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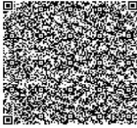

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Effect of Inoculum Density and Different Media on the Growth of Hairy Roots and Production of Withanolide-A from *Withaniasomnifera*

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Abstract

Withaniasomnifera (L.) Dunal. (Indian ginseng) is one of the most important medicinal plants used as a crude drug for its preventive and therapeutic purposes. Among the diverse constituents of *Withania*, withanolides are found to be the major components responsible for their biological and pharmacological actions. The present study deals with the effect of inoculum density and different media on the growth of hairy roots and withanolide-A production from *Withaniasomnifera*. An inoculum size of 10 g/L FW favoured the biomass accumulation(120.42 g/L of FW and 11.98 g/L DW) and withanolide-A production(11.96 mg/g DW) inthe tested range of 2.5, 5.0, 10.0 and 20.0 g/L FW. Among different media tested [Murashige and Skoog (MS), Gamborg's (B5), Nitsch and Nitsch (NN) and Chu's (N6)], MS medium favoured both biomass accumulation (121.15 g/L FW and 11.96 g/L DW) and withanolide-A production (11.50 mg/g DW).

Keywords: *Withaniasomnifera*, Withanolide-A, inoculum density, Media strength, MS media, Gamborg's media, Nitsch and Nitsch Media, Chu's media.

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1. Introduction

Withania somnifera, also known as ashwagandha, Indian ginseng is an important medicinal plant, which is cultivated in India for the medicinal purposes. The roots and leaves of ashwagandha contain various alkaloids, viz., withanolides and withaferins. The withanolides are steroidal compounds and bear resemblances, both in action and appearance to the active ginsenosides of Asian ginseng. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumor, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia [1]. 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 [2]. Recently, withanolide-A has attracted interest due to its strong neuropharmacological properties of promoting outgrowth and synaptic reconstruction [3], [4]. Withanolide-A is therefore important candidate for the therapeutic treatment of neurodegenerative diseases, like Alzheimer's disease, Parkinson's disease, convulsions, cognitive function impairment, as it is able to reconstruct neural networks [4].

For commercial withanolide production, field grown plant material has generally been used but the quality of these products may be highly affected by different environmental conditions, pollutants and fungi, bacteria, viruses and insects, which can result in heavy loss in yield and alter the medicinal content of plant. Plant cell and organ cultures are promising technologies to obtain plant specific valuable metabolites [5]. Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cell/organs and to a condensed biosynthetic cycle. Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organs thus proliferate at higher growth rates than whole plant in cultivation [6]. In the native plant, withanolide-A represents a very minor proportion of withanolide complement and therefore alternative exploration of condition optimized cultures for efficient in vitro biogeneration of such withanolides, which are pharmacologically promising but are severely limited in production, is important. Production of

withanolide D, withanolide-A, withaferin A, withanone have been reported in organogenic cultures including hairy roots [7-15]. However, optimization of culture conditions has not been worked out. By proper manipulation of culture medium and conditions, it is possible to obtain valuable secondary metabolites in larger scale. In view of this, in the present study, we have established hairy root cultures of *W. somnifera* and examined the effect of inoculum density and different media [16-19], on biomass accumulation and withanolide-A production.

2. Materials and methods

2.1 Hairy root culture

The hairy root cultures were initiated by culturing 500 mg *Withania somnifera* hairy roots in 250 ml Erlenmeyer's flasks each containing 50 ml of MS medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose [9]. The initial medium pH was adjusted to 5.8 ± 0.2 before autoclaving (at 121°C and 1.2 kg cm^2 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±2°C, with a 16 h photoperiod (40 µ mol m^{-2} s^{-1}) provided by 40W white fluorescent lamps (Philips, Kolkata, India).The roots were subcultured every 15 days.

