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BRIEF COMMUNICATION

IN VITRO-SEED GERMINATION OF DIFFERENT CHEMOTYPES OF WITHANIA SOMNIFERA

Vibha Pandey[1,2], Manoj K. Mishra[1,2], Neelam Atri[2], Pratibha Misra[1*]
[1]Council of Scientific and Industrial Research - National Botanical Research Institute, Rana Pratap Marg, Lucknow-226 001, India;
[2]Department of Botany, Banaras Hindu University, Varanasi-221005, India.

***Abstract*— Seed germination potential of *W. somnifera* is very low under in vitro conditions. During optimization of seed germination conditions, it had been found that half strength Murashige and Skoog's medium was better than Hoagland's medium. Similarly, incision in the overnight soaked seeds was observed necessary to improve the germination rate. Culture room conditions of 16 h light and 8 h dark was found beneficial for seed germination. Fresh but dried seeds of the same season when grown on the above optimized conditions, percentage of germination for selected chemotypes i.e. NMITLI-130, NMITLI-101 and NIMITLI-108 were observed as 84.3%, 78.7% and 71%, respectively. Within a chemotype, regenerative potentiality varied among different plant parts. Highest regeneration of shoots was observed in leaf segments as compared to internodes and nodal segments**

Key Words: *Withania somnifera*, chemotypes, medicinal plant, seed germination, regenerative potentiality.

I. INTRODUCTION

Withania somnifera (L.) Dunal (Ashwagandha), also known as Indian ginseng, contains a number of medicinally important metabolites, like tropane alkaloids and withanolides. These metabolites have been isolated mainly from the roots and leaves of the plant [5, 14, 19, 23]. These withanolides and alkaloids are being used in the treatment of various ailments, as antitumor agent, as immunosuppressive, as rejuvenating agent improving vitality and aid recovery after chronic illness [7, 10, 24]. Such pharmacological properties of *W. somnifera* ultimately raised the plant towards widespread medicinal uses [1, 2, 4, 7, 10, 15, 24].

Self-propagation of *W. somnifera* have been reported through seeds [9]; while the germination potential of the seeds has been reported to be very low [25], i.e. only 46% under field conditions, although seed viability is 78.8% [8]. Seeds sown on moist sand will germinate in 14 to 21 days at 20ºC. Seed germination is still more difficult under in vitro conditions and is required for selection of transgenic plants on antibiotic selection medium. This requires optimization and development of protocol so as to ease the selection process of transgenic plants and also in order to ensure its sustainable utilization for commercial cultivation for which this plant has high potential [11]. Earlier reports on seed germination were focused on the influence of growth regulators [25], effect of temperature, pre-chilling and light [8]. But for in vitro seed germination these parameters did not work in the same order, making the present study necessary.

Being a member of solanaceae family, regeneration in *W. somnifera* is not a problem. There are reports which show direct regeneration from leaf explants [12,18], from node, internode, hypocotyl and embryo explants [12, 21]; also indirect organogenesis via callus from various seedlings explants [3,17, 22]. Although Sharda et al. [21] had identified 5 different withanolides from in vitro cultures of different parts from field-grown *W. somnifera* cv. WSR. Similarly, biogeneration of withanolide A has been discussed in detail from shoot cultures of two chemotypes of *W. somnifera* [20], but to our knowledge there is no report on difference in the regenerative potentiality of various vegetative parts of the seedlings. In the present communication the in vitro-seed germination of three selected chemotypes and regeneration of shoots from leaf, stem internode and node of best germinated chemotype has been compared.

II. MATERIALS AND METHODS

COLLECTION OF CHEMOTYPE OF *W. SOMNIFERA*

The experimental materials were obtained from CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India; where the lines have been deposited at the National Gene Bank, CSIR-CIMAP, India. For in vitro seed germination three chemotypes of *W. somnifera*; NIMITLI-130, NIMITLI-101 and NIMITLI-108 were selected.

STERILIZATION OF SEEDS

After washing with 5% Teepol solution, seeds were washed with distilled water and kept overnight in 0.1N HCl solution. Surface sterilization of HCl treated seeds was performed using 70% alcohol for 30 seconds followed by 0.1% $HgCl_2$ solution for 15 min. Traces of alcohol and $HgCl_2$ were removed by 4-5 washings with autoclaved distilled water. Initial experiments of comparative seed germination were conducted in 9 cm sterile petri dishes.

CONDITIONS FOR SEED GERMINATION

Two different media; half strength semisolid Murashige and Skoog's [16] medium and Hoagland's medium [6] on sterilized thin foam sheet were used to analyze effect of media composition on comparative seed germination. Treatments were arranged in a factorial experiment (randomized complete block) with three replicates of 20 seeds each.

Similarly, in another experiment sterilized seeds were inoculated directly or with a fine incision given with the help of a blade in the margins of seeds protecting the embryo for germination. The incision was given on the thicker seed coat region delicately under a microscope to avoid rupture of the embryo.

Half set of petri plates were kept in the continuous dark while remaining half set in the 16 h light and 8 h dark in the culture room for germination. Freshly isolated, freshly isolated and dried seeds of the same season and one year old seeds of previous season were taken for experiment and their germination was observed under in vitro conditions.

REGENERATION OF DIFFERENT PLANT PARTS OF SELECTED CHEMOTYPE

Regenerative potentiality of different vegetative parts of one chemotype (NIMITLI-130) was analyzed. NIMITLI-130 has been analyzed due to its best response towards germination. Percentage germination of NIMITLI-130 was greater than NIMITLI-101 as well as NIMITLI-108 chemotype. Internodes and nodal segments were obtained from the germinated seedlings after attaining a height of 4 to 5 cm having 3 to 4 nodes and subcultured separately for proliferation of shoots in MS.

Shoot tips, nodal segments, internodes and leaves of NIMITLI-130 were used to see the difference in their regenerative potentiality. Regeneration efficiency of different parts of NIMITLI-130 was analyzed by subjecting different concentrations of BAP and kinetin (kn) (0.5, 1.0, 1.5 and 2.0 mg/l), supplemented in MS media to obtain the best suited concentration.

All the regenerated shoots whether from shoot tips, nodal segments, intermodal segments or leaf segments were further proliferated in MS basal media supplemented with 0.5 mg/l BAP and 0.1 mg/l IAA (Fig. 5). Isolated shoots were rooted in 0.2 mg/l α-naphthaleneacetic acid (NAA), where 100% rooting could be achieved within 15 days (Fig. 6). Rooted shoots/plantlets were hardened for 15 days and transferred in the pots (Fig. 6c) