

# Withanolide A production from *Withania somnifera* hairy root cultures with improved growth by altering the concentrations of macro elements and nitrogen source in the medium

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**Abstract** *Withania somnifera* is an important medicinal plant that contains withanolides as bioactive compounds. We have investigated the effects of macroelements and nitrogen source in hairy roots of *W. somnifera* with the aim of optimizing the production of biomass and withanolide A content. The effects of the macroelements  $\text{NH}_4\text{NO}_3$ ,  $\text{KNO}_3$ ,  $\text{CaCl}_2$ ,  $\text{MgSO}_4$  and  $\text{KH}_2\text{PO}_4$  at concentrations of 0, 0.5, 1.0, 1.5 and 2.0× strengths and of nitrogen source [ $\text{NH}_4^+/\text{NO}_3^-$  (0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60 mM)] in Murashige and Skoog medium were evaluated for biomass and withanolide A production. The highest accumulation of biomass (139.42 g l<sup>-1</sup> FW and 13.11 g l<sup>-1</sup> DW) was recorded in the medium with 2.0× concentration of  $\text{KH}_2\text{PO}_4$ , and the highest production of withanolide A was recorded with 2.0×  $\text{KNO}_3$  (15.27 mg g<sup>-1</sup> DW). The  $\text{NH}_4^+/\text{NO}_3^-$  ratio also influenced root growth and withanolide A production, with both parameters being larger when the  $\text{NO}_3^-$  concentration was higher than that of  $\text{NH}_4^+$ . Maximum biomass growth (148.17 g l<sup>-1</sup> FW and 14.79 g l<sup>-1</sup> DW) was achieved at  $\text{NH}_4^+/\text{NO}_3^-$  ratio of 14.38/37.60 mM, while withanolide A production was greatest (14.68 mg g<sup>-1</sup> DW) when the  $\text{NH}_4^+/\text{NO}_3^-$  ratio was 0.00/18.80 mM. The

results are useful for the large scale cultivation of *Withania* hairy root culture for the production of withanolide A.

**Keywords** Hairy root culture · Macro elements · Nitrogen source · *Withania somnifera* · Withanolide A

## Introduction

*Withania somnifera*, also known as ashwagandha, Indian ginseng and winter cherry is an important medicinal plant in ayurvedic medicine, the traditional medicinal system of India (Gupta and Rana 2007). It has been used as a tonic and antistress supplement. Pharmacological activities include physiologic and metabolic restoration, antiarthritic, antiaging, nerve tonic, cognitive function improvement in geriatric states, and recovery from neurodegenerative disorders (Bhattacharaya et al. 2002; Dhuley 2000). Various alkaloids, withanolides and sitoindosides have been isolated from this plant. Of the various withanolides reported, withaferin A and withanone are customary major withanolides of the plant whereas the amount of withanolide A is usually very low (Zhao et al. 2002). Recently, withanolide A has attracted interest due to its strong neuropharmacological properties of promoting outgrowth and synaptic reconstruction (Kuboyama et al. 2005; Tohda et al. 2005a, Tohda et al. 2005b). Withanolide A is therefore an important candidate for the therapeutic treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, convulsions, cognitive function impairment, as it is able to reconstruct neural networks (Tohda et al. 2005a).

Plant cell and organ culture systems are promising methodologies for obtaining valuable plant-specific metabolites (Verpoorte et al. 2002). Cell and organ cultures have a higher rate of metabolism than field-grown plants because

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the initiation of cell and organ growth in culture leads to the rapid proliferation of cells/organs and to a condensed biosynthetic cycle (Ramachandra Rao and Ravishankar 2002). Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions, and the cells/organs can thus proliferate at higher growth rates than the cultivated whole plant. Several biotechnological advances have been made in tissue culture methodologies that have improved secondary metabolite production, such as the optimization of cultural conditions, the selection of high-producing strains of lines, precursor feeding, elicitation, metabolic engineering, transformed root cultures, micro-propagation, and bioreactor cultures, among others (Sarin 2005). Production of withanolide A has been reported in hairy root cultures of *W. somnifera* (Murthy et al. 2008). In an earlier publication, we reported that withanolide A production depends on carbon source and medium pH in hairy root culture of *W. somnifera* (Praveen and Murthy 2012). Further enhancement of biomass and withanolide A accumulation can also be achieved by manipulation of the medium composition. To date, there have been no reports on the effect of macro elements and nitrogen source on the biomass accumulation and withanolide A production in hairy root cultures of *W. somnifera*. Therefore, we have investigated the effects of different macro elements and nitrogen source on hairy root growth of *W. somnifera* in terms of biomass accumulation and withanolide A production.

