

# Induction and Proliferation of *in vitro* Mass of Callus of *Withania somnifera* (L.) Dunal

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**Abstract** *Withania somnifera* (L.) Dunal, commonly known as “Ashwaganda”, belongs to the family Solanaceae is an important medicinal plant and a major source of alkaloids and steroids. Induction of callus was observed from stem explants on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of growth hormones viz, 6-Benzylaminopurine (BAP),  $\alpha$ -Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxy acetic acid (2, 4-D) and kinetin. All the hormone supplemented media gave response for callus growth. The callus was compact and yellowish brown color in all culture condition. The best growth of callus was observed in the MS medium supplemented with  $0.5 \text{ mgL}^{-1}$  BAP +  $1.5 \text{ mgL}^{-1}$  NAA; followed by MS medium supplement with  $0.5 \text{ mgL}^{-1}$  BAP +  $0.5 \text{ mgL}^{-1}$  NAA,  $0.5 \text{ mgL}^{-1}$  BAP +  $2.0 \text{ mgL}^{-1}$  NAA and  $1.0 \text{ mgL}^{-1}$  BAP +  $1.5 \text{ mgL}^{-1}$  NAA at 8 weeks. Mass production of callus might be used for production and isolation of secondary metabolites for medicinal propose in *W. somnifera*.

**Keywords:** *Withania somnifera*, phytohormone, callus, NAA, BAP, 2,4-D, kinetin

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## 1. Introduction

*Withania somnifera* popularly known as ‘Ashwagandha’ belongs to family Solanaceae. This plant grows wildly in all drier parts of subtropical region of India, Congo, South Africa, Egypt, Jordan, Pakistan, Afghanistan etc [1]. In Nepal, it is found in cultivated state. In the traditional system this plant is used to have potent aphrodisiac, treatment of nervous exhaustion, memory related conditions, insomnia, tiredness potency issues, skin problems and coughing [2]. The biologically active chemical constituents of *Withania somnifera* are alkaloids, steroidal compounds, saponins, glucosides, starch, reducing sugar, a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, tryptophan, and high amount of iron [3].

The applications of plant tissue culture to medicinal plant are micropropagation for conservation and mass production and *in-vitro* production of desired phytochemicals [4]. There are various advantages of tissue culture technology in medicinal plants over conventional method of propagation. Cell of any plants, tropical or alpine could easily be multiplied to yield their specific metabolites in a large scales, extraction of phytochemicals from callus and suspension cultures is easier than from the plant parts. *In vitro* propagated medicinal plants have been found to be uniform, showing less variation in their content of secondary metabolites than their wild/cultivated counterparts [5]. Crude medicines derived from plants, in

contrast to synthetic drugs, play an important role in the basic health care systems of various developing countries. Plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants [6].

Callus culture consists of an undifferentiated, proliferating mass of cells usually arising on wounds of differentiated tissues and cells. Normally juvenile and hence physiologically the most active tissues give better callus formation. The exogenous plant growth regulator is required for callus formation [7]. The strongest callus induction factor is the growth/nutrient medium supplemented with plant growth regulators [8].

There are various reports on *in vitro* induction of callus and production of secondary metabolites in medicinal plants [6]. Callus was initiated from the shoot tips of *Zingiber officinale* using MS medium supplemented with combination of  $0.5 \text{ mgL}^{-1}$  NAA and  $1-2 \text{ mgL}^{-1}$  Kinetin [9]. High percentage of callus was induced from the root tips of two varieties of garlic on MS medium supplemented with  $1.5 \text{ mgL}^{-1}$  2,4-D and Kinetin [10]. Successfully induced callus from the leaf explants of *Eurycoma longifolia*, a woody plant, using MS medium supplemented with only NAA but at high level ( $10 \text{ mgL}^{-1}$ ); in some case, a cytokinin was also added together with the auxin to the medium for stimulation of callus grown [11].

