2015). The RNA yield was determined by using the NanoDrop (Thermo Scientific), and simultaneously cDNA was prepared using 2 μg of total RNA by following the manufacturer's instructions (Invitrogen). Consequently, the cDNA of all the samples was diluted for qRT-PCR reactions, which was carried out following the published protocol (Misra et al. 2015) using 7900HT Fast Real-Time PCR (Applied Biosystems). The oligonucleotide primers used for the present study are listed in Table 1. The relative gene expression was analysed using actin as the endogenous control, following the $2^{-\Delta\Delta}$Ct (cycle threshold) method.

### Statistical analysis

The data were expressed as mean ± SD of three independent tests ($n = 3$). Mean and SD were calculated with the help of MS Office Excel version 2007.

## Results and discussion

### In vitro multiplication and callus induction

The nodal explants of *A. paniculata* demonstrated axillary bud proliferation response under in vitro conditions with all the three tested concentrations of BAP (0.5 to 1.5 mg/l), but the higher concentration of BAP proved ineffective on prolongation of the culture period as hyperhydricity occurred (data not presented). This observation substantiates earlier report where lower concentrations of BAP proved effective for the successful in vitro multiplication of *A. paniculata* (Dandin and Murthy 2012). Callus cultures were initiated from the leaves of the in vitro raised plants upon their inoculation on MS semi-solid medium, supplemented with NAA/2,4-D (1.0 to 3.0 mg/l), along with the fixed concentration of Kn (0.25 mg/l). All the applied concentrations resulted in the formation of calli (Table 2) with distinct differences in their textures and morphologies.

Amongst the different tested hormonal combinations, the NAA (3 mg/l) + Kn (0.25 mg/l) combination was found to be

**Table 1** Oligonucleotides utilized for qRT-PCR

| Genes | Forward primers (5′–3′) | Reverse primers (5′–3′) |
|---|---|---|
| MVA pathway | | |
| *HMGR1* | CATGGAAGTGATCGGCTTATCC | GGCTTCGCACACCACTGATT |
| *HMGR2* | CGGTCAATGACGGAAAGGA | CCAACCGTACCAACCTCTATGG |
| MEP pathway | | |
| *DXS1* | TGGCTGTGGGAAGGGATCT | GCCGGCTATCATAGCACCAT |
| *DXS2* | GACATTCGCTGCGGGTCTAG | CCCGGTCCATGATGAACCT |
| *HDR1* | TTGACCTAGAATGGCGATTTCTC | GGTATCCGGCAAGGAAAGCT |
| *HDR2* | GGGCTGTCCAGATTGCTTATG | TCCATCTCTTCGAGCCTCTGA |
| Central pathway | | |
| *GGPS1* | GGCCCACAAACCACAAGGT | TGGCCCTGACTACTCTGCAA |
| *GGPS2* | GGAAACCGACGAACCACAAG | CAACTCACCAATGGCGGATAC |
| *IPP* | GTCCCCGTCGATCAATTCAC | CGTGCTCTCCCCATTTTCC |
| Endogenous control | | |
| *Actin* | ACGATGTTCACGGGCATTG | GAGCCACCACCTTGATCTTCA |

the best optimized medium for the induction of callus on which green compact calli could be obtained (96 ± 2.7%) after 4 weeks of cultivation (Table 2; Fig. 2a). On the other hand, all the tested concentrations of 2,4-D with Kn in the MS semi-solid medium demonstrated fragile white callus formation after the same time intervals amongst which the supplementation with 2,4-D (3 mg/l) and Kn (0.25 mg/l) showed the maximum (86 ± 1.9%) response (Table 2). Literature survey reveals that MS medium supplemented either with 2,4-D or NAA (alone or in combination) proved effective for the induction of callus and suspension cultures in *A. paniculata* (Gandi et al. 2012; Sharma and Jha 2012; Sharma et al. 2015; Sharmila et al. 2012; Vidyalakshmi and Ananthi 2013).

The NAA (3 mg/l) + Kn (0.25 mg/l)-supplemented MS semi-solid medium-derived calli demonstrated spontaneous adventitious root induction upon their transfer to half-strength liquid MS medium supplemented with the same hormonal formulation (Fig. 2b). Such submergence-induced adventitious root initiation from calli is in agreement with already studied facts illustrating cell-fate reprogramming by the use of auxin-mediated cell signalling (Steffens and Rasmussen 2016). As a result, the predetermined cells switch over from their morphogenetic path to act as root primordia for the de novo adventitious root emergence (Murthy et al. 2016; Yu et al. 2017). Such redifferentiation of the calli towards the development of adventitious roots upon their transfer to liquid medium might have been triggered by submergence-mediated entrapment of ethylene production as documented previously (Steffens and Rasmussen 2016).

**Table 2** Percentage of callus induction in leaf explants of *A. paniculata* under different concentrations of NAA or 2,4-D in combination with kinetin (Kn) after 4 weeks of culture

| Growth regulators (mg/l) | | Callus induction (%) |
|---|---|---|
| NAA | Kn | |
| 1 | 0.25 | 91 ± 2.9 |
| 2 | 0.25 | 93 ± 3.1 |
| 3 | 0.25 | 96 ± 2.7 |
| 2,4-D | Kn | |
| 1 | 0.25 | 79 ± 2.1 |
| 2 | 0.25 | 85 ± 2.4 |
| 3 | 0.25 | 86 ± 1.9 |

Then again, contrary to the above observation, the calli derived from the 2,4-D (3 mg/l) and Kn (0.25 mg/l)-supplemented MS semi-solid medium remained unresponsive in terms of adventitious root formation even on their transfer to the half-strength liquid MS medium containing either 2,4-D (3 mg/l) + Kn (0.25 mg/l) or NAA (3 mg/l) + Kn (0.25 mg/l). This is in corroboration with the earlier established reality that a species-specific regulation of the accurate type of auxin remains operational in steering the molecular and biochemical changes in the cells towards their dedifferentiation for adventitious root initiation (Yan et al. 2017). Additionally, literature survey has also revealed that NAA has proven to be the most preferred plant growth regulator for cultivation of adventitious roots of diverse plant systems in the liquid medium over semi-solid due to its longer half-life, faster uptake and slower degradation in the plant cells (Saeed et al. 2017; Khan et al. 2017).

During the present course of the study, further optimization was also carried out concerning the multiplication of the established adventitious roots by their cultivation in half-strength liquid MS medium containing NAA (3.0 mg/l) + Kn (0.25 mg/l) along with/without the addition of IBA (0.5 to 1.5 mg/l). The results demonstrated that the addition of IBA at its lowest concentration (0.5 mg/l) stimulated the maximum proliferation of the established adventitious roots (Fig. 2c),

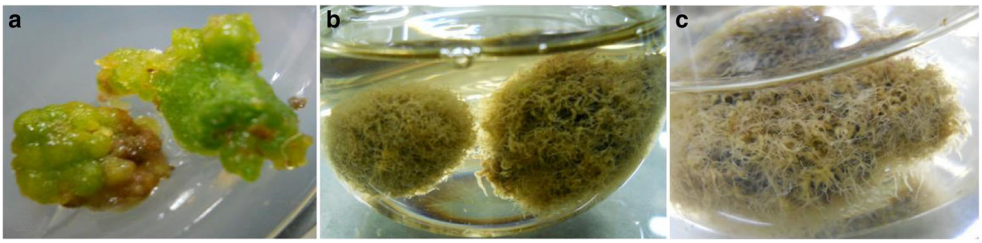


**Fig. 2** **a** Callus culture cultivated in semi-solid MS medium supplemented with 3 mg/l NAA + 0.5 mg/l Kn. **b** Adventitious root cultivated in "N" medium (i.e. half-strength liquid MS medium with 3 mg/l NAA + 0. 5 mg/l Kn). **c** Adventitious root cultivated in "P" medium (i.e. half-strength liquid MS medium with 3 mg/l NAA + 0. 5 mg/l Kn + 0.5 mg/l IBA)

while its higher concentrations caused hyperhydricity. The stimulatory role of IBA in the induction and lateral growth of adventitious roots has been illustrated earlier (Murthy et al. 2016).

## Growth kinetics of callus and adventitious root cultures

The growth kinetic analysis revealed that both the calli and the adventitious root cultures demonstrated their optimum growth indices (GI) on the 8 weeks of cultivation in their respective best medium formulations (Fig. 3). The adventitious roots attained their maximum GI upon their cultivation in the "P" medium (1501.05 ± 19.5), which is 1.1-fold higher than that in the "N" medium (1408.01 ± 18.67), which indicated that the supplementation of IBA in the N medium (resulting to P medium) might have stimulated faster growth and higher biomass yield of the adventitious roots. The superior role of IBA over NAA towards the lateral root proliferation and higher biomass production has earlier been documented in the case of *Panax ginseng* adventitious root cultures (Kim et al. 2003). Then again, the calli showed the maximum GI (684.47 ± 23.8) in the optimized semi-solid MS medium with NAA (3 mg/l) + Kn (0.25 mg/l) (Fig. 3), which is 2.2-fold inferior to the maximum GI of the adventitious root at the maximum growth phase.

## Quantification of AD and NAD through HPLC analysis

The HPLC analysis of the crude samples of adventitious roots (cultivated in N and P media) and calli (cultivated in semi-solid medium containing 3 mg/l NAA + 0.25 mg/l Kn) revealed the presence of both AD and NAD during both the tested growth phases (4 and 8 weeks of culture) with retention times of 3.16 and 5.20 min respectively (Fig. 4). The production of only AD from the callus and cell suspension cultures of *A. paniculata* has earlier been documented in several reports (Gandi et al. 2012; Sharma and Jha 2012; Sharmila et al. 2012; Vidyalakshmi and Ananthi 2013; Zaheer and Giri 2015). Then again, the potential of multiple shoot cultures of *A. paniculata* for AD synthesis had also been explored earlier accompanied by its yield enhancement strategy through elicitation with abiotic elicitors—jasmonic acid and salicylic acid (Zaheer and Giri 2015).