## Materials and methods

### Hairy root culture

*Withania somnifera* (Indian ginseng) was transformed by *Agrobacterium rhizogenes*. Explants from seedling cotyledons and young leaves were inoculated with *A. rhizogenes* strain R1601, and hairy roots were induced from these explants. The transgenic status of hairy roots was confirmed by PCR using *nptII* and *rolB* specific primers and, subsequently, by Southern analysis for the presence of *nptII* and *rolB* genes in the genomes of transformed roots (Murthy et al. 2008). The hairy root cultures were initiated by culturing 500 mg *W. somnifera* hairy roots in 250 ml Erlenmeyer's flasks each containing 50 ml of MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose (Murthy et al. 2008). The initial medium pH was adjusted to  $5.8 \pm 0.2$  before autoclaving (at 121 °C and 1.2 kg cm<sup>2</sup> pressure for 15 min), and the cultures were kept under continuous agitation at 110 rpm in an orbital shaker (Orbitek, Scigenics, Chennai, India) and incubated at  $25 \pm 2$  °C with a 16 h photoperiod (40 μmol m<sup>-2</sup> s<sup>-1</sup>) provided by 40 W white fluorescent lamps (Philips, Kolkata, India). The roots were subcultured every 15 days.

### Effect of varying concentrations of macro elements in the culture medium

Five hundred milligram of hairy roots was cultured in 250 ml Erlenmeyer's flasks containing 50 ml of MS medium supplemented with 3 % sucrose. The levels of NH<sub>4</sub>NO<sub>3</sub>, KNO<sub>3</sub>, CaCl<sub>2</sub>, MgSO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> in the MS medium were each varied at 0, 0.5, 1.0, 1.5 and 2.0× strengths, respectively, of the normal concentration.

### Effects of nitrogen source on hairy root growth and withanolide A production

Hairy root cultures were established as described above, and the medium was altered with respect to nitrogen source; the following ratios of NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> (mM/mM) were used: 0.00/18.80, 7.19/18.80, 14.38/18.80, 21.57/18.80, 28.75/18.80, 14.38/0.00, 14.38/9.40, 14.38/18.80, 14.38/28.20 and 14.38/37.60. All the cultures were kept under continuous agitation at 110 rpm in an orbital shaker (Orbitek) and incubated as described for the initiation of hairy root cultures. Root biomass growth and withanolide A productivity were measured after 4 weeks of culture.

### Determination of root biomass

The roots were separated from the media by passing them through a 0.45 μm stainless steel sieve (Sigma, USA). Their fresh weights (FW) were determined after they were washed with distilled water and the excess surface water blotted away. Dry weights (DW) were recorded after the roots were dried at 60 °C till constant weight is recorded. The growth rate was determined as GR = (harvested dry weight-inoculated dry weight)/inoculated dry weight.

### Extraction and HPLC analysis

Extraction and HPLC analysis of withanolide A was carried out by following the method of Ganzera et al. (2003). Hundred milligram of powdered hairy root material was extracted with 2 ml of methanol by sonication for 20 min after centrifugation (5 min at 3,000 rpm), the extracts were combined and diluted with equal volume of methanol. The samples were filtered through a 0.45 μm nylon membrane filters and then subjected for the HPLC analysis. The withanolide fractions were analyzed using HPLC system (Waters 2487, Milford, CT, USA) equipped with Phenomenex C18, 5 μm (4.6 × 250 mm) column. The mobile phase was a mixture of reagent alcohol and water (80:20, v/v) at flow rate of 1 ml/min and the column temperature was maintained at 30 °C. The detection wave length was set at 230 nm. The injection volume was 20 μl. The chromatography system was equilibrated by the mobile

phase. Withanolide A standard was obtained from Chromadex Inc. (Laguna Hills, CA, USA). All the experiments were set up in a completely randomized design, and the data were subjected to Duncan's multiple range test using SPSS software version 9.0.

