Effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins NAA and IBA in B5 medium on growth of callus derived from embryonic cotyledon explants of *Withania somnifera* (cultivated)

[1]Naveen Gaurav, [2] AP Singh, [3] Abhishekh Srivastava, [4]Arun Kumar, [5]Shefali Bindiya, [6] Komal, [7]Hira Singh Gariya

1. *Assistant Professor Department of Biotechnology, S G R R P G College Dehradun, U.K.*
2. *Professor, Department of Botany Govt. P.G. Science College, Rewa, M.P.*
3. *Assistant Professor, Department of Botany Govt. S.V. College Teonthar, M.P.*
4. *Assistant Professor, Department of Biotechnology, S G R R P G College Dehradun, U.K.*
5. *M.Sc, Department of Biotechnology, S G R R P G College Dehradun, U.K.*
6. *M.Sc, Department of Biotechnology, S G R R P G College Dehradun, U.K.*
7. *B.Sc Biotechnology, Department of Biotechnology, SGRRPG College Dehradun, U.K.*

1. ABSTRACT

Withania somnifera is mainly distributed from the Southern mid region to the Canary Islands and to South and East Africa, from Palestine upto North India, also having, Baluchistan, Sudan, Jordan, Egypt, Afghanistan, Iran, Israel and Pakistan. In India the plant grows and present in wild in North Western regions to mountainous and hills regions of Himachal Pradesh, Punjab and Jammu upto an altitude of 1500 m above the sea level. In India they are cultivated in Madhya Pradesh, Andhra Pradesh, Rajasthan and Uttar Pradesh. It is used as a bactericide, abortifacient, contraceptive, amoebocide, anodyne and diuretic. *Withania somnifera* has various properties against different diseases like nervous disorders, leprosy, rheumatism, diseases of respiratory and reproductory tract, inflammation, venereal disorders, psoriasis, scabies, consumption, ulcers, asthma, bronchitis, marasmus of children, insomnia, cancer, epilepsy, diabetes etc. *Withania somnifera* (L.) Dunal or Ashwagandha can be propagated by either asexual method (*in vitro* propagation of cotyledonary and nodal explants) or sexual. Seed propagation in *Withania* is not always satisfactory, since the heterogenetically strain produces a great deal of variation.

Key words: *Withania somnifera*, distributed, sea level, properties, asexual method, sexual, Seed propagation etc.

2. INTRODUCTION

When Ashwagandha is conventionally micro-propagated by seeds the percentage of growth & germination is minimum, due to the presence of certain inhibitory components in the fruit (De Silva and Senarath, 2009). There must be conventional agrotechnology to meet out the demand of the people. However, the conventional and old micro-propagation technique cannot be increasing demand of this herb which is used as raw material for the manufacture of pharmaceutical products in industries. Due to these reason the application of *in vitro* micro-propagation methods can be the other effective process for the continuous and regular possibility of supply or distribution of plantlet which is in stock for large scale field cultivation i.e. mass propagation in culture room. *In vitro* shoot differentiation of *Withania* from many different explants like as cotyledonary leaf and hypocotyl (Rani, *et.al*., 2003), leaf (Logesh, *et.al*., 2010;

Sharma, 2010; Owk, *et.al*., 2011) shoot tip (Rani & Grover, 1999; Ahuja, *et.al*., 2009; Furmanowa, et.al., 2001; Choudhary & Trivedi, 2012) node (Siddique, *et.al*., 2004; Shukla, *et.al*., 2010; Aniel, *et.al*., 2011) internode (Valizadeh & Valizadeh, 2009; Rout, *et.al*.,2011) successfully takes placed (Gupta & Sahu, 2015). It is maintain that in all of the above examines plants were re-prepared via morphogenesis from callus induced from the explants of Ashwagandha. One drawback of such method reported recalcitrance of the callus for differentiation which is negatively reduced the regeneration and multiplication tendency. They has been prepared few reports on *in vitro* shoot multiplication of Ashwagandha via direct organogenesis (Kulkarni,*et.al*., 1996; Kulkarni,*et.al*., 2000; Govindaraju, *et.al*., 2003; Supe,*et.al*., 2006; Sivanesan and Jeong, 2007; Sivanesan and Murugesan, 2008; Joshi and Padhya, 2010; Kanungo and Sahoo, 2011). Therefore, the recent studies have been providing best applicable traits for direct herb regeneration and multiplication from nodal explants of *Withania somnifera* (L.) Dunal. The comparative account has been prepared for natural and propagated traits for qualitative properties.