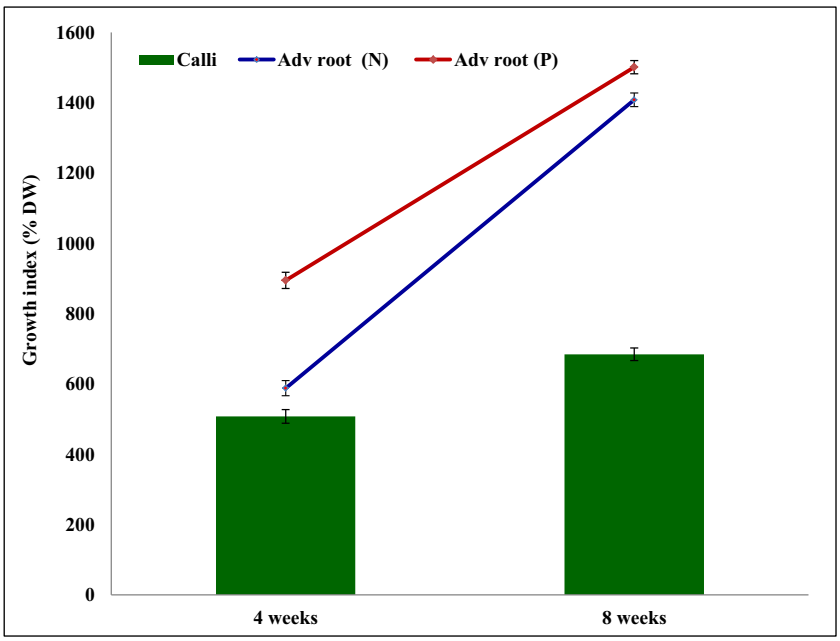


**Fig. 3** Growth indices (% DW) of the calli and adventitious roots cultivated at two different time intervals (4 and 8 weeks). The values are mean of three replicates ± SD

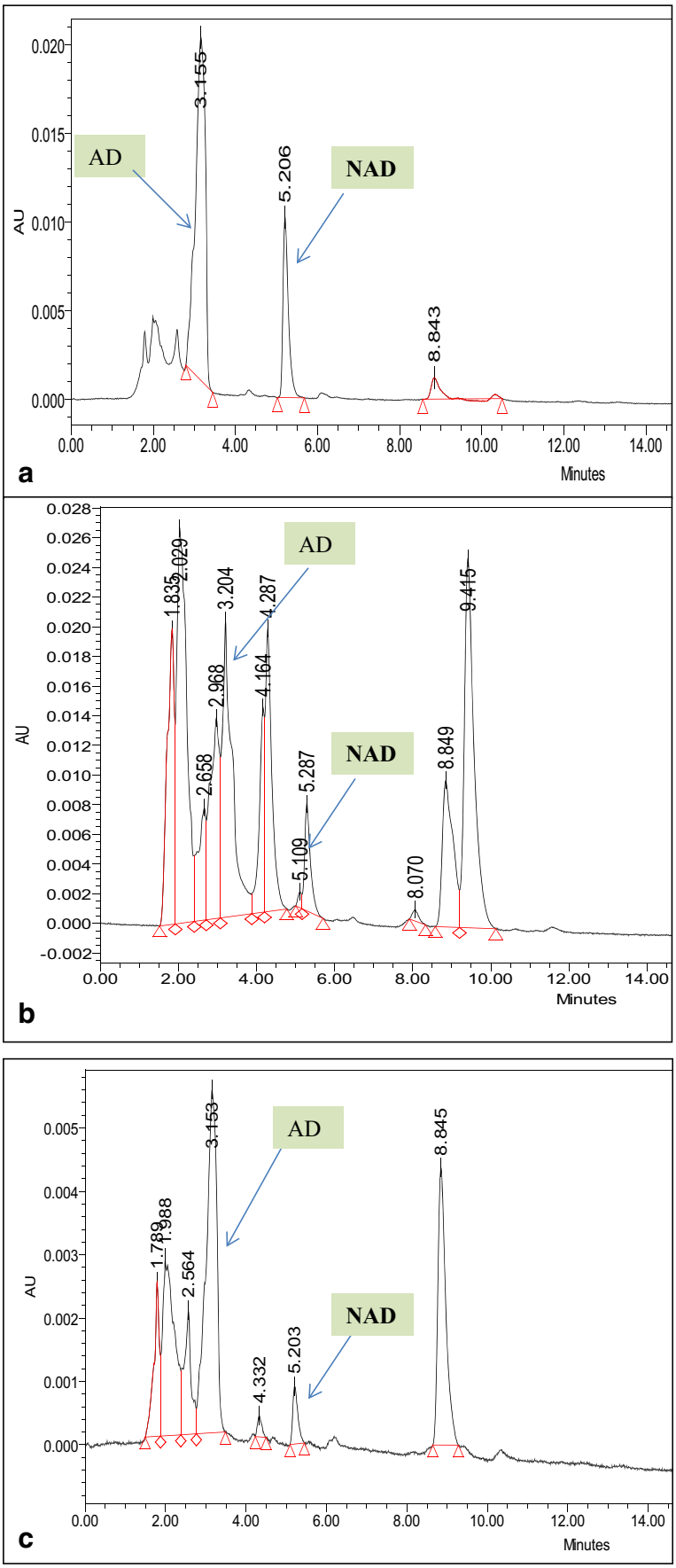


**Fig. 4** Representative HPLC chromatograms of andrographolide (AD) and neoandrographolide (NAD) in the methanolic extract of *A. Paniculata*. **a** Standards of AD and NAD. **b** Crude extract of 8-week-old adventitious roots culture. **c** Crude extract of 8-week-old callus culture