Many reports are available on *in vitro* regeneration of *W. somnifera* species using different explants [12]. However there are very few reports are available on callus induction using leaf/stem explants of *W. somnifera* [13]. Rani *et al.* reported the callus cultures of *Withania*

*somnifera* from axillary leaves, axillary shoots, hypocotyls, and root segments on MS, 1962 medium supplemented with 2 mg L<sup>-1</sup> 2,4-D and 0.2mg L<sup>-1</sup> kinetin [14]. Callus induction, root organ culture, plant-let regeneration, and withanolide production in multiple shoots and roots have been reported by Ray and Jha, 1999 [15]. In our previous studies, we have reported the successful mass propagation of *W. somnifera* using nodal and shoot tip explants [12], and production of secondary metabolites on callus of different ploidy level in *W. somnifera* [16].

## 2. Methodology

The required glassware's for *in vitro* culture of *W. somnifera* were cleaned and sterilized by dry heat in a hot air oven at 121°C for 1-2 hours; the metal instruments were wrapped with aluminum foil prior to keep inside the hot air oven for sterilization at 250°C. Laminar airflow chamber was thoroughly sterilized by spirit. All the necessary material such as glassware, metal instruments were kept in the laminar flow with ultraviolet (UV) light irradiation ensuring sterile condition. Air blower was turned on after turning off UV light and kept running while working inside the laminar air flow. Then, the inoculation of explants was carried out aseptically. Surface-sterilized Stem explants of *W. somnifera* were inoculated on MS medium supplemented with plant hormones alone or in combination and 3% (w/v) sucrose,

and solidified with 0.8% (w/v) agar. Leaves (0.5cm×0.5cm) size from *in vitro* grown plantlets were inoculated on MS medium supplemented with four different plant hormone  $\alpha$ -naphthalene acetic acid (NAA), 6-Benzylaminopurine (BAP), kinetin and 2,4-dichlorophenoxy acetic acid (2,4-D) alone (0.5, 1.0, 1.5 and 2 mg L<sup>-1</sup>) or in combination of NAA and BAP (0.5,1.0, 1.5 and 2 mg L<sup>-1</sup>). Similarly callus was sub-cultured on MS medium supplemented with four different plant hormones alone or in combination. The cultures were maintained at 25 ± 2 °C, 8 weeks, 12-16 h Photoperiods.

## 3. Result and Discussion

Callus induction and proliferation from leaves were obtained by inoculating sterile leaves on MS medium supplemented with different concentrations of BAP and NAA (0.5, 1.0, 1.5 and 2 mg L<sup>-1</sup>) All the media gave positive response for callus growth. The best callusing 1 medium were MS media supplemented with BAP 0.5 mg L<sup>-1</sup> + NAA 1.5 mg L<sup>-1</sup> in which callus was obtained in at 8 weeks of primary culture. Similarly MS media supplemented with BAP 0.5 mg L<sup>-1</sup> + NAA 0.5 mg L<sup>-1</sup>, 0.5 mg L<sup>-1</sup> BAP+2.0 mg L<sup>-1</sup> NAA and 1.0 mg L<sup>-1</sup> BAP + 1.5 mg L<sup>-1</sup> NAA were also found to be good condition for callus induction.