2.2 Optimization of culture conditions

Five hundred milligrams of hairy roots was inoculated in 250 mL Erlenmeyer flasks containing 50 mL of MS medium supplemented with 30 g/L sucrose. The initial concentration of root inoculum was adjusted to 2.5, 5.0, 10.0 and 20.0 g/LFW for determining its optimum density for promoting root growth. Various media like [Murashige and Skoog (MS), 1962, Gamborg's (B5) 1968, Nitsch and Nitsch (NN) 1969, and Chu's (N6) 1978][16-19], were tested for the maximum growth of root biomass were applied depending on the objective of the experiment. All shake flask cultures were kept in 16 h photoperiod (40 µm mol^{-1} s^{-1}) at 25±2°C and 110 rpm. After four weeks of culture, the growth of hairy roots was assessed in terms of fresh weight, dry weight, growth ratio and content of withanolideA production was also determined.

2.3 Determination of root biomass

The roots were separated from the media by passing them through a stainless steel sieve. Their fresh weights were determined after they were washed with distilled water and the excess surface water blotted away. Dry weights were recorded after the roots were dried at 60°C till constant weight is recorded. The growth ratio was determined as GR = Growth ratio is the quotients of the dry weight of harvested biomass and the dry weight of the inoculum.

2.4 Extraction and HPLC analysis

Extraction and HPLC analysis of withanolide-A was carried out by following the method of Ganzera et al., (2003)[20] with few modifications. Hundred milligram of dried root material was extracted with 2 mL of methanol by sonication for 20 mins, after centrifugation (5 min at 3000 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 equipped with Phenomenex C18, 5 µm (4.6 x 250 mm) column, LC 10ATVP lamps, SCL-10AVP system controller, SIL-10AD VP auto injector, SPDM-10AVP photodiode array detector. The mobile phase was a mixture of reagent alcohol and water (80:20, v/v) at flow rate of 1 ml/min and column temperature was maintained at 30°C. The detection wavelength 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.

3. Results and discussion

3.1 Effect of inoculum density on biomass accumulation and withanolide-A production

Plant suspensions are initiated using relatively high cell concentrations, as there is a minimum inoculation density below which growth does not occur or is preceded by a lag phase. Medium conditioning can be used to reduce the minimum

inoculum density; however, the chemical basis of the conditioning effect has not been fully defined and it is primarily empirical [21]. The effect of inoculum density (2.5, 5.0, 10.0 and 20.0 g/L) on biomass accumulation and withanolide-A production from hairy root suspension cultures is presented in Figure 1. There was increase in the accumulation of biomass with increase in the inoculum density and reached maximum (120.42 g/Lof FW and 11.98 g/LDW) with a growth ratio of 9.51 when inoculum density was 10.0 g/L(Figure 1A) and thereafter there was decrease in the biomass with increase in the inoculum density. The lowest accumulation of biomass (77.00 g/Lof FW and 7.69 g/Lof DW) was recorded with the inoculum density of 2.5 g/L. The highest production of withanolide-A (11.96 mg/g DW) was recorded at 10.0 g l^{-1} of inoculum density (Figure 1B). Higher and lower inoculum density than 10.0 g/Lresulted in the decrease in the production of withanolide-A content. The lowest production of withanolide-A (10.88 mg/g DW) was recorded at 2.5 g/Lof inoculum density. Similar to our results, in adventitious roots of *E. angustifolia*, the maximum biomass accumulation was recorded at inoculum density of 10–20 g/LFW. However, large density resulted in low phenol and flavonoid contents. Therefore, the best inoculum density for biomass accumulation and secondary metabolite production was determined to be 10 g/LFW [22]. Whereas in ginseng adventitious roots, 5 g/Lof inoculum density favoured the accumulation of biomass and ginsenoside production [23]. This optimization of density is a known fundamental factor in determining the success of tissue cultures [21].

3.2 Effect of different media on biomass accumulation and withanolide-A production

The levels of secondary metabolites produced by in vitro cultures can vary dramatically. Most reports focus on the composition of the medium nutrients to achieve optimizedaccumulation of metabolites in cultured cells[6]. In the present study differentmedia like MS, B5, NN and N6 were employed for hairy root culture and production of withanolide-A and the results revealed that MS medium was found to be superior when compared with other media for both biomass accumulation and withanolide-A production. Highest accumulation of biomass (121.15 g/LFW and