3. MATERIAL AND METHODS

Composition of B5 medium

Composition :	Ingredients milligrams/litre
Sucrose	20000.00
Potassium nitrate	2500.00
Calcium chloride.$2H_2O$	150.00
Ammonium sulphate $(NH_4)_2SO_4$	134.00
sulphate monobasic	130.42
Magnesium sulphate	122.09
myo - Inositol	100.00
EDTA disodium salt.2H2O	37.30
Ferrous sulphate.$7H_2O$	27.80
um pho Sodi Manganese sulphate.H_2O	10.00
Thiamine hydrochloride	10.00
Boric acid	3.00
Zinc sulphate.$7H_2O$	2.00
Pyridoxine hydrochloride	1.00
Nicotinic acid (Free acid)	1.00
Potassium iodide	0.75
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Molybdic acid (sodium salt).$2H_2O$	0.25
TOTAL gm/litre	23.23

Preparation of B5 medium:-

Added 23.23 grams of dehydrated powdered B5 medium in 600ml of distilled water and to wash or clean the media vial by suitable and small quantity of double distilled water to remove out the traces of powder. Apply constant gentle animation to the solution in a proper way till the powder dissolves completely. Add heat stable supplements to obtain after autoclaving. Maintain the obtain pH of the medium by using 1N HCl/1N NaOH/1N KOH. Make up 1000ml final volume with continuous adding distilled water. Sterilize the medium or make the medium free from contamination by the process of autoclaving at 15 lbs or 121ºC for 15 minutes. Then cool the autoclaved medium to 45ºC prior addition of the filter sterilized heat sensitive supplements. Store the prepared medium at 2-8ºC away from direct light.

PLANT GROWTH REGULATORS

Plant growth regulators (PGR's) were prepared separately as stock solutions. Some naturally occurring PGR's are heat sensitive so they should be sterilized either by filter sterilization or other by autoclaving at 1.04 k.g/cm^2 at 121^{o}C for 20 min depending on heat liability of substances. Some of the commonly used PGR's are listed as below :-

Commonly Used Planted Growth Regulators.

Substances (PGR's)	Solvent	Stock concentration	Sterilization	Storage conditions
1BA	1N Na OH	0.5 mg/1	CA	$0^{O}C$
BAP	1N Na OH	20 mg/1	CA	$0^{O}C$
NAA	1N Na OH	2 mg/1	CA	$4^{O}C$

CA = Co-autoclavable with other media components
F = Filter sterilization with 0.22 micro Millipore filter

Medium and glassware sterilization

All the tissue culture media and vessels were steam sterilized by autoclaving at 15psi (1.04 kg/cm2) pressure at $121^{0}C$ for 20 min. thermolabile substance were sterilized separately filtration (0.22µm Millipore)then added to the autoclaved media when it was cooled at 40-$45^{0}C$ and mixed thoroughly.

