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In vitro tissue culture studies from nodal and petiole explants of wild and cultivated traits of *Withania somnifera* in MS and B5 medium

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Abstract
However, the traditional or conventional and old micro-propagation technique cannot be applicable in increasing and maintaining of demand of this herb which is used as very important raw substance for the preparation of therapeutic and pharmaceutical products in medicinal industries because of these reason the application and cultivation of *in vitro* micro-propagation methods of shwagandha plants can be the another most effective method for the regular and continuous possibility of maintaining demand and supply or distribution of several plantlet which is in stock of *Withania* for large scale field mass cultivation i.e. mass propagation *in vitro* culture room. *In vitro* shoot differentiation and micro-propagation of *Withania somnifera* from various different small excised explants such as hypocotyl and cotyledonary leaf, shoot tip, node, internode. Optimal normal growth, multiplication and development of *Withania somnifera* through morphogenesis *in vitro* processes of many tissues may vary for several different plants based on important nutritional requirements of plants. Basic nutrient basal media that are most frequently applicable and used include MS (Murashige and Skoog) medium, Gamborg (B5) medium, Linsmaier and Skoog (LS) medium and Nitsch and Nitsch (NN) medium.

Keywords: Ashwagandha, bio-generation, withanolide-A, *in vitro* shoots, hormonal combinations.

1. Introduction
Winters (2006) [22] did work on Ashwagandha, has been appleid for past centuries in Rasayana and Ayurvedic medicine to increase longevity life and vitality. Root part of Ashwagandha is highly rich in several alkaloids like withanine (Majumdar, 1955; Mohan, *et al*, 2004; Das, *et al*, 2010) [10, 11, 1] and these are value added substances uses in many traditional Ayurvedic and Rasayana drug or medicine preparations prevent many diorder such as female disorders, rheumatism, cough, anti-inflammatory and dropsy (Kiritikar & Basu, 1975; Naveen Gaurav *et al*, 2016) [7]. In addition to fresh roots, the various parts of *Withania* herb having anti-bacterial, anti-tumor property & improvement in many disorder of the animal body system (Devi, *et. al*, 1992; Devi, 1996; Singh & Kumar, 2012) [21, 3, 17]. When Ashwagandha is conventionally or traditionally cultivated or propagated by seeds the percentage of growth, multiplication & germination is lowest or minimum, because of the occurrence of certain many inhibitory substances in the fruit (De Silva, *et. al*, 2009; Naveen Gaurav *et. al*, 2015) [2, 13]. It is regularly maintain that in all examines plants part were re-isolated via morphogenesis from callus (unorganized and undifferentiated part) induced from the small excised explants of *Withania somnifera*. One of the important drawback of these process examined re-calcitrance of the callus (unorganized and undifferentiated part) for differentiation which is negatively decreased the multiplication and regeneration tendency. They has been identified several reports on *in vitro* shoot or stem multiplication and growth of *Withania somnifera* via direct organogenesis (without formation of callus) (Kulkarni, *et al*, 1996; Kulkarni, *et al*, 2000; Govindaraju, *et al*, 2003; Supe, *et al*, 2006; Sivanesan, 2007; Sivanesan & Murugesan, 2008; Joshi & Padhya, 2010; Naveen Gaurav *et al*, 2015) [8, 9, 5, 20, 18, 19, 6, 13].

2. Material and Methods
An efficient effective and improved shoot regeneration technique for the *in vitro* propagation micro-propagation of *Withania somnifera* L. through micro-propagation *(in vitro)* culture of nodal segments (cut small part) with axillary meristematic buds using by thidiazuron (TDZ) is

prepared. The *in vitro* plantlets of *Withania* went through hardening stage in a growth chamber and green house, before to transfer in *ex vitro*. Normal maturity and flowering takes place in micro-propagated plants. The present regeneration and multiplication process favoured large-scale production and *in vitro* conservation of *W. somnifera* for many years. (Fatima & Anis, 2011) [4]

Specimen collections

The seeds of the cultivated variety of *Withania somnifera* L. (Dunal) were obtained from the local nurseries while the seeds of wild varieties obtained from several research institutes like Forest Research Institute, National Botanical Research Institute and CIMAP.