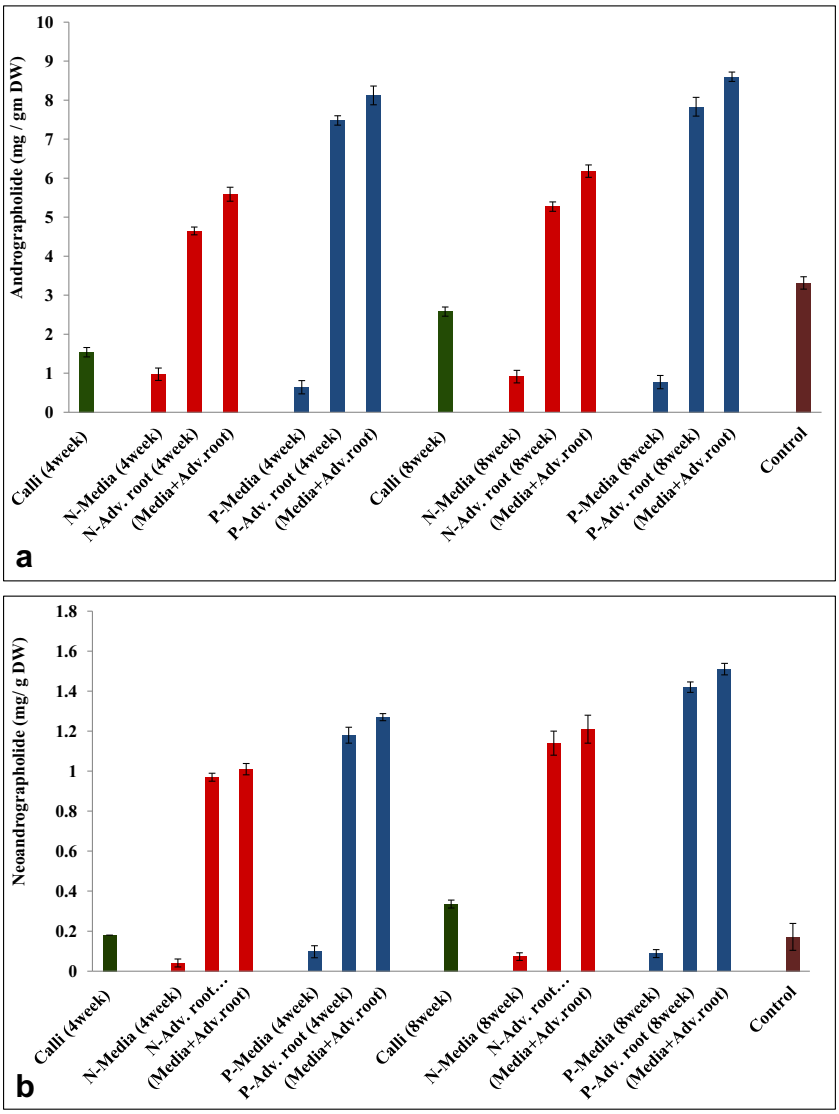


**Fig. 5** **a**, **b** Content of andrographolide (AD) and neoandrographolide (NAD) in the callus culture, adventitious roots and its corresponding medium at different growth phases with respect to that with the control leaves. The values are mean of three replicates ± SD

In the present study, the content of AD remained higher in the adventitious roots which increased from 5.59 ± 0.16 to 6.18 ± 0.24 mg/g DW during the 4 and 8 weeks of cultivation in the liquid N medium (Fig. 5a). Alternatively, the calli showed an increase in the contents of AD from 1.54 ± 0.12 to 2.58 ± 0.17 mg/g DW at the same time intervals upon cultivation in semi-solid NAA (3 mg/l) + Kn (0.5 mg/l)-supplemented MS medium (Fig. 5a). These observations indicated that the content of AD is 2.4-fold higher in the adventitious roots compared to that in the calli of the same age (8 weeks) upon their cultivation with the identical hormonal supplementation. Consecutively, the AD production potential of the adventitious roots could further be improved (8.12 ± 0.18 mg/g DW) upon its cultivation in the P medium (through the addition of 0.5 mg/l IBA in the N medium) which demonstrated a 1.32-fold higher yield with 50% reduction in the cultivation time (4 weeks) as compared to that in the N medium (8 weeks) (Fig. 5a). In accordance with the present findings, the superior influence of IBA over other auxins towards the yield enhancement of secondary metabolites in the adventitious roots had also been documented earlier involving diverse medicinal plants (Kalarija and Paric 2011; Murthy et al. 2016; Singh et al. 2015).

Noticeably, in the present study, the contents of AD remained 2.45-fold higher with 50% times reduction (4 weeks) in the adventitious roots upon its cultivation in the P medium in comparison to that in the 8-week-old control leaves of field-grown plants (Fig. 5a). An analogous observation relating to the elevated production of AD through the adventitious root cultures of *A. paniculata* had also been demonstrated previously, which showed 3.6-fold superior accumulation of AD as compared to that in the control leaves after 5 weeks of cultivation (Praveen et al. 2009).