11.96 g/LDW) was recorded in the MS medium with a growth ratio of 9.49 (Figure 2A). The highest production of 11.50 mg/g DW of withanolide-A was recorded in the MS medium (Figure 2B). It was followed by B5 medium which accumulated biomass of 93.45 g/Lof FW and 9.17 g/Lof DW (Figure 2A) with a withanolide-A production of 10.76 mg/g DW. The lowest biomass accumulation was recorded in N6 medium with 71.32 g/Lof FW and 6.94 g/Lof DW. The withanolide-A production was lowest in the N6 medium with 7.93 mg/g DW.Similar to our results MS medium was used for culturing of hairy roots in *W. somnifera*[9], for the production of taxane diterpene from *T. chinensis*[24]. Betsui et al., (2004)[25] also reported that MS medium favored the accumulation of biomass and production of anthocyanins from the adventitious root cultures of *Raphanussativus*L. cv. Peking Koushin. In contrast to our results, Min et al., (2007) [26] reported that Gamborg's B5 medium was suitable for the culturing of adventitious roots and production of tropane alkaloids in *Scopoliaparviflora*.

4. Conclusion

Hairy root culturing is an efficient method for producing useful bioactive molecules. In the present study of flask scale system, we found that in vitro conditions strongly affected root growth and the accumulation of secondary metabolites from Withania hairy roots. The best performance overall was obtained in a MS medium with inoculum density of 10g/L. The above results are useful for the optimization of the cultural conditions and for large-scale cultivation of *Withaniasomnifera* hairy root culture for the production of withanolide-A.

References

[1] G. L. Gupta and A. C. Rana, "*Withaniasomnifera* (Ashwagandha):a review," Pharmacog Rev., vol. 1, pp. 129–136, 2007.

[2] J. Zhao, N. Nakamura, M. Hattori, T. Kuboyama, C. Tohda, K. Komatsu, "Withanolidederivatives from roots of *Withaniasomnifera* and their neurite outgrowth activities," Chem. Pharm. Bull., vol. 50, pp. 760–765,2002.

[3] T. Kuboyama, C. Tohda, K. Komatsu,"Neuritic regeneration and synaptic reconstruction induced by withanolide-A," Br. J.Pharmacol.,vol.144, pp. 961–971, 2005.
[4] C. Tohda, K. Komatsu, T. Kuboyama, "Scientific basis of anti-dementia drugs of constituents from ashwaghanda (*Withaniasomnifera*)," J. Tad. Med., vol. 22, pp. 176–1822005.
[5] R. Verpoorte, A. Contin, and J.Memelink,"Biotechnology for the production of plant secondary metabolites," Phytochem. Rev.,vol. 1, pp. 13–25, 2002.
[6] S. Ramachandra Rao, G. A. Ravishankar, "Plant cell cultures: chemical factories of secondary metabolites," Biotech. Adv., vol.20, pp. 10–153, 2002.
[7] S. Banerjee, A. A. Naqvi, S. Mandal, and P. S. Ahuja, "Transformation of *Withaniasomnifera* (L.) Dunal by *Agrobacterium rhizogenes*: Infectivity and phytochemical studies," Phytother. Res., vol. 8, pp. 452-455, 1994.
[8] M. Furmanowa, D.Gajdzis-Kuls, J.Ruszkowska, Z. Czarnocki, G. Obidoska, A. Sadowska, R. Rani, S. N. Upadhyay, "Invitro propagation of *Withaniasomnifera* and isolation of withanolides with immunosuppressive activity," Planta Med., vol.67, pp. 146–149, 2001.
[9] H.N. Murthy, C. Dijkstra, P. Anthony, D. A. White, M. R. Davey, J. B. Power, E. J. Hahn,K. Y. Paek, "Establishment of *Withaniasomnifera* hairy root cultures for the production of withanolide A," J. Int. Plant Biol., vol. 50, pp. 975–981, 2008.
[10] S. Ray,S.Jha, "Withanolide synthesis in cultures of *Withania somnifera* transformed with *Agrobacterium tumefaciens,*" Plant Science, vol. 166, pp. 1-7, 1999.
[11] S. Ray, S. Jha, "Production of withaferin A from shoot cultures of *Withaniasomnifera*,"Planta Med., vol. 67, pp. 432-436, 2001.
[12] S. Ray, B. Ghosh, S.Sen, and S. Jha, "Withanolide production by root cultures of *Withania somnifera* transformed with *Agrobacterium rhizogenes*," Planta Med., vol. 62, pp. 571-573, 1996.
[13] G. Roja, M. R. Heble, "Tissue cultures of *Withania somnifera*: morphogenesis and withanolide synthesis," Phytother. Res.,vol. 5, pp. 185–187, 1991.
[14] R. S. Sangwan, N. S. Chaurasiya, P. Lal, L. Misra, G. C. Uniyal, R. Tuli, and N. S. Sangwan,"Withanolide A biogeneration in vitro shootcultures of ashwagandha (*Withania somnifera*Dunal), a main medicinal plant in Ayurveda," Chem. Pharm. Bull., vol. 55, pp. 1371–1375, 2007.

[15] G. Vitali, L. Conte, M. Nicoletti, "Withanolide composition and invitro culture of Italian *Withaniasomnifera*,"Planta Med., vol. 62, pp. 287–288, 1996.

[16] T. Murashige, and F.Skoog, "A revised medium for rapid growth andbioassays with tobacco tissue cultures," Physiol. Plant. Vol. 15, pp. 473–497, 1962.

[17] O. L. Gamborg, R. A. Miller, K. Ojima,"Nutrient requirements ofsuspension cultures of soybean root cells," Exp. Cell Res.,vol. 50, pp. 151–158, 1968.

[18] J. P. Nitsch, and C. Nitsch, "Haploid plants from pollen grains," Science, vol.163, pp. 85–87, 1969.

[19] C. C. Chu, "The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of symposium plant tissueculture," Science Press, Beijing, pp. 43–50, 1978.

[20] M. Ganzera, M. I. Choudhary, I. A. Khan, "Quantitative HPLC analysis of withanolides in *Withaniasomnifera*,"Fitoterapia, vol. 74, pp. 68–76,2003.

[21] C. W. T. Lee, and M. L. Shuler, "The effect of inoculum density and conditioned medium on the production of ajmalicine and catharanthine from immobilized *Catharanthusroseus* cells," Biotechnol. Bioeng., vol. 67, pp. 61–67,2000.

[22] [22]C. H. Wu, Y. H. Dewir, E. J. Hahn, K. Y.Paek,"Optimization ofculturing conditions for the production of biomass and phenolicsfrom adventitious roots of *Echinacea angustifolia*," J. Plant Biol.Vol. 49, pp. 193–199, 2006.

[23] C. S. Jeong, H. N. Murthy, E. J. Hahn, H. L. Lee, K. Y. Paek, "Inoculumsize and auxin concentration influence the growth of adventitiousroots and accumulation of ginsenosides in suspension cultures of ginseng (*Panax ginseng* C.A. Meyer),"Acta. Physiol. Plant.Vol. 31, pp. 219–222, 2009.

[24] Z. W. Pan, H. Q. Wang, J. J. Zhong,"Scale-up study on suspension cultures of *Taxus chinensis* cells for production of taxanediterpene," Enz.Microb. Technol. Vol. 27, pp. 714-723, 2000.

[25] F. Betsui, N. Tanaka-Nishikawa, K. Shimmomura, "Anthocyaninproduction in adventitious root cultures of *Raphanussativus*L.cv. Peking Koushin," Plant Biotechnol., vol. 21, pp. 387–391, 2004.

[26] J. Y. Min, H. Y. Jung, S. M. Kang, Y. D. Kim, Y. M. Kang, D. J. Park, D. T. Prasad, M. S. Choi, "Production of tropane alkaloids by small scale bubble column bioreactor cultures of *Scopoliaparviflora* adventitious roots, "Bioresource Technol., vol. 98, pp. 1748–1753, 2007.