Preparation of MS and B5 medium

Medium Used For Tissue Culture for *in vitro* growth and regeneration of Ashwagandha was the standard MS medium (Murashige and Skoog, 1962) [12] containg macronutrient salts, micronutrient salts, vitamins, Fe-EDTA, 0.01% (w/v) myo-inositol along with 3% (w/v) sucrose. 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 it is light sensitive. The above stock solutions were kept at 4 ºC after autoclaving. During media preparation, the final concentration of each component was kept 1x and pH was adjusted to 5.8+0.1.

B5 or Gamborg basal medium has been some time considered for the growth of Ashwagandha cells in the presence of several growth hormones. Dehydrated B5 medium of 23.23 grams added in 600 ml of distilled water and to wash or clean the media 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 suitable pH of the medium by using 1N HCl/1N NaOH/1N KOH. Make up the final volume to 1000 ml with continuous adding distilled water. Sterilize the medium or make the medium free from contamination by the process of autoclaving at 15 lbs or 121 ºC for 15 minutes. Then cool the autoclaved medium to 45 ºC prior addion of the filter sterilized heat sensitive supplements. Store the prepared medium at 2-8 ºC away from direct light.

Medium and glassware sterilization

All the media of tissue culture and glass vessels were steam sterilized by autoclaving at 15psi (1.04 kg/cm^2) pressure at 121 ^{0}C for 20 min. Thermolabile substance were sterilized separately by filtration (0.22 µm Millipore) then added to the autoclaved media when it was cooled at 40-45 ^{0}C and mixed thoroughly. The media were then dispensed into autoclave culture tubes of radiations sterilized Petri dishes at allot to solidify. The glassware subjected to the solutions of biodegradable detergent (labolene, India) and rinsed with double distilled water, oven dried at 80 ^{0}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 (Naveen Gaurav *et al*, 2015) [13].

Solidifying agents

Hardness of the culture medium for *in vitro* multiplication greatly influences and affects the growth of micro-propagated cultured tissues. There are many solidifying or gelling agents uses in tissue culture like as agarose, agar, and gellatin gum.

3. Results and Discussion

Ms Medium

In recent *in vitro* study, we identify to know the influence of Kinetin, BAP and IAA on the excised nodal small segments of *Withania somnifera.* It was having an admirable interest to identify that the 1.5 mg/l Kinetin + 0.5 mg/l BAP + 1.5 mg/l IAA growth hormones perform the maximum useful effect on the *in vitro* formation of callus followed by 0.5 mg/l Kinetin + 1.5 mg/l BAP + 0.5 mg/l IAA, where the formation of callus (unorganized) lowers from +++ to ++. The formation of callus was least visualized at the concentration of 1.5 mg/l Kinetin + 0. 5 mg/l BAP + 0.5 mg/l IAA. Result has shown in table 4.1.

Maximum % and number of shoots formation takes placed in 1.5 mg/l Kinetin + 0.5 mg/l BAP + 1.5 mg/l IAA with MS basal medium while lowest % of and number of shoots formation through callus occurs in MS medium supplemented with 2 mg/l mg/l BAP alone through nodal segments as examined in table 4.1.

When the hormonal combinations of 1 mg/l BAP and 0.5 mg/l IAA to 2 mg/l IAA is introduced on the petiole of *Withania somnifera* the formation of callus gets lower as the concentration of IAA rises while the number of shoot bud and callus formation maximum on MS medium with 1 mg/l BAP + 1 mg/l IAA as shown in table 4.2.

When MS medium with 2 mg/l kinetin alone induces the frequency of callus as well as shoots formation are always high as shown in table 4.3.

Table 4.1: Influence of Kinetin, BAP and IAA on the excised nodal segments of *Withania somnifera.*

S. No.	Medium	Hormone Taken(mg/L)	No of Explants	No. of Shoots/explants	Callus Formation
1.	MS	1.5 Kinetin + 0.5 BAP + 0.5 IAA	15	1-2	++
2.	MS	1.5 Kinetin+ 0.5 BAP + 1.5IAA	10	5-6	++++
3.	MS	0.5 Kinetin + 1.5 BAP + 0.5 IAA	10	1-2	++
4.	MS	2 BAP + 0.5 IAA	30	1-4	+++
5.	MS	1.0 BAP + 2.O Kinetin	30	3-5	++
6.	MS	2.0 BAP	30	1-2	+

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.2: Effect of BAP, IAA on Petiole of *Withania somnifera*

S.No.	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	1 BAP + 0.5 IAA	15	2-3	+++
2.	MS	1 BAP + 1 IAA	15	4-5	++++
3.	MS	1 BAP + 2 IAA	10	1-3	++

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.3: Influence of kinetin (Kn) on the excised nodal small segment of *Withania somnifera*

S. No.	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	2.0 Kinetin	30	4-6	++++

Table 4.4: Influence of of Kinetin, BAP and IAA on the excised small nodal segments of *Withania somnifera.*

S. No.	Medium	Hormone Taken(mg/L)	No of Explants	No. of Shoots/explants	Callus Formation
1.	MS	1.5 Kinetin + 0.5 BAP + 0.5 IAA	15	2-3	++
2.	MS	1.5 Kinetin + 0.5 BAP + 1.5 IAA	10	5-7	++++
3.	MS	0.5 Kinetin + 1.5 BAP + 0.5 IAA	10	3-4	++
4.	MS	2 BAP + 0.5 IAA	30	2-3	++
5.	MS	1.0 BAP + 2.0 Kinetin	30	5-6	+++
6.	MS	2.0 BAP	30	3-4	++

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.5: Effect of BAP, IAA on Petiole of *Withania somnifera*

S No	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	1 BAP + 0.5 IAA	15	5-7	++++
2.	MS	1 BAP + 1 IAA	15	3-4	++
3.	MS	1 BAP + 2 IAA	10	2-3	++

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.6: Influence of kinetin (Kn) on the excised nodal segment of *Withania somnifera*

S. No.	Medium	Hormone taken mg/L	No. of Explants	No. of Shoots/Explants	Callus Formation
1.	MS	2.0 Kinetin	30	5-7	++++

Table 4.7: Overall conclusion of Influence of Kinetin, BAP and IAA on the excised nodal segments of *Withania somnifera* (both W & C):-

S. No.	Conc. of GRs mg/l (max. gth conc.)	Cultivars	No. of explants	No. of shoots	Callus formation
1.	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Wild	10	5-7	++++ Very Good
2.	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Cultivated	10	5-6	++++ Very Good

Table 4.8: Overall conclusion of Influence of kinetin, BAP and IAA on excised Petiole of *Withania somnifera* (W & C):-

S. No.	Medium	Conc. of GRs mg/l (max. gth conc.)	Cultivars	No. of explants	No. of shoots	Callus formation
1.	MS	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Wild	15	5-7	++++ Very Good
2.	MS	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Cultivated	1	4-5	++++ Very Good

Table 4.9: Influence of kinetin, BAP and IAA on excised small nodal segments of *Withania somnifera.*

S. NO.	Medium	Hormone Taken(mg/L)	No. of explants	No. of Shoots/explants	Callus Formation
1.	B5	1.5 Kinetin + 0.5 BAP + 0.5 IAA	15	0-1	+
2.	B5	1.5 Kinetin + 0.5 BAP + 1.5 IAA	10	1-3	++
3.	B5	0.5 Kinetin + 1.5 BAP + 0.5 IAA	10	1-2	++
4.	B5	2 BAP + 0.5 IAA	30	1-2	++
5.	B5	1.0 BAP + 2.O Kinetin	30	1-3	++
6.	B5	2.0 BAP	30	0-1	+