## Results and discussion

### Effect of macro elements on hairy root growth and withanolide A production

The effect of different macroelements and different concentrations of these macroelements play an important role in biomass accumulation, and secondary metabolite production in suspension culture systems (Wu and Zhong 1999). In our study, the highest accumulation of biomass (139.42 g l<sup>-1</sup> FW and 13.11 g l<sup>-1</sup> DW) was recorded in the medium containing 2.0× concentration of KH<sub>2</sub>PO<sub>4</sub>, followed by that containing 2.0× KNO<sub>3</sub>, (137.87 g l<sup>-1</sup> FW and 13.69 g l<sup>-1</sup> DW) (Table 1). The highest production of withanolide A content was recorded in the medium with 2.0× KNO<sub>3</sub>, (15.27 mg g<sup>-1</sup> DW), followed by 2.0× KH<sub>2</sub>PO<sub>4</sub> (14.68 mg g<sup>-1</sup> DW) (Fig. 1).

### Effect of NH<sub>4</sub>NO<sub>3</sub> on hairy root growth and withanolide A production

Medium supplemented with 0.5× NH<sub>4</sub>NO<sub>3</sub> was responsible for maximum hairy root biomass (127.65 g l<sup>-1</sup> of FW and 12.74 g l<sup>-1</sup> of DW) with a growth rate of 10.11 and resulted in the optimal production of withanolide A (14.37 mg g<sup>-1</sup> DW). Yu et al. (2001) also reported that 0.5× concentration of NH<sub>4</sub>PO<sub>3</sub> in SH medium favored the biomass accumulation and the NH<sub>4</sub><sup>+</sup> free medium resulted in the maximum production of ginsenoside from adventitious roots of ginseng. Murthy and Praveen (2012) also reported that 0.5× concentration of NH<sub>4</sub>NO<sub>3</sub> in the MS medium favored the biomass accumulation and withanolide A production from adventitious roots of *W. somnifera*.

### Effect of KNO<sub>3</sub> on hairy root growth and withanolide A production

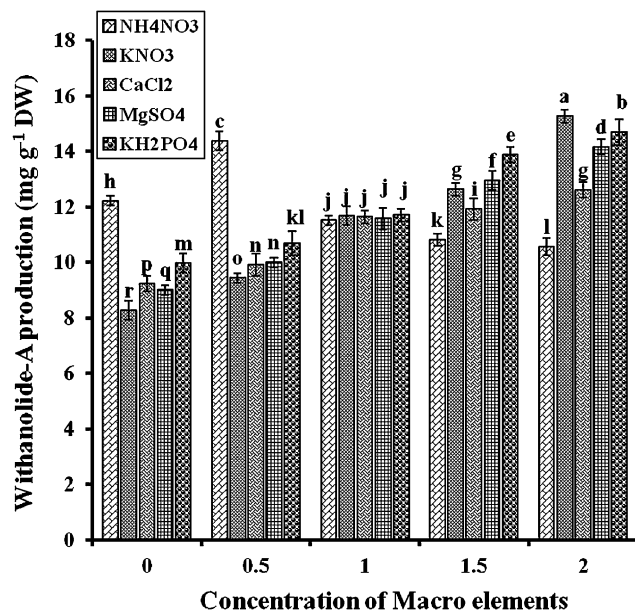
Higher strength of 2.0× KNO<sub>3</sub> was responsible for optimal accumulation of biomass (137.87 g l<sup>-1</sup> FW and 13.69 g l<sup>-1</sup> DW) and withanolide-A content (15.27 mg g<sup>-1</sup> DW). Similar results were reported with the adventitious root cultures of *W. somnifera* for the production of withanolide A

**Table 1** Biomass growth of *Withania somnifera* hairy root cultures as affected by concentrations of macro elements in the MS medium