4. RESULTS

Table 4.1: Effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins NAA in B5 medium on growth of callus derived from embryonic cotyledon explants of *Withania somnifera* (Cultivated)

S. No.	Cyto–kinins	Conc. (mg/l)	Auxins (NAA) (mg/l)	Callus (Fresh Weight) Gram	Callus Dry Weight Gram	Frequency of formation of callus (%)
01	BAP	0.5	1.0	5.21±0.08	0.39±0.005	71±1.78
		1.0	1.0	5.99±0.11	0.51±0.011	75±2.03
		1.5	1.0	5.02±0.06	0.40±0.006	66±0.99
02	Kinetin	0.5	1.0	7.75±0.21	0.61±0.016	66±1.19
		1.0	1.0	7.26±0.18	0.55±0.014	61±0.73
		1.5	1.0	7.01±0.15	0.50±0.009	69±1.45

(Mean [+ or -] Standard error).

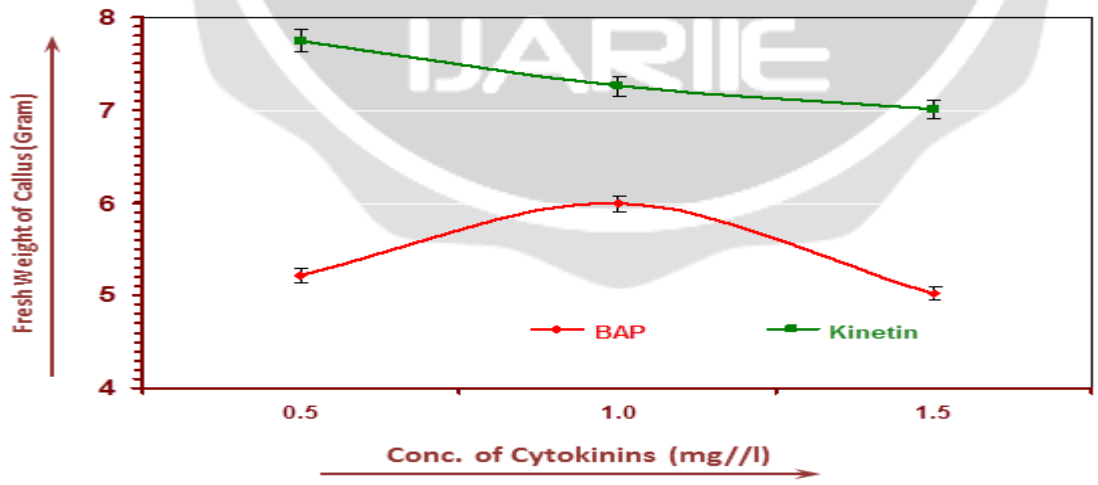

Table-4.1a

Bar diagram showing effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins NAA on growth of Callus (fresh weight) in B5 medium of *Withania somnifera* (Cultivated)

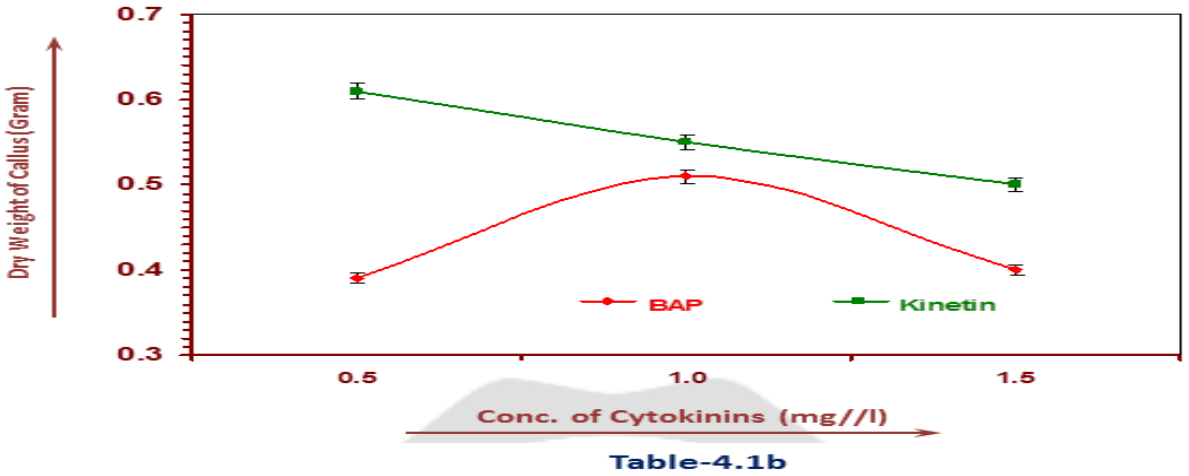

Table-4.1b

Bar diagram showing effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins NAA on growth of Callus (dry weight) in B5 medium of *Withania somnifera* (Cultivated)