**Table 1. Combined effect of BAP and NAA in MS medium on the growth and development of callus**

S.N.	BAP	NAA	Weight of callus explants	Weight of callus at 8 <sup>th</sup> week.	Dry wt. of callus at 8 <sup>th</sup> week.	% Increase of callus after 8 <sup>th</sup> week.
1	0.5	0.5	0.171 ± 0.026	1.347 ± 0.099	0.194 ± 0.015	787
2	1.0	0.5	0.198 ± 0.034	0.677 ± 0.076	0.085 ± 0.010	341
3	1.5	0.5	0.213 ± 0.034	0.849 ± 0.042	0.103 ± 0.003	398
4	2.0	0.5	0.177 ± 0.048	0.585 ± 0.098	0.071 ± 0.009	330
5	0.5	1.0	0.340 ± 0.015	0.857 ± 0.137	0.110 ± 0.017	252
6	1.0	1.0	0.221 ± 0.031	1.101 ± 0.068	0.130 ± 0.008	498
7	1.5	1.0	0.198 ± 0.031	0.591 ± 0.147	0.080 ± 0.018	298
8	2.0	1.0	0.150 ± 0.053	0.675 ± 0.027	0.090 ± 0.004	450
9	0.5	1.5	0.136 ± 0.027	1.091 ± 0.026	0.144 ± 0.006	802
10	1.0	1.5	0.145 ± 0.030	1.058 ± 0.034	0.145 ± 0.002	729
11	1.5	1.5	0.181 ± 0.046	0.435 ± 0.032	0.061 ± 0.004	240
12	2.0	1.5	0.178 ± 0.019	0.747 ± 0.087	0.094 ± 0.010	419
13	0.5	2.0	0.131 ± 0.019	1.015 ± 0.046	0.128 ± 0.008	774
14	1.0	2.0	0.135 ± 0.022	0.708 ± 0.032	0.084 ± 0.005	524
15	1.5	2.0	0.200 ± 0.059	0.699 ± 0.022	0.089 ± 0.003	349
16	2.0	2.0	0.212 ± 0.061	0.394 ± 0.035	0.051 ± 0.005	185

Culture condition: 25 ± 2 °C, 8 weeks, 12-16 h Photoperiods

Callus development was also observed in the MS media supplemented with individual various concentration of Kin, BAP, 2, 4-D and NAA from callus explants. The best growth of callus during subculture was observed in the

MS medium supplemented with 1.5 mg L<sup>-1</sup> NAA and then 1.0 mg L<sup>-1</sup> NAA, 2.0 mg L<sup>-1</sup> NAA, 2.0 mg L<sup>-1</sup> 2,4-D under 25 ± 2 °C, 8 weeks, 16 h Photoperiods conditions.

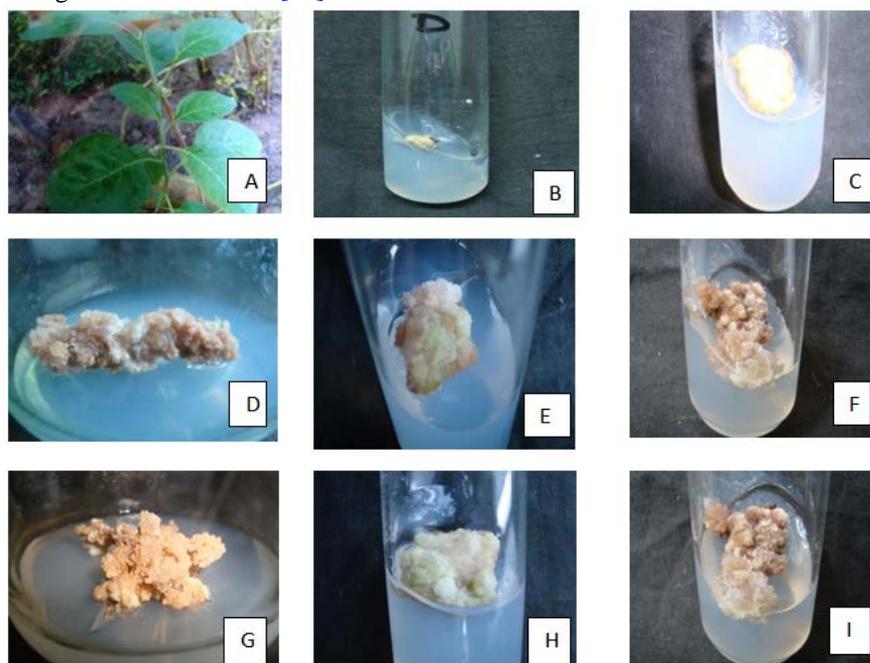
**Table 2. Individual effects of different phytohormones (Kin, BAP, NAA, 2, 4-D) on the growth and development of callus**