Another noteworthy aspect of the present study includes the NAD-synthesizing potentials of both the calli and the adventitious roots of *A. paniculata*, which has not been reported so far (to the best of our knowledge). Noticeably, the adventitious

roots yet again proved superior to the calli towards in vitro NAD accumulation with a trend towards its upturn from 1.01 ± 0.02 to 1.21 ± 0.03 mg/g DW in the former (upon its cultivation in liquid N medium) and 0.18 ± 0.01 to 0.33 ± 0.02 mg/g DW in the latter (grown on semi-solid MS medium with matching hormonal combination) during their 4th and 8th weeks of cultivation respectively (Fig. 5b). This observation clearly demonstrated that the maximum NAD-synthesizing potential of the adventitious root culture is 3.67-fold higher compared to that in the calli during the maximum production phase (Fig. 5b). Then again, compared to the control leaves of field-grown plants, the adventitious root culture outshined in terms of its NAD-synthesizing potential upon its cultivation in the P and N media with 8.8- and 7.12-fold gains respectively at the same time span of 8 weeks (Fig. 5b).

So far, to the best of our knowledge, the adventitious root cultures of *A. paniculata* had only been credited for their potential to synthesize the single diterpene (AD) in NAA-supplemented MS medium with no assertion of any concurrent NAD production potential (Praveen et al. 2009; Sharma et al. 2013; Zaheer and Giri 2017). Recently, Zaheer and Giri (2017) have demonstrated the best affirmative influence of jasmonic acid amongst four tested abiotic elicitors (jasmonic acid, salicylic acid, acetyl salicylic acid and methyl salicylic acid) on the adventitious root cultures of *A. paniculata* relating to its growth and AD-synthesizing ability. In contrast to all the previously reported observations relating to the in vitro production sources (i.e. callus, suspension, whole plant regeneration and adventitious root cultures) of the targeted diterpenoids, the present study for the first time unravelled the NAD-synthesizing ability of the adventitious root cultures of *A. paniculata* other than AD. Noticeably, the medium of the currently established adventitious roots also demonstrated the exudation and accumulation of AD and NAD in minor amounts (Fig. 5a, b), which highlights the prospect of exudation of such metabolites in the surrounding medium by strategic procedural manipulations that facilitate the downstream processing (Cai et al. 2012).

In the background of the present overall findings, it seems pertinent to emphasize that, notwithstanding the incessantly escalating research attention that is being focussed towards harnessing the maximum biosynthetic potentials of diverse culture forms of *A. paniculata* for serving as the bio-factories of its molecules, in vitro production of NAD has never embarked on the intended contemplations equal to that of AD. To the best of our understanding, the present observations not only for the first time reported the superior NAD-synthesizing potentials of *A. paniculata* adventitious root culture on top of its AD production competence, but also have illustrated the prominent role of the form of the medium (liquid over semi-solid) and the recipe of auxin composition in enhancing their in vitro yield.

In this context, it seems prudent to mention that the presently obtained AD productivity from adventitious roots of *A. paniculata* is considerably lesser than that reported by Sharma et al. (2013), in which the NAD productivity remained obscured. On the basis of established published information, it would be solicitous to acknowledge that several underlying factors (such as inherent metabolite-yielding potential of the explant source, the growth/production stage of the explants, the divergence of the genetic makeup of the induced individual culture forms, influence of the culture conditions/biosynthetic mechanism) essentially govern the overall biosynthetic competence of the established/selected culture forms (Gaillochet and Lohmann 2015). The amazing developmental plasticity of plant cells under differentiation and de-differentiation states eventually contributes towards the biosynthetic competence of the concerned in-vitro culture forms. This might be the prevailing factor that distinguishes our results from that of Sharma et al. (2013) concerning the higher AD synthesis in the latter with no mention of NAD.

As the overall biosynthetic potential of any cell culture system is reputedly governed by the expression kinetics of major pathway enzymes, it becomes imperative to study the involvement of the underlying key genes through real-time analysis to correlate their expression profiles with the AD and NAD accumulation trend in the adventitious roots and calli of *A. paniculata* to envisage their fundamental mechanism for better exploitation.

## qRT-PCR analysis of biosynthetic pathway genes

The MEP and MVA pathways are designated to supply 5C precursors as the building blocks for the biosynthesis of diverse terpene compounds in plants (Srivastava and Akhila 2010). Moreover, the putative plastidial MEP and cytosolic MVA genes for the biosynthesis of terpene metabolites in *A. paniculata* have been explicitly determined earlier through transcriptome analysis (Garg et al. 2015; Fig. 1). The first dedicated rate-limiting enzyme of the cytosolic MVA pathway has been elucidated to be regulated by the *HMGR* gene, while *DXS* had been designated for the same function concerning the plastidial MEP pathway (Cordoba et al. 2009). The *HDR* gene is the other key regulatory gene of the MEP pathway that regulates the penultimate step of the MEP pathway towards the synthesis of IPP (Cordoba et al. 2009). In order to elucidate the underlying molecular dynamism of the major rate-limiting genes towards the biosynthesis of AD and NAD in the presently studied in vitro culture forms at different growth periods, the *HMGR* (one and two isoforms) of the MVA pathway, *DXS* and *HDR* (one and two isoforms of both) of the MEP pathway and *GGPS* (one and two isoforms) and *IPP* of the central pathway have been analysed through qRT-PCR (Fig. 1). On the other hand, the variable biosynthetic potentials of the adventitious roots in the two optimized culture media offered an opportunity to elucidate the relative expression profiles of the above stated pathway genes, which have also been studied to understand their overall regulatory function.