Cotyledon explants (size 1.5cm) were inoculated in full strength B5 medium supplemented with 0.8% agar-agar and same concentration 1mg/l of NAA with 0.5mg/l to 1.5mg/l BAP, same concentration 1mg/l of NAA with 0.5mg/l to 1.5mg/l Kn. After two to three weeks of inoculation greenish colored callus was observed in different frequencies in different harmone concentration in B5 medium. The results observed are depicted by the table 4.1.

B5 medium having same concentration of NAA (1mg/l) with different concentration of cytokinins (0.5 to 1.5mg/l) also initiates the formation of callus. Maximum formation of fresh and dry callus takes placed in B5 medium having 1.0mg/l BAP with 1.0mg/l NAA with and 1.0mg/l NAA with 1.5mg/l kinetin, in which maximum frequency of callus formation takes placed with BAP (1.0mg/l BAP) as shown in table 4.1.

Table 4.2: Effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins IBA in B5 medium on growth of callus derived from embryonic cotyledon explants of *Withania somnifera* (Cultivated)

S. No.	Cyto–kinins	Conc. (mg/l)	Auxins (IBA) (mg/l)	Callus		Frequency of formation of callus (%)
				(Fresh Weight) Gram	Dry Weight Gram	
01	BAP	0.5	1.00	3.01±0.04	0.19±0.002	65±1.17
		1.0	1.00	4.89±0.12	0.30±0.006	77±2.08
		1.5	1.00	4.11±0.06	0.28±0.004	63±0.76
02	Kinetin	0.5	1.00	4.43±0.08	0.32±0.008	69±1.45
		1.0	1.00	5.00±0.14	0.34±0.009	73±1.83
		1.5	1.00	4.80±0.10	0.29±0.005	64±0.96

(Mean [+ or -] Standard error).