S.N.	Kin	BAP	2,4-D	NAA	Weight of callus explants	Weight of callus at 8 <sup>th</sup> week.	Dry wt. of callus at 8 <sup>th</sup> week.	% Increase of callus after 8 <sup>th</sup> week.
1	0.5				0.442 ± 0.079	0.696 ± 0.063	0.092 ± 0.009	157
2	1.0				0.309 ± 0.044	0.679 ± 0.077	0.090 ± 0.009	219
3	1.5				0.406 ± 0.076	0.871 ± 0.036	0.110 ± 0.004	214
4	2.0				0.456 ± 0.084	0.967 ± 0.118	0.131 ± 0.018	212
5		0.5			0.448 ± 0.147	1.034 ± 0.178	0.135 ± 0.023	230
6		1.0			0.352 ± 0.085	0.931 ± 0.106	0.128 ± 0.013	264
7		1.5			0.498 ± 0.046	1.113 ± 0.152	0.152 ± 0.023	223
8		2.0			0.347 ± 0.080	0.972 ± 0.181	0.132 ± 0.024	280
9			0.5		0.227 ± 0.047	0.899 ± 0.064	0.119 ± 0.006	396
10			1.0		0.220 ± 0.036	0.845 ± 0.035	0.115 ± 0.006	384
11			1.5		0.234 ± 0.031	0.763 ± 0.087	0.100 ± 0.011	326
12			2.0		0.181 ± 0.006	0.820 ± 0.045	0.113 ± 0.007	453
13				0.5	0.248 ± 0.064	1.071 ± 0.090	0.140 ± 0.008	431
14				1.0	0.155 ± 0.014	0.986 ± 0.075	0.140 ± 0.008	636
15				1.5	0.141 ± 0.026	0.917 ± 0.029	0.124 ± 0.003	650
16				2.0	0.166 ± 0.027	0.922 ± 0.031	0.135 ± 0.004	555

Culture condition: 25 ± 2 °C, 8 weeks, 12-16 h Photoperiods

Callus proliferation was also observed in MS medium containing different concentration and combination of NAA, BAP, 2,4-D, Kin within 12-14 days of incubation of callus explants. Early callus induction and proliferation was observed with 3 mg L<sup>-1</sup> 2,4-D and 0.5 mg L<sup>-1</sup> kinetin. Whereas at higher concentration of kinetin (more than 1.0 mg L<sup>-1</sup>) with 3 mg L<sup>-1</sup> 2,4-D very compact, dark brown colored and slow growing calli were induced [17]. In most

herbaceous plant only one types of auxin is sufficient and required to be incorporated into the nutrient medium for the induction of callus from the explants [18]. Similarly, in our study the best callusing condition was found to be MS medium containing 1.5 mg L<sup>-1</sup> NAA in which 0.917 gm callus was produced after 8 weeks from 0.141 gm initial callus explants.



**Photo plate.** *In-vitro* study of *Withania somnifera*. Shoot part of *Withania somnifera*(A); stem culture in 0.5 mg L<sup>-1</sup> NAA + 0.5 mg L<sup>-1</sup> BAP (B); callus grow on 2.0 mg L<sup>-1</sup> NAA + 2.0 mg L<sup>-1</sup> BAP. At 8 weeks (C); callus grow on 1.5 mg L<sup>-1</sup> NAA + 1.0 mg L<sup>-1</sup> BAP at 8 weeks (D); callus grow on 0.5 mg L<sup>-1</sup> BAP + 1.5 mg L<sup>-1</sup> NAA at 8 weeks(E); callus grow on 1.0 mg L<sup>-1</sup> BAP (F); callus on 0.5 mg L<sup>-1</sup> BAP + 1.5 mg L<sup>-1</sup> NAA , after 8 week culture(G); callus on 0.5 mg L<sup>-1</sup> BAP + 2.0 mg L<sup>-1</sup> NAA after 8 weeks(H); Callus on 1.0 mg L<sup>-1</sup> BAP + 1.0 mg L<sup>-1</sup> NAA at 8 weeks (I).