Macro elements	Concentration (× times)	Fresh weight (g l <sup>-1</sup> )	Dry weight (g l <sup>-1</sup> )	Growth rate
NH <sub>4</sub> NO <sub>3</sub>	0.0	96.10 ± 1.30h	9.34 ± 0.07ij	7.41
	0.5	127.65 ± 0.75d	12.74 ± 0.08d	10.11
	1.0	118.23 ± 1.71f	11.70 ± 0.19g	9.29
	1.5	88.30 ± 1.94jk	8.74 ± 0.16k	6.94
	2.0	79.50 ± 1.20l	7.83 ± 0.06l	6.21
KNO <sub>3</sub>	0.0	77.18 ± 0.67l	7.69 ± 0.10l	6.10
	0.5	86.84 ± 1.13k	8.83 ± 0.02k	7.01
	1.0	115.68 ± 0.62fg	11.54 ± 0.11g	9.16
	1.5	124.07 ± 0.35e	12.34 ± 0.02ef	9.79
	2.0	137.87 ± 0.75ab	13.69 ± 0.06a	10.86
CaCl <sub>2</sub>	0.0	78.51 ± 1.76l	7.76 ± 0.18l	6.16
	0.5	95.66 ± 0.68h	9.51 ± 0.09i	7.55
	1.0	124.08 ± 1.63e	12.23 ± 0.13f	9.71
	1.5	127.39 ± 1.27de	12.62 ± 0.12de	10.02
	2.0	133.18 ± 1.14c	13.28 ± 0.11bc	10.54
MgSO <sub>4</sub>	0.0	76.15 ± 0.47lm	7.48 ± 0.06l	5.94
	0.5	91.25 ± 0.74ij	9.08 ± 0.03jk	7.21
	1.0	118.04 ± 0.59f	11.70 ± 0.08g	9.29
	1.5	134.85 ± 0.82bc	13.50 ± 0.18ab	10.71
	2.0	118.35 ± 0.91f	11.71 ± 0.06g	9.29
KH <sub>2</sub> PO <sub>4</sub>	0.0	73.24 ± 0.42m	6.97 ± 0.02m	5.53
	0.5	91.82 ± 0.26i	8.93 ± 0.01k	7.09
	1.0	113.16 ± 1.12g	10.75 ± 0.11h	8.53
	1.5	117.45 ± 0.67f	11.41 ± 0.26g	9.06
	2.0	139.42 ± 2.46a	13.11 ± 0.23c	10.40

Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at  $P \leq 0.05$ . Cultures were grown in 250 ml Erlenmeyer's flasks containing 50 ml medium for 4 weeks. The same letters in different columns have similar statistical significance.

(Murthy and Praveen 2012). Whereas, Yu et al. (2001) reported that  $1.0\times$   $\text{KNO}_3$  resulted in the highest accumulation of biomass, and  $2.0\times$  strength medium favored ginsenoside production from adventitious root suspension cultures of *Panax ginseng*.



**Fig. 1** Withanolide A content in *Withania* hairy root cultures as affected by different concentrations of macroelements. Hairy roots (500 mg) were cultured in 250 ml Erlenmeyer flasks containing 50 ml of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values  $\pm$  SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at  $P \leq 0.05$  according to Duncan's multiple range test

Effect of  $\text{CaCl}_2$  on hairy root growth and withanolide A production

Among different concentrations of  $\text{CaCl}_2$  tested,  $2.0\times$  strength resulted in the highest accumulation of biomass ( $133.18 \text{ g l}^{-1}$  FW and  $13.28 \text{ g l}^{-1}$  DW) and withanolide A ( $12.62 \text{ mg g}^{-1}$  DW). Similar to our results,  $2.0\times$   $\text{CaCl}_2$  favored the highest biomass accumulation and ginsenoside production from ginseng adventitious root culture (Yu et al. 2001) and withanolide A production from *W. somnifera* adventitious root culture (Murthy and Praveen 2012).

Effect of  $\text{MgSO}_4$  on hairy root growth and withanolide A production

A higher strength of  $1.5\times$   $\text{MgSO}_4$  favored the highest accumulation of biomass ( $134.85 \text{ g l}^{-1}$  FW and  $13.50 \text{ g l}^{-1}$  DW), and  $2.0\times$   $\text{MgSO}_4$  favored the highest production of withanolide A ( $14.17 \text{ mg g}^{-1}$  DW). Similarly,  $1.5\times$   $\text{MgSO}_4$  favored the highest accumulation of biomass, whereas  $2.0\times$   $\text{MgSO}_4$  favored the maximum production of ginsenoside (Yu et al. 2001).