++++= Very High; +++= Good; ++= Low; += Poor, -= Absent

Table 4.10: Effect of BAP, IAA on Petiole of *Withania somnifera*

S. No.	Medium	Hormone taken mg/L	No. of Explants	No. of Shoots/Explants	Callus Formation
1	B5	1 BAP + 0.5 IAA	15	1-3	++
2.	B5	1 BAP + 1 IAA	15	0-1	+
3.	B5	1 BAP + 2 IAA	10	0-1	+

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.11: Influence of hormone kinetin on the excised small nodal segment of *Withania somnifera*

S. No.	Medium	Hormone taken mg/L	No. of Explants	No. of Shoots/Explants	Callus Formation
1.	B5	2.0 Kinetin	30	1-2	++

Table 4.12: Influence of Kinetin, BAP and IAA on the excised nodal segments of *Withania somnifera.*

S. NO.	Medium	Hormone Taken(mg/L)	No of Explants	No. of Shoots/explants	Callus Formation
1.	B5	1.5 Kinetin + 0.5 BAP + 0.5 IAA	15	-	-
2.	B5	Kinetin + 1.5 0.5 BAP + 1.5 IAA	10	1-2	++
3.	B5	0.5 Kinetin + 1.5 BAP + 0.5 IAA	10	0-1	+
4.	B5	2 BAP + 0.5 IAA	30	-	-
5.	B5	1.0 BAP + 2.O Kinetin	30	-	-
6.	B5	2.0 BAP	30	0-1	+

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.13: Effect of BAP, IAA on Petiole of *Withania somnifera*

S No	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	B5	1 BAP + 0.5 IAA	15	0-1	+
2.	B5	1 BAP + 1 IAA	15	-	-
3.	B5	1 BAP + 2 IAA	10	-	-

++++ = Very High, +++ = Good, ++ = Low, + = Poor, - = Absent

Table 4.14: Influence of hormone kinetin on the excised nodal segment of *Withania somnifera*

S No	Medium	Hormone taken mg/L	No of Explants	No. of Shoots/Explants	Callus Formation
1.	B5	2.0 Kinetin	30	0-1	+

Table 4.15: Overall conclusion of Effect of BAP, Kinetin and IAA in B5 medium on the nodal segments of *Withania somnifera* (both W & C):-

S. No.	Conc. of GRs mg/l (max. gth conc.)	Cultivars	No. of explants	No. of shoots	Callus formation
1.	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Wild	10	1-2	++ Low
2.	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Cultivated	10	1-3	++ Low

Table 4.16: Overall conclusion of Effect of BAP, IAA in B5 medium on Petiole of *Withania somnifera* (W & C):-

S. No.	Medium	Conc. of GRs mg/l (max. gth conc.)	Cultivars	No. of explants	No. of shoots	Callus formation
1.	B5	0.5 BAP + 1.5 Kinetin + 1.5	Wild	15	0-1	+ Poor
2.	B5	0.5 BAP + 1.5 Kinetin + 1.5 IAA	Cultivated	1	1-3	++ Low

In the following research we have evaluated the the influence of Kinetin, BAP and IAA on the excised nodal small segments of *Withania somnifera*. It was having an admirable interest to identify that the 1.5 mg/l Kinetin + 0.5 mg/l BAP + 1.5 mg/l IAA growth hormones perform the maximum useful effect on the *in vitro* formation of callus followed by 2.0 mg/l Kinetin + 1.0 mg/l BAP + 0.5 mg/l IAA, where the callus formation lowers from ++++ to +++. The formation of callus was least visualized at the concentration of 1.5 mg/l Kinetin + 0.5 mg/l BAP + 0.5 mg/l IAA same as cultivated variety of *Withania somnifera* but frequency of formation of callus and shoots are higher than cultivated variety of *Withania* explants. Result has shown in table 4.4.

When the combination of growth regulators 1 BAP (benzene aminopurine) and hormone 2 mg/l IAA (indole 3-acetic acid) is applied on excised small petiole of Ashwagandha the *in vitro* callus formation is reduces when amount of IAA (indole 3-acetic acid) increases but frequency of formation of callus and shoots are higher than cultivated variety of *Withania* explants. Result has shown in table 4.5.