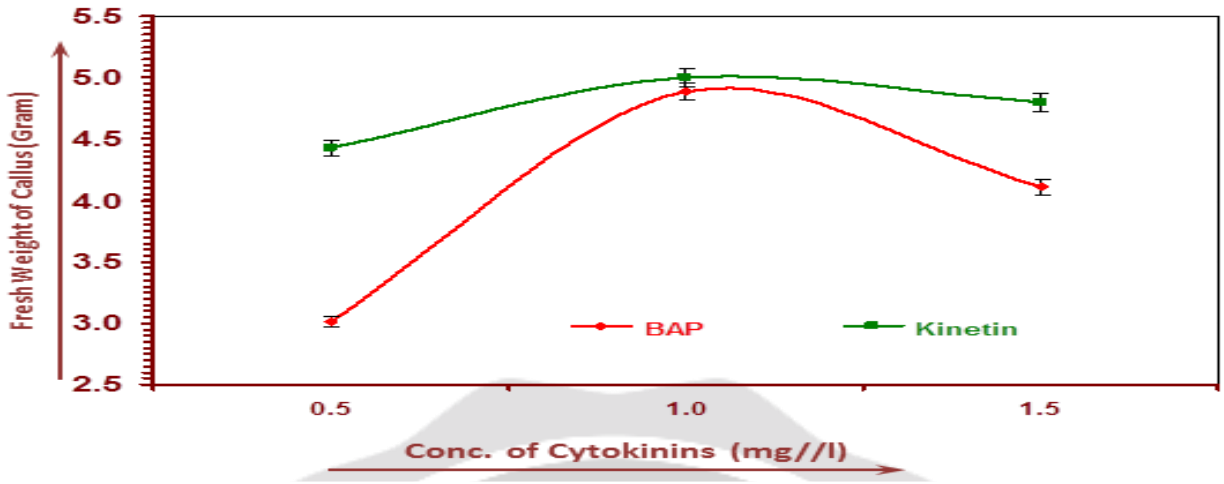

Table-4.2a

Bar diagram showing effect of supplementing mixture of Benzene aminopurine (BAP) and Kinetin (Kn) along with auxins IBA on growth of Callus (fresh weight) in B5 medium of *Withania somnifera* (Cultivated)

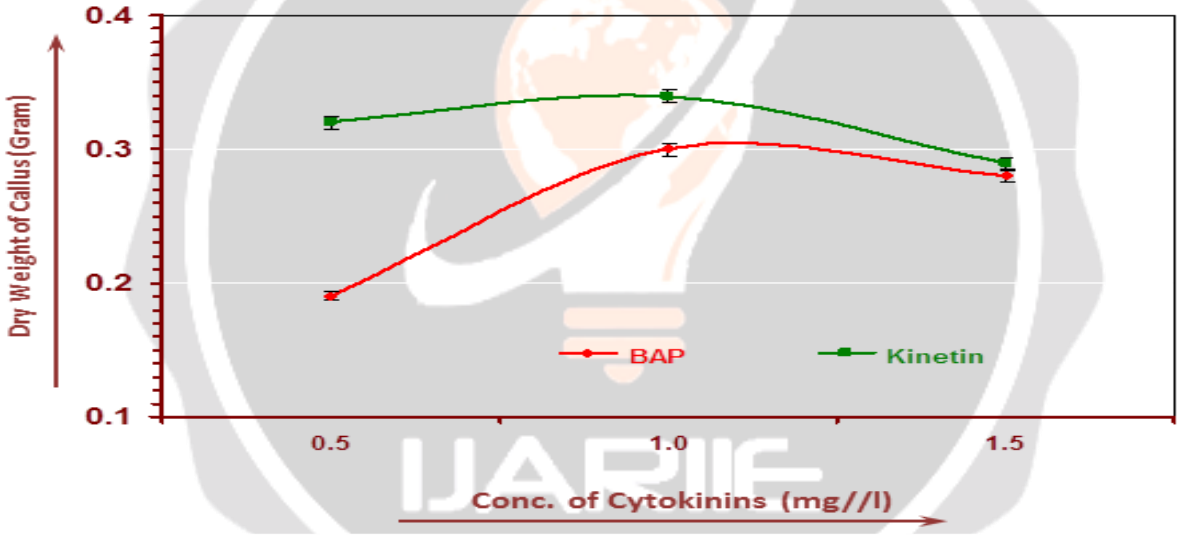

Table-4.2b

Bar diagram showing effect of supplementing mixture of Benzene aminopurine (BAP) and kinetin (Kn) along with auxins IBA on growth of Callus (dry weight) in B5 medium of *Withania somnifera* (Cultivated)

Cotyledon explants (size 1.5cm) were inoculated in full strength B5 medium supplemented with 0.8% agar-agar and same concentration 1mg/l of IBA with 0.5mg/l to 1.5mg/l BAP, same concentration 1mg/l of IBA with 0.5mg/l to 1.5mg/l Kn. After two to three weeks of inoculation greenish colored callus was observed in different frequencies in different harmone concentration in B5 medium. The results observed are depicted by the table 4.2.

B5 medium having same concentration of IBA (1.0mg/l) with different concentration of cytokinins (0.5 to 1.5mg/l) also initiates the formation of callus. Maximum formation of fresh and dry callus takes placed in B5 medium having 1.0mg/l IBA with 1.0mg/l BAP and 1.0mg/l IBA with 1.0mg/l kinetin, in which maximum frequency of callus formation takes placed with BAP (1.0mg/l) as shown in table 4.2.