Effect of  $\text{KH}_2\text{PO}_4$  on hairy root growth and withanolide A production

Among various concentrations of  $\text{KH}_2\text{PO}_4$  tested,  $2.0\times$  favored the highest accumulation of biomass ( $139.42 \text{ g l}^{-1}$  FW and  $13.11 \text{ g l}^{-1}$  DW) and withanolide A ( $14.68 \text{ mg g}^{-1}$  DW; Table 1, Fig. 1). Similar to our results, higher concentrations of  $\text{KH}_2\text{PO}_4$  favored the biomass accumulation and withanolide A production in adventitious root cultures of *W. somnifera* (Murthy and Praveen 2012). In cell suspension

**Table 2** Biomass growth of *Withania somnifera* hairy root cultures as affected by  $\text{NH}_4^+/\text{NO}_3^-$  ratios in the MS medium

$\text{NH}_4^+/\text{NO}_3^-$ ratios in MS medium ( $\mu\text{M}$ )	Fresh weight ( $\text{g l}^{-1}$ )	Dry weight ( $\text{g l}^{-1}$ )	Growth rate
0.00/18.80	$110.12 \pm 1.04\text{e}$	$10.47 \pm 0.10\text{d}$	8.31
7.19/18.80	$140.45 \pm 1.92\text{b}$	$13.29 \pm 0.16\text{b}$	10.55
14.38/18.80	$127.31 \pm 1.10\text{d}$	$12.05 \pm 0.20\text{c}$	9.56
21.57/18.80	$85.22 \pm 0.65\text{f}$	$8.54 \pm 0.07\text{e}$	6.78
28.75/18.80	$75.09 \pm 0.58\text{g}$	$7.15 \pm 0.08\text{f}$	5.68
14.38/0.00	$36.66 \pm 1.21\text{h}$	$3.70 \pm 0.22\text{g}$	2.94
14.38/9.40	$74.06 \pm 1.07\text{g}$	$7.32 \pm 0.06\text{f}$	5.81
14.38/18.80	$127.31 \pm 1.10\text{d}$	$12.05 \pm 0.20\text{c}$	9.56
14.38/28.20	$135.46 \pm 1.61\text{c}$	$13.27 \pm 0.08\text{b}$	10.53
14.38/37.60	$148.17 \pm 0.19\text{a}$	$14.79 \pm 0.06\text{a}$	11.74

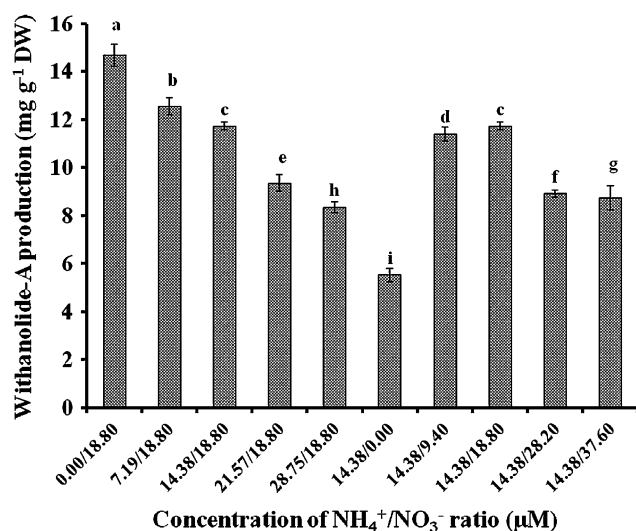
$\text{NH}_4^+/\text{NO}_3^- = \text{NH}_4\text{Cl}/\text{KNO}_3$  (mM/mM)

Data represents mean values  $\pm$  SE of three replicates; each experiment was repeated twice. Mean separation within column by Duncan's multiple range test at  $P \leq 0.05$

Cultures were grown in 250 ml Erlenmeyer's flasks containing 50 ml medium for 4 weeks

The same letters in different columns have similar statistical significance





**Fig. 2** Withanolide A content in hairy root cultures as affected by different ratio of  $\text{NH}_4^+/\text{NO}_3^-$  in the MS medium. Hairy roots (500 mg) were cultured in 250 ml Erlenmeyer flasks containing 50 ml of MS medium supplemented with 3 % sucrose for 4 weeks. Data represents mean values  $\pm$  SE of three replicates; each experiment was repeated twice. Means with common letters are not significantly different at  $P \leq 0.05$  according to Duncan's multiple range test

cultures of *P. ginseng* and *P. quinquefolium*, a low initial concentration of phosphate in the medium sufficiently promoted both cell growth and ginsenoside accumulation (Liu and Zhong 1998). Mantell and Smith (1983) reported that lack of phosphate stimulated secondary metabolite biosynthesis. Therefore, optimizing macro elements concentration, especially for nitrogen and phosphate in the culture media is a key step towards higher production of secondary metabolites in plant cell, tissue and organ cultures.