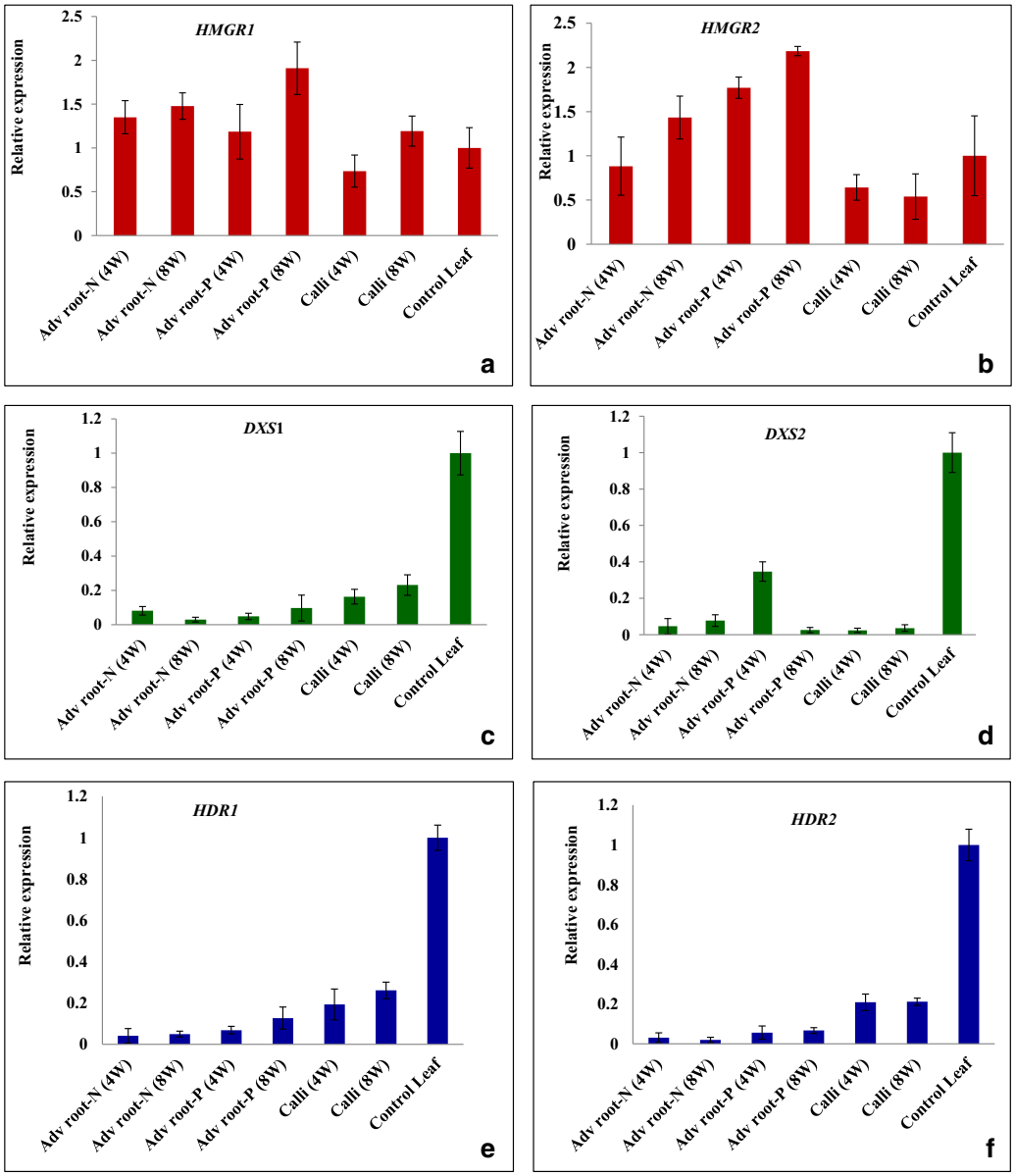


**Fig. 6** **a**–**i** Relative expressions of nine selected genes (*HMGR1/2*; *DXS1/2*; *HDR1/2*; *GGPS1/2* and *IPP*) of MVA and MEP pathways involved in andrographolide biosynthesis in different in vitro cultures of *A. paniculata* as determined through quantitative real-time PCR (qRT-PCR). The values are mean of three replicates ± SD

While considering the MVA pathway under the present scenario through comparative analysis of the qRT-PCR results, observable variations in the expression profiles of *HMGR1* and *2* genes could be noted in the adventitious roots as compared to that in the control leaves of field grown *A. paniculata* plants. The 8-week-old P medium-grown adventitious roots revealed 1.91- and 2.18-fold higher expressions of *HMGR1* and *HMGR2* respectively as compared to that in the control leaves of the same age (Fig. 6a, b). Furthermore, compared to that of N medium-grown adventitious roots, the P medium-grown counterpart showing better biosynthetic potential exhibited 1.29- and 1.52-fold higher expressions of *HMGR1* and *HMGR2* respectively (Fig. 6a, b).