Callus induction of *W. somnifera* was observed from hypocotyl, root, and cotyledonary leaf segments on MS medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D)

and kinetin (KN) [14]. In our study, the best callus was induction from leaf segment and rapid growth from sub-culturing the callus on MS medium supplemented with various concentrations and combination of 6-

Benzylaminopurine (BAP) and  $\alpha$ -Naphthalene acetic acid (NAA). Various worker reported the induction of callus from various explants of *W. somnifera*. Sharma *et al.* 2010 reported that maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on MS medium supplemented with a combination of 2mgL<sup>-1</sup> 2,4-D and 0.2 mgL<sup>-1</sup> Kinetin [19]. The best callus production of *W. somnifera* was observed by Silva *et al.* 2009 in MS medium supplemented with 1.0  $\mu$ M (Kin), 4.5  $\mu$ M BAP, and 1.5  $\mu$ M NAA within a 14 day dark period [20]. In previous study callus initiation was observed best in MS media with 1.0-5.0 mg/L 2,4- D after 16-20 days (93%) [21]. In our study, the highest mass of callus was developed 1.347  $\pm$  0.009 gm callus on MS medium containing 0.5 mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> NAA and followed 1.101  $\pm$  0.068 gm callus in MS media containing 1.0 mg L<sup>-1</sup> BAP + 1.0 mg L<sup>-1</sup> NAA. Only 0.585  $\pm$  0.098 gm of callus was formed from the initial callus within the same duration when they were culture on MS supplemented with 2.0 BAP and 0.5 NAA. The present of high level of NAA (1.0 mg L<sup>-1</sup> or more) in the culture media formation of callus as similar with same concentration of BAP then the formation of callus gradually reduce as the concentration of BAP add was increased. The amount of callus induced gradually reduces as the concentration of BAP added increase.

#### 4. Conclusion

In MS-medium with 0.5mg L<sup>-1</sup> BAP + 0.5 mg L<sup>-1</sup> NAA large callus were developed from subculturing callus and 1.347  $\pm$  0.99 gm of fresh callus was produced after the 8 week of culture from the 0.171  $\pm$  0.026 gm of initial callus mass. The dry weight of callus after the 8 week of culture is 0.194  $\pm$  0.015 gm. The best callusing medium was 0.5 mg L<sup>-1</sup> BAP + 1.5 mg L<sup>-1</sup> NAA; in which 1.091  $\pm$  0.026 gm of fresh callus was produced after the 8 week of culture from the 0.136  $\pm$  0.027 gm of callus then the dry weight of callus after the 8 week of culture was 0.144  $\pm$  0.006 gm. The amount of callus produced gradually decrease as the concentration of BAP added is increased; only 0.585  $\pm$  0.098 gm of callus was formed from the callus explants within the same duration when they were culture on MS-medium supplemented with 2.0 mg L<sup>-1</sup> BAP and 0.5 mg L<sup>-1</sup> NAA. In MS-medium with single phytohormones condition the formation of large callus were found 1.5 mg L<sup>-1</sup> BAP, 1.113  $\pm$  0.152 gm callus was produced from 0.498  $\pm$  0.046 gm callus explants after 8 weeks. The best callusing medium was 1.0 mg L<sup>-1</sup> NAA; 0.986  $\pm$  0.075 gm callus was produce after 8 weeks from 0.155  $\pm$  0.014 gm callus explants.

We have developed an efficient protocol for induction and proliferation of callus of *W. somnifera* using stem explants. This protocol might be useful for the production and isolation of metabolites in callus culture, because the natural propagation of *W. somnifera* is time taking because of long germination period and low levels of seed germination. We are undertaking the analysis of phytochemicals in culture (data not shown) of various ploidy levels. Furthermore this standardized callus induction and proliferation protocol might be used in

further research for mass propagation of *W. somnifera* via indirect regeneration methods.