Appendix

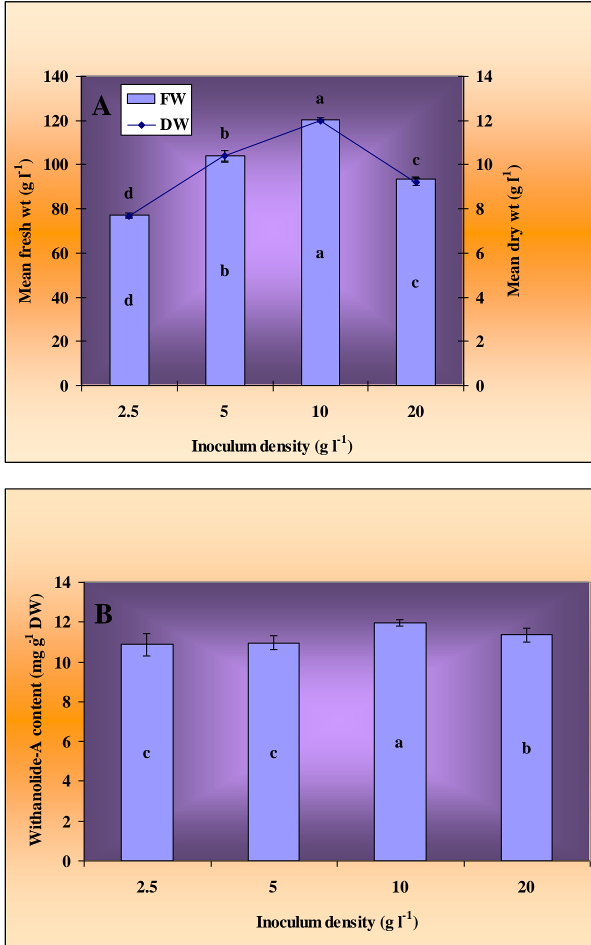

Figure 1. *Withania somnifera* hairy root suspension culture: effect of inoculum density on biomass accumulation (A) and withanolide-A production (B)[z, y].

[z] Hairy roots were cultured in 50 ml of MS basal medium for 4 weeks.

[y] Data represents mean values ± 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 (DMRT).

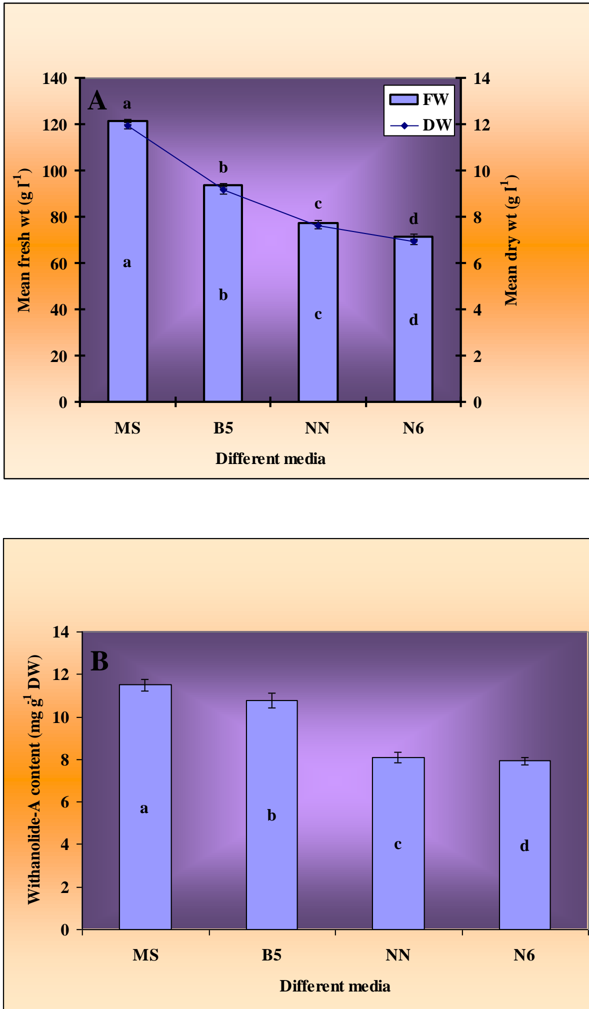

Figure 2. *Withania somnifera* hairy root suspension culture: effect of different media on biomass accumulation (A) and withanolide-A production (B)[z, y].

[z] 0.5 g of hairy roots was cultured in 50 ml of basal medium for 4 weeks.

[y] Data represents mean values ± 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 (DMRT).