On the basis of the present observations, it is pertinent to mention that, in comparison to the control leaves of field-grown plants as well as callus culture, the elevated expression profiles of *HMGR1* and *HMGR2* genes in the adventitious roots (irrespective of their media of cultivation) agreed with the superior biosynthetic trend of the targeted metabolites in the latter, as substantiated with the HPLC analysis data. In corroboration to our present findings, the exclusive role of the *HMGR* gene towards the biosynthesis of AD in the callus culture of *A. paniculata* had also been documented recently

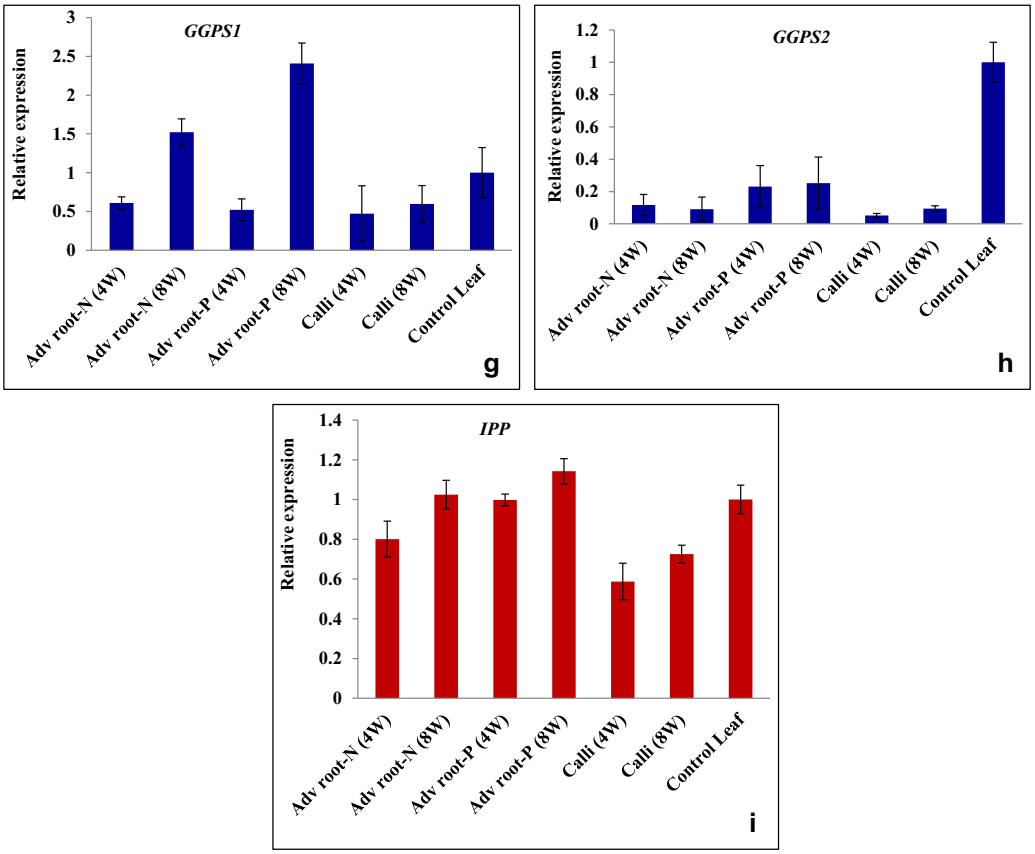


**Fig. 6** (continued)

through in silico and HPLC studies (Bindu et al. 2017). Analogous observation citing the distinctiveness of *HMGR* genes towards improved production of AD by elicitation had also been documented previously (Jha et al. 2011).

On the other hand, while considering the expression profiles of the MEP pathway genes, the roles of *DXS* and *HDR* genes in their respective two isoforms have been evaluated in the present study as their key role towards the production of diterpenes are well renowned and emphasized earlier (Cordoba et al. 2009; Srivastava and Akhila 2010). Relatively higher expression levels of *DXS1*/*DXS2* and *HDR1*/*HDR2* genes could be noted in the control leaves of the field-grown plant compared to that in the adventitious roots and calli. The adventitious roots in its most productive cultivation medium (P) revealed striking differences in the expression levels of the *DXS1*/*DXS2* and *HDR1*/*HDR2* genes compared to that in the normal leaves of the same age with 11.11-/38.46- and 7.69-/14.29-fold downregulations respectively (Fig. 6c–f). Correspondingly, the calli also showed 4.35-/25.0- and 3.85-/4.76-fold downregulations of the *DXS1*/*DXS2* and *HDR1*/*HDR2* genes respectively with respect to that of the control leaves (Fig. 6c–f). The higher expressions of all the isoforms of both these genes of the MEP pathway in the leaves of the field-grown plant compared to that in the in vitro grown culture forms are logically comprehensible because of their major involvement in the plastidial pathway (Cordoba et al. 2009; Srivastava and Akhila 2010; Garg et al. 2015).

Nevertheless, the role of the isoforms of the *DXS* and *HDR* genes towards the AD and NAD synthesis in the in vitro raised cell and tissues would be better understandable through analysis of their expression profiles at inter-media production phases. Noticeably, the adventitious roots in its best secondary metabolite-yielding medium (P) demonstrated 4.5-fold upregulation of the *DXS1* isoform and 2.6-fold downregulation of the *DXS2* isoform in comparison to their respective expression profiles in the same adventitious roots upon cultivation in the lesser productive (N) medium (Fig. 6c, d). On the other hand, moderately higher expressions of both the isoforms of the *HDR1*/*HDR2* genes (2.6-/3.5-fold) could be recorded in the P medium-grown adventitious roots compared to its N medium-grown counterpart at the same growth stage of 8 weeks (Fig. 6e, f). These findings imply that, although the MEP pathway gene expression profiles remained relatively downregulated in the adventitious roots in comparison to that in the control leaves with an efficient plastidial pathway, their subtle share in completing the metabolic pathways cannot be presumably overruled in the in vitro culture states as their expressions are getting upregulated with the increase in biosynthetic potentials as indicated above.