#### Effects of nitrogen source on hairy root growth and withanolide A production

Nitrogen concentration affects the level of proteinaceous or amino acid products in cell suspension cultures. The ratio of the ammonia/nitrate-nitrogen and overall levels of total nitrogen markedly affect the production of secondary plant products. In the present study, maximum root biomass (148.17 g l<sup>-1</sup> FW and 14.79 g l<sup>-1</sup> DW) was achieved at a  $\text{NH}_4^+/\text{NO}_3^-$  ratios of 14.38/37.60 mM, followed by 7.19/18.80 mM of  $\text{NH}_4^+/\text{NO}_3^-$  ratios which produced the biomass of 140.45 g l<sup>-1</sup> FW and 13.29 g l<sup>-1</sup> DW (Table 2). The maximum production of withanolide A (14.68 mg g<sup>-1</sup> DW) was found with an  $\text{NH}_4^+/\text{NO}_3^-$  ratios of 0.00/18.80 mM, followed by 7.19/18.80 mM of  $\text{NH}_4^+/\text{NO}_3^-$  ratios which produced the withanolide A content of 12.56 mg g<sup>-1</sup> DW (Fig. 2). In contrast, root growth and the accumulation of withanolide A were severely inhibited when the  $\text{NH}_4^+$  concentration was increased. Liu and Zhong

(1997) also reported increased root growth of *P. ginseng* at low  $\text{NH}_4^+/\text{NO}_3^-$  ratios, as well as the greatest ginsenoside productivity in the absence of  $\text{NH}_4^+$ . Maximum levels of saponin and polysaccharide were also obtained when  $\text{NO}_3^-$  was the sole nitrogen source (Zhong and Wang 1998). These results suggest that the nitrate and ammonium ions have differential effects on secondary metabolism in plant cell and tissue cultures. Many reports have confirmed that root growth as well as secondary metabolite accumulation is promoted under the influence of  $\text{NO}_3^-$  in hairy root cultures of *Artemisia annua* (Wang and Tan 2002) and in cell suspension cultures of *W. somnifera*, where the biomass accumulation was maximum at lower concentration of  $\text{NH}_4^+$  and the withanolide A production was increased under the influence of  $\text{NO}_3^-$  (Nagella and Murthy 2011). Whereas in *Camptotheca acuminata* cell suspension cultures, the cell dry weight was improved in the medium with the higher  $\text{NO}_3^-/\text{NH}_4^+$  ratio (60:0 mM), and for secondary metabolism, a high ratio of  $\text{NH}_4^+/\text{NO}_3^-$  (5:1) was favourable for camptothecin production (Pan et al. 2004).

#### Conclusion

It is evident from our present study on flask scale hairy root cultures from *W. somnifera* that both the biomass and secondary metabolite accumulation were influenced by macro elements and ammonia-nitrate ratio. The highest accumulation of biomass was recorded in the medium with 2.0 $\times$  concentration of  $\text{KH}_2\text{PO}_4$  and withanolide A was recorded in the medium with 2.0 $\times$  concentration of  $\text{KNO}_3$ . The moderate concentration of 14.38 mM of  $\text{NH}_4^+$  with higher concentration of 37.60 mM of  $\text{NO}_3^-$  favoured the highest accumulation of biomass and maximum production of withanolide A was recorded in the absence of  $\text{NH}_4^+$  with moderate concentration of  $\text{NO}_3^-$  (18.80 mM). The above results are useful for the large scale cultivation of *Withania* hairy root culture for the production of withanolide A.

**Author contributions** H. N. Murthy provided the idea and supervised the research work, N. Praveen designed and performed the experiments. H. N. Murthy and N. Praveen wrote the manuscript and approved it.

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