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# *IN VITRO* CALLUSING AND EFFECT OF GROWTH REGULATORS ON *IN VITRO* PROPAGATED *WITHANIA* (CULTIVATED & WILD) THROUGH COTYLEDONARY LEAFS

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## Abstract

Leafs as well as Cotyledonary excised small explants of Ashwagandha were responsible and introduced to evaluate the effect of various growth regulators upon the *in vitro* micropropagation processes. Explants were applied to generate callus, shoot and root regeneration. In the first experiment small segments of leaf were cultured on MS basal medium added with mixture of 2,4 – Dichlorophenoxyaceticacid (2,4–D), naphthalene acetic acid (NAA) and indole butyric acid (IBA). The agile pharmacological components of *Withania somnifera* components are withanolides a Steroidal lactones ring with ergostane skeleton framework and alkaloids (Elsakka *et al* 1990; Gavande *et al* 2015). The active contents or substances of Indian *Withania somnifera* or Ashwagandha are normally withaferin-A and withanolide-D, both are generally present in leaves, stems and roots of the plant in high quantity, are used as a source of drugs. This procedure was standardized and stablished for very easy large scale mass propagation of ashwagandha medicinal plant. Callus formation was seen best in Murashige and Skoog media supplemented with NAA (1.0-3.0mg/L) after 16-20 days.

**Key words:** Cotyledonary explants, micropropagation, growth regulators, alkaloids, MS medium, withaferin-A.

## Introduction

A numbers of medicinal plants, continuously used for many past of years, now in a group of plant preparations of the Indian Ayurveda health care system named Rasayana given for their interesting antioxidant properties. Ashwagandha herb also possesses anti-stress properties, immunomodulatory properties, and antibacterial properties (Devi *et al* 1992; Devi *et al* 1993). Murthy *et al* (2010) worked on Ayurveda is a Sanskrit word, which means "the scripture for longevity" (Winters 2006). *Withania somnifera* (L.) Dunal (Family: Solanaceae, commonly known as Ashwagandha, English name: Winter cherry) is an important perennial plant species with immense therapeutic uses in traditional as well as modern system of medicine (Datta *et al* 2010). Due to restorative property of roots, the species is also known as 'Indian Ginseng' (Tripathi *et al 1996;* Andallu & Radhika 2000; Matsuda 2001; Winters 2006).

*Withania somnifera* consists of very high concentration of secondary metabolites that can be also known as bioreactors like steroidal lactones, alkaloids and flavonoides, which have effective properties and they used in ninety commercially ayurvedic formulations (Sreerekha *et al* 2004). *Withania somnifera* are propagated in northern western region of Madhay Pradesh in India, on about 400 ha. (Khare 1996; Thapliyal & Thapliyal 2001). But the risk of fungal infections are very high in this plants. Because of its medicinal properties, these plants are collected and subjected as very important raw substance for large or mass-scale medicinal industry, which leads to the over exploitation of plants and thus becomes plants move towards an endangered extinct plant species. One of the problems associated with Ashwagandha for its commercial cultivation and propagation, it takes very long periods for germination of seed and its strains productivity. Micro-propagation of *Withania somnifera* (Darwesh *et al* 2014) introduces various (excised pieces) such as shoot or stem tips (Sen & Sharma 1991), auxiliary highly division phase meristems (Roja *et al* 1991), auxiliary tip leaves, auxiliary or apical shoot, hypocotyls, cotyledonary leaves (embryonic leaves) and root (small) segments (Rani & Grover 1999) has been demonstrated. Propagation use by seed, but seed viability is limited to normally more than one year studied by Roja and Heble (1991) reported, callus formation from excised explants.

## Materials and Methods

**Chemicals:-** All chemicals were mostly of Hi Media, India and Sigma, USA and some of the chemical were also obtained from SRL, Qualigens and E. Merck, India.

**Medium Used For Tissue Culture:-** Medium Used For Tissue Culture for *in vitro* growth and regeneration of ashwagandha was the standard MS medium (Murashige & Skoog 1962) contain macronutrient salts, micronutrient salts, vitamins, Fe-EDTA, 0.01%(w/v) myo-inositol along with 3%(w/v) sucrose.

**For MS media, four stock solution were prepared as follows:**

| | | |
|---|---|---|
| Stock I | macronutrients | 10x |
| Stock II | micronutrients | 100x |
| Stock III | Fe-EDTA | 100x |
| Stock IV | Vit and AA | 100x |

The stock solutions I, II and IV were prepared by dissolving appropriate amounts of salt in MQ water but stock solution III was prepared by weighing FeSO4 7H2O and sodium salt of EDTA 2H2O separately in the required quantities, dissolving them separately by slight warming together and stored in dark container, because I is light sensitive. The above stock solutions were kept at 4$^{o}$C after autoclaving. During media preparation, the final concentration of each component was kept 1x and pH was adjusted to 5.8+0.1.

**Medium and glassware sterilization**

All the tissue culture media and vessels were steam sterilized by autoclaving at 15psi (1.04 kg/cm2) pressure at 121.c for 20 min. thermolabile substance were sterilized separately filtration (0.22um Millipore) then added to the autoclaved media when it was cooled at 40-45.c and mixed thoroughly. The media were then dispensed into autoclave culture tubes of radiations sterilized Petri dishes at allot to solidify. The glassware the solutions biodegradable detergent (labolene, India) and rinsed with double distilled water, over dried at 80.c for 2 hours, followed by most heat sterilization the instrument used for tissue culture, viz. forceps, needles, scalpels, spatula

etc. which is make contamination less by washing or dipping in 70% ethanol followed by burner flaming and then cooling in sterilized water at regular intervals while using.

**Surface-Sterilizing Plant Material**

Collected explants washed thoroughly in tap water to remove dust particles

Washed with distilled water for 4 to 5 times

Washed in Tween-20 for 30 min

Kept in Bactalon 10% (V/V) for 10 min

Washed with D.D water 4 or 5 time

Kept in 0.4% Bavistin for 1½ hours

Rinsed by autoclaved distilled water (4-5 time)

Treated with 70% ethanol for 30 sec

Rinsed by autoclaved distilled water (4-5 time)

Kept in 0.2% $Hgcl_2$ for 5 min

Washed by autoclaved distilled water (3-4 time)

Half procedures of sterilization were takes place in media preparation room and half in aseptic conditions, i.e., laminar air flow room.

**Results**

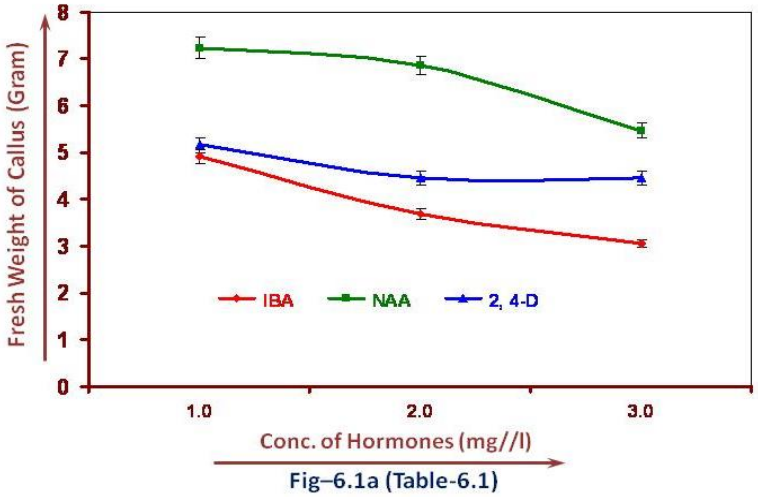
**Callus Culture initiation**

Cotyledon explants of cultivated ashwagandha (size 1.5cm) were inoculated in full strength MS medium supplemented with 0.8% agar-agar and same concentration (1mg/l - 3mg/l) of 2, 4-D, NAA and IBA. After two weeks of inoculation greenish colored callus was observed in different frequencies in different hormone concentration in MS medium. The results observed are depicted by the table below.

**Table 1-Effect of different auxins on *in vitro* growth and multiplication of callus in MS medium derived from embryonic cotyledon excised explant of *Withania somnifera* or ashwagandha (Cultivated):-**

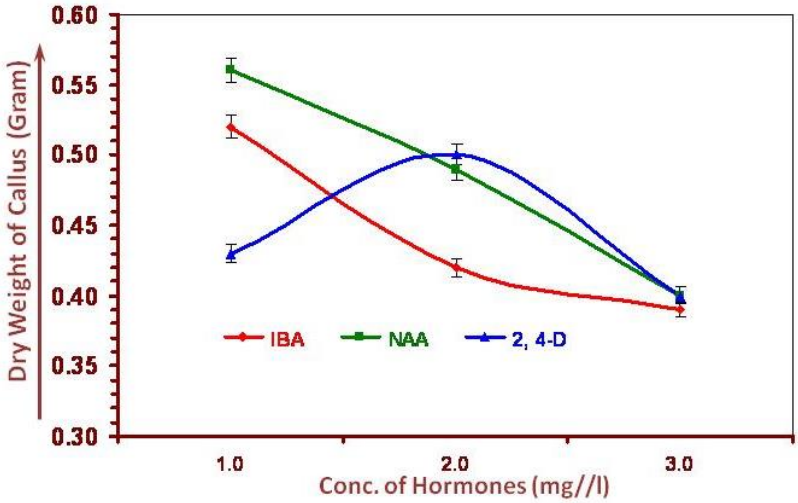
| S. No | Hormones (Auxins) | Conc. (mg/l) | Callus | | Frequency of formation of callus (%) |
|---|---|---|---|---|---|
| | | | Fresh Weight (Gram) | Dry Weight (Gram) | |
| 01 | IBA | 1.0 | 4.91±0.083 | 0.52±0.011 | 85±1.45 |
| | | 2.0 | 3.68±0.048 | 0.42±0.007 | 78±1.01 |
| | | 3.0 | 3.05±0.034 | 0.39±0.004 | 75±0.83 |
| 02 | NAA | 1.0 | 7.23±0.202 | 0.56±0.016 | 100±2.80 |
| | | 2.0 | 6.86±0.151 | 0.49±0.009 | 95±2.09 |
| | | 3.0 | 5.47±0.104 | 0.40±0.005 | 89±1.60 |
| 03 | 2, 4-D | 1.0 | 5.16±0.093 | 0.43±0.007 | 80±1.28 |
| | | 2.0 | 4.46±0.067 | 0.50±0.010 | 90±1.71 |
| | | 3.0 | 4.46±0.071 | 0.40±0.006 | 78±1.17 |

(Mean ± Standard error).




Fig–6.1a (Table-6.1)

**Bar diagram showing effect of different auxins on *in vitro* growth of excised cotyledons and formation of fresh weight of Callus in MS medium of *Withania somnifera* (Cultivated)**




Fig–6.1b (Table-6.1)

**Bar diagram showing effect of different auxins on *in vitro* growth of excised cotyledons and formation of dry weight of Callus in MS medium of *Withania somnifera* (Cultivated)**

## Callus Culture initiation

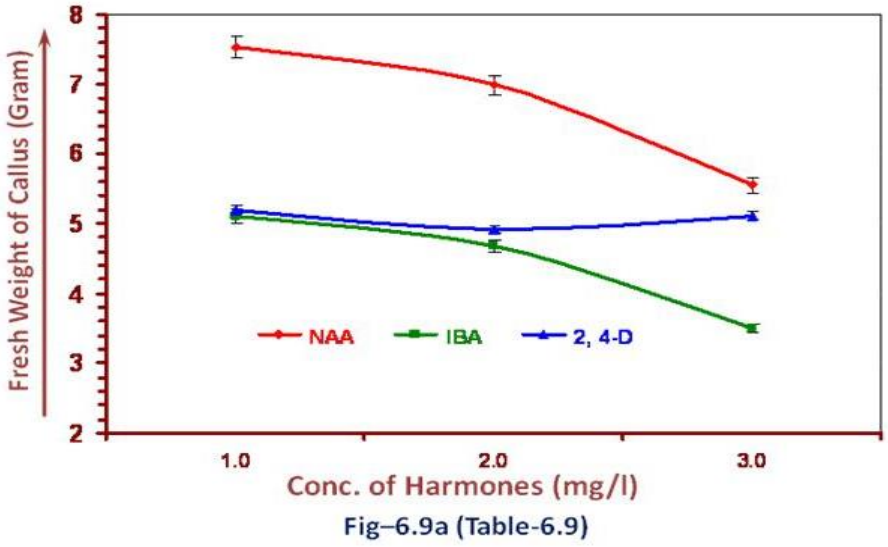
Cotyledon explants of wild ashwagandha (size 1.5cm) were inoculated in full strength MS medium supplemented with 0.8% agar-agar and same concentration (1mg/l - 3mg/l) of 2, 4-D, NAA and IBA . After two to three weeks of inoculation greenish colored callus was observed in

different frequencies in different harmone concentration in MS medium. The results observed are depicted by the table below.

**Table 2-Effect of auxins on growth of callus in MS medium derived from embryonic cotyledon excised explant of *Withania somnifera* or ashwagandha *(Wild):-***

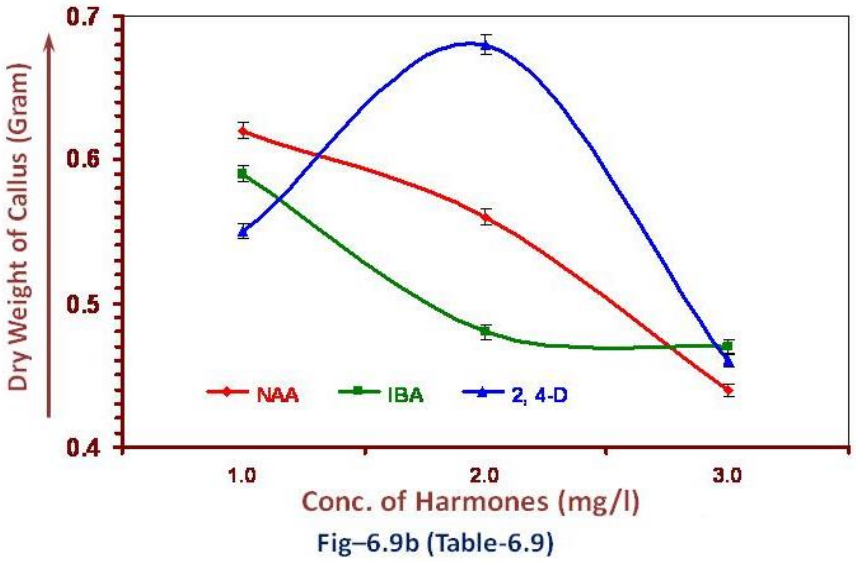
| S. No | Hormones (Auxins) | Conc. (mg/l) | Callus | | Frequency of formation of callus (%) |
|---|---|---|---|---|---|
| | | | Fresh Weight (Gram) | Dry Weight (Gram) | |
| 01 | NAA | 1.0 | 7.52±0.21 | 0.62±0.014 | 99±2.77 |
| | | 2.0 | 6.99±0.15 | 0.56±0.010 | 96±2.11 |
| | | 3.0 | 5.55±0.11 | 0.44±0.005 | 90±1.62 |
| 02 | IBA | 1.0 | 5.11±0.08 | 0.59±0.011 | 86±1.38 |
| | | 2.0 | 4.68±0.06 | 0.48±0.008 | 80±1.20 |
| | | 3.0 | 3.50±0.04 | 0.47±0.007 | 78±0.86 |
| 03 | 2, 4-D | 1.0 | 5.20±0.09 | 0.55±0.009 | 88±1.50 |
| | | 2.0 | 4.92±0.07 | 0.68±0.019 | 92±1.75 |
| | | 3.0 | 5.11±0.09 | 0.46±0.006 | 79±1.03 |

(Mean ± Standard error).




Fig–6.9a (Table-6.9)

**Bar diagram showing effect of different auxins on *in vitro* growth of excised cotyledons and formation of fresh weight of Callus in MS medium of *Withania somnifera* (Wild)**




Fig–6.9b (Table-6.9)

**Bar diagram showing effect of different auxins on *in vitro* growth of excised cotyledons and formation of dry weight of Callus in MS medium of *Withania somnifera* (Wild)**

## Discussion

*In vitro* propagation of plant easily achieved by researchers due to totipotency nature of plant cells in aseptic condition with suitable nutrient culture media. The unorganized and undifferentiated mass of plant cell referred to as callus which can be easily formed in *in vitro* condition when plant cells culture in a suitable medium. Plant growth regulator increases the efficiency of *in vitro* organogenesis. In this work callus formation occurs in nutrient medium with IBA, NAA, 2, 4-D due to dedifferentiation but the maximum callus formation takes placed with NAA in both cultivated and wild variety. Frequency of formation of callus in wild Withania is more than cultivated variety has been observed.

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