5. DISCUSSION

Chaudhry et.al.,(2009) did work on Ashwagandha, the medicinal plant of India it will be used as an "adaptogen" and has been referred to as the 'ayurvedic ginseng due to their different effective property'. The recent methodology deals with the showing effect of different media explants, carbon sources, different concentration of agar

and varying salt concentrations on in-lab propagation of Ashwagandha and its phytochemical effects. From taking different explants (node, internode, apical bud, petiole) and media (MS, B5) which is responsible for growth, the best results in terms of percent regeneration and growth index were present or obtained on MS media with cotyledonary leaves, nodal and apical bud explants. Callusing was obtained on MS+ NAA+ Kn with petiole and internodes as explants. Among the given different carbon sources like (sucrose, fructose, glucose), sucrose at three percent concentration was assume to be optimum for shoot induction. Among the certain decrease in agar concentration from 0.8% to 0.16%, there was increase in shoot regeneration and production and also the size of leaf.

The nodal segments showed bud break response when it cultured in both the basal medium. The MS medium fortified with BAP induced bud break in 100% explants whereas B5 medium supplemented with BAP induced bud break in 80-100% of explants. The nodal segments (Aniel, et.al, 2011) produced 3.0±0.2 shoots per explants on MS medium as compared to 1.0±0.02 on B5 medium. The shoots elongated from nodal explants on different media shows variation in shoot length.

Sabir, *et.al., (*2008) completed work on Ashwagandha which is the one of most honourable medicinal plants of Ayurveda, in the time of ancestors to posterity medicinal system. Many of its proclaimed medicinal properties coming from ancient time to work in combination with another by recent molecular pharmacological examination and research which is proved to be associated with its definite secondary metabolites i.e. the properties that characterize a species, the novel group of ergostane skeletal phytosteroids known as withanolides, named after the plant.

Schliebs, *et.al.,* (1997) worked on some medicinal plants and promising results have been identified by the presence of acetylcholinesterase inhibitors, an useful treatment mediation in Alzheimer's disease shows an affective effect and it still remains an important goal. Withaferin-A, obtained mainly from the aqueous or soluble methanol extract obtain from the roots of cultivated varieties of Ashwagandha (called as Indian ginseng), act of exuding from a slope rocks of the Himalaya mountain, are widely used in Indian medicine to make slender cerebral functional deficits.

Sahana, *et.al.,* (2012) worked on most of the Neuro-defective disorders that play a great role in detecting maximum healthy age and also act as important factors in detecting the maximum healthy age and the mortality in recent times. Some of the neurological disorders which act an important role in disorders are Parkinson's, Creutzfeldt-Jakob's, Alzheimer's, Huntington's, amyotrophic lateral sclerosis. According to the recent study the World Health Organization (WHO), the UN's health agency, described that the mental and neurological diseases may increase from depression to Alzheimer's currently strike 400 million people all around the world and are set to a powerful rush in the next twenty years. The methanolic extracts obtained from the roots of these plantlets were subjected to HPLC for identification of compounds. Phytochemical screening showed the presence of various types of bioactive components such as alkaloids, steroids, tannins, flavonoids, glycosides, terpenoids and saponins (Sankar, 2012).

Malik, *et.al.,* (2007) published paper on a standardized root extract of *Withania somnifera* and its principal constituent withanolide-A elicit immune response such as cell and humoral-mediated immune responses.

Chatterjee, et.al., (2010) published paper on comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts and worked on Profiling of metabolites which is a rapid intended area of research for resolving metabolic pathways. Metabolic fingerprinting in terms of medicinal plants is crucial to establishing the importance of herbal medicines. In recent studies, metabolic profiling of crude extracts of leaf and root of *Withania somnifera* (Ashwagandha), an important medicinal plant of Indian system of medicine (ISM) were carrying out through NMR and HPLC techniques.

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