Consequently, the roles of the central pathway genes, such as *GGPS1/GGPS2* and *IPP*, also revealed a similar trend at the intra-media cultivation states of the adventitious root. The adventitious roots cultivated in the P medium exhibited prominent upregulation of *GGPS1*/*GGPS2* and *IPP* with 1.59-/2.5- and 1.12-fold higher expressions, respectively, as compared to that in adventitious root cultivated in N medium (Fig. 6g–i). Concurrently, it is interesting to note that, compared to the control leaves of field-grown plants, the expression levels of *GGPS1* and *IPP* genes showed relative upregulation in the adventitious roots of the same age, irrespective of their cultivation media (Fig. 6g–i). The 2.41-fold higher expression of the *GGPS1* gene in the P medium-grown adventitious root over that of the control leaves might account for the higher AD/NAD productivity of the former as has earlier been corroborated specifying the key role of *GGPS* in the first committed step towards controlling the metabolic flux through the branch point of the biosynthetic pathway (Sharma et al. 2015). The prominent involvement of *HMGR* and *GGPS* genes in the cell suspension culture of *A. paniculata* had earlier been studied, and a direct correlation with the accumulation of AD in the in vitro tissues had been elucidated (Sharma et al. 2015).

The overall analysis of the qRT-PCR observations evidently drew attention to two fundamental elements as categorized below, which might be responsible in adding force to the 2.61- and 8.8-fold higher biosynthetic potential of AD and NAD, respectively, in the adventitious roots over that of the control leaves: (i) ascendancy of the MVA pathway route as corroborated by the elevated expression of *HMGR1* (1.91-fold) and *HMGR2* (2.18-fold) gene and (ii) convincing command of the central pathway with *GGPS1* (2.41-fold) in the adventitious roots—the first accredited branch-point regulatory gene routing the flux towards AD biosynthesis as illustrated earlier (Sharma et al. 2015). Moreover, the better functionality of *HMGR2* (1.14-fold), *DXS2* (7.0-fold), *HDR1* (1.86-fold) and *GGPS1* (9.64-fold) isoforms over that of their respective counter isoforms, i.e. *HMGR1*, *DXS1*, *HDR2* and *GGPS2*, in the adventitious root at its highest metabolite-yielding culture medium (P) may also have straightened up the possible reason for the comparatively higher accumulation of AD and NAD in the above-stated in vitro culture form. Evidently, the lesser expressions of the studied MEP pathway genes with higher AD/NAD-synthesizing potential of the adventitious roots indicated the crucial role of MVA pathway genes over that of the MEP ones towards the accumulation of the presently targeted two diterpenoids—AD/NAD. Such observation further highlights an important revelation that in vitro cultures devoid of green tissues/cells can serve as efficient production alternatives for AD/NAD as the cytosolic pathway is competent enough to supersede the plastidial one, provided the culture requirements are optimally fulfilled.

## Conclusion

The present study unravelled the consequence of hormonal and other culture conditions towards the first time production of NAD along with the predominantly occurring AD in the established adventitious roots and calli of *A. paniculata*. The maximum production of AD and NAD was found in the adventitious roots cultivated in P medium after 8 weeks of cultivation. Amongst all the selected nine pathway genes, the qRT-PCR analysis displayed the upregulation of *GGPS1*, *HMGR1*, *HMGR2* and *IPP* genes in the adventitious roots cultivated in the P medium as compared to the control plant. The correlations between the gene expression of the selected pathway genes and accumulation of the AD/NAD in the in vitro cultures displayed that the higher expression of not only one gene might have regulated the andrographolide biosynthesis; instead, a series of genes regulate the entire diterpene biosynthetic pathway. The present study for the first time revealed the significant role of MVA (cytosolic) pathway in the adventitious roots and calli towards the biosynthesis of the targeted diterpenoids as compared to the other MEP (plastidial) pathway. Additionally, the present findings also offer a promising opportunity towards the advancement of an alternative juncture to produce NAD and AD in the in vitro cultures of *A. paniculata*. Furthermore, to meet the escalating demand of this pharmaceutically important phytomolecules, enhancement in the productivity of the presently targeted metabolites can be accomplished through elicitation and bioreactor upscaling, for which the study is under progress.

**Acknowledgements** The authors wish to express their sincere thanks to the Director, CSIR-CIMAP, for providing the facilities to carry out this research. SB is thankful to the National Academy of Sciences (NASI, Allahabad, India), for honouring her as a Platinum Jubilee Senior Scientist and providing the financial support to continue the research. SS is also thankful to NASI, Allahabad, India, and PP to the Department of Science and Technology (DST, New Delhi, India), for financial support in the form of fellowships. This work is carried out under a NASI project (GAP-365).

## Compliance with ethical standards

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

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