#### References

- [1] Saldanha, C.J., Nicolson, D. H. "Flora of Hassan District Karnataka", India; Amerind Publishing Co. Pvt. Ltd., Lucknow. 1978.
- [2] Vaidyaratnam, P.S. "Indian Medicinal Plants, a compendium of 500 species", (Warrier.P.K. Nambiar V.P.K, Ramankutty Eds.), PartII, Orient Longman Publications, Hyderabad., 52-55. 1994.
- [3] Abraham, A., Kirson, I., Glotter, E., Lavie, D. A. "A chemotaxonomic study of *Withania somnifera* (L.) Dunal." *Phytochem.*, 7. 957-962. 1968.
- [4] Mohammad, Y. K., Aliabbas, S., Kumar, V., Rajkumar, S., "Recent advances in medicinal plant biotechnology." *Indian journal of Biotechnology*, 8(1). 9-22. 2009.
- [5] Yamada, Y., Shoyama, Y., Nishioka, I., Kohda, H., Namera, A. and Okamoto, T., "Clonal micropropagation of *Gentiana scabra* Bunge var. buregeri Maxim. And examination of the homogeneity concerning the gentiopicroside content." *Chemical and pharmaceutical Bulletin*. 39. 204-220. 1991.
- [6] Hina, T., Ali, S., Asi, M.R., "Appraisal of an important Flavonoid, Quercetin, in callus cultures of *Citrullus colocynthis*." *International Journal of Agriculture and Biology*, 14(4). 528-532. 2012.
- [7] Pierik, R. L. M., "In-vitro culture of higher plants" Martinus Nijhoff Publishers, Dordrecht, The Netherlands, 1987.
- [8] Gibson, D. M., Ketchum, R. E. B., Hirasuna, T. J., "Shuler, M. L., Potential of plant cell culture for Taxol production." *Science and Applications*, 1995.
- [9] Jamil *et al.*, "Regeneration of ginger plant from callus culture through organogenesis and effect of CO<sub>2</sub> enrichment on the differentiation of regenerated plant." *Biotechnplopy*. 6. 101-104. 2007.
- [10] Khan *et al.*, "In-vitro regeneration of garlic through garlic culture." *J. Biol. Sci.*, 4.189-191. 2004.
- [11] Luthfi *et al.*, "Selection of cell source and the effect of pH and MS macronutrients on biomass production in cell culture of Tongkat ali (*Eurycoma longifolia* Jack)." *Journal of Plant Biotechnol.*, 5. 131-135. 2003.
- [12] Shukla, D.D., Bhattarai, N., Pant, B., "In vitro Mass Propagation of *Withania somnifera* (L.) Dunal." *Nepal Journal of Science and Technology* 11.101-106. 2010.
- [13] Rani, G., Grover, I.S., "In vitro callus induction and regeneration studies in *Withania somnifera*." *Plant Cell Tiss Org Cult*, 57. 23-27. 1999.
- [14] Rani *et al.*, "Callus induction and plantlet regeneration in *Withania somnifera* (L.) Dunal. In Vitro Cell." *Dev. Biol. Plant* 39. 468-474. 2003.
- [15] Ray, S., Jha, S., "Withanolide synthesis in cultures of *Withania somnifera* transformed with *Agrobacterium tumefaciens*." *Plant Sci* 146. 1-7. 1999.
- [16] Paudel, S., Adhikari, S. R., Pant, B., "Effect of colchicines on production of secondary metabolites from callus of *Withania somnifera* (L)." *Journal of Nepal Biotechnology*, 3(1). 15-18. 2013.
- [17] Sangwan, N., "In vitro Withanolide Production by *Withania somnifera* L. Cultures." *Z. Naturforsch* 63. 409-412. 2008.
- [18] George, E.F., Sherrington, P.D., "Plant propagation by tissue culture Handbook and Dictionary of commercial Laboratories." Exegetics limited, Eversley, Basingstoke, Hants, England. 1984.
- [19] Sharma, P. K., Singh, G., Dudhe, R. and Singh, S., "Biological activities of *Withania somnifera*." *Anul of Bio Research* 1 (3). 56-63. 2010.
- [20] Silva, M.A.N. and Senarath, W.T.P.S.K., "In vitro mass propagation and greenhouse establishment of *Withania somnifera* (L.) Dunal (Solanaceae) and comparison of growth and chemical compounds of tissue cultured and seed raised plants." *J.Natm.Sci.Foundation Sri Lanka*, 37(4). 249-255. 2009.
- [21] Arumugam, A., Gopinath, K., "Micro propagation and tissue culture of the endangered medicinal plant *withania somnifera* by the direct shoot and root initiation method," *International Journal of Applied Biology and Pharmaceutical Technology*, 2(3). 2011.