When MS medium supplemented with 2mg/l kinetin alone then the frequency of callus as well as shoots formation are always high in comparison to cultivated variety of explants as shown in table 4.6.

Comparison between wild and cultivated explants in MS medium with 0.5 mg/l BAP + 1.5 mg/l Kinetin + 1.5 mg/l IAA hormones concentration shows that the frequency of formation of callus as well as shoots higher in wild explants (nodal) of Withania. Results have been shown in table 4.7.

Comparison between wild and cultivated explants in MS medium with 0.5 mg/l BAP + 1.5 mg/l Kinetin + 1.5 mg/l IAA hormones concentration shows that the frequency of formation of callus as well as shoots higher in wild explants (petiole) of Withania. But frequency of formation of callus as well as shoots higher with nodal explants in comparison with petiole. Results has shown in table 4.8.

B5 Medium

In the following research we have evaluated the influence of Kinetin, BAP and IAA on B5 medium the excised nodal small segments of *Withania somnifera*, the 1.5 mg/l Kinetin + 0.5 mg/l BAP + 1.5 IAA growth hormones perform the maximum useful effect on the *in vitro* formation of callus. Result has shown in table 4.9 Maximum % and number of shoots formation takes placed in 1.5mg/l Kinetin + 0.5 BAP + 1.5 mg/l IAA hormones with B5 basal medium while lowest % of and number of shoots formation through callus occurs in B5 medium supplemented with 2 mg/l BAP alone through nodal segments. But the frequency of formation of callus as well as shoots in B5 medium are lower than MS medium as examined in table 4.9.

When the combination of growth regulators, 1 mg/l BAP (benzene aminopurine) and 0.5 mg/l IAA to 2 mg/l IAA (indole 3-acetic acid) is applied on excised small petiole of Ashwagandha the *in vitro* callus formation is reduces when amount of IAA (indole 3-acetic acid) increases. While the number of shoot bud and callus formation maximum on B5 medium supplemented with 1 mg/l BAP + 0.5 mg/l IAA. But the frequency of formation of callus as well as shoots in B5 medium are lower than MS medium as shown in table 4.10.

When B5 medium supplemented with 2 mg/l kinetin alone then the frequency of callus as well as shoots formation are always high but in comparison to MS medium frequency of callus and shoots formation is very low as shown in table 4.11.

In the following research we have evaluated the the influence of Kinetin, BAP and IAA on the excised nodal small segments of *Withania somnifera*. It was having an admirable interest to identify that the 1.5 mg/l Kinetin + 0.5 mg/l BAP + 1.5 mg/l IAA growth hormones perform the maximum useful effect on the *in vitro* formation of callus same as cultivated variety of *Withania somnifera* but frequency of formation of callus and shoots are higher than cultivated variety of *Withania* explants but frequency of formation of callus and shoots are lower than MS medium as shown in table 4.12.

In B5 medium callus and shoot formation occurs in 1 mg/l BAP + 0.5 mg/l IAA hormone concentrations but in very poor and minimum amount in comparison to MS medium as shown in table 4.13.

When B5 medium supplemented with 2 mg/l kinetin alone then the frequency of callus as well as shoots formation are always poor and in minimum amount but in comparison to MS medium frequency of callus and shoots formation is very low as shown in table 4.14.

Comparison between wild and cultivated explants in B5 medium with 0.5 mg/l BAP + 1.5 mg/l Kinetin + 1.5 mg/l IAA hormones concentration shows that the frequency of formation of callus as well as shoots low in wild as well as cultivated explants (nodal) of *Withania*. Results have shown in table

4.15.

Comparison between wild and cultivated explants in B5 medium with 0.5 mg/l BAP + 1.5m g/l Kinetin + 1.5 mg/l IAA hormones concentration shows that the frequency of formation of callus as well as shoots poor and low in wild and cultivated explants (petiole) of *Withania* in comparison to MS medium. Results have shown in table 4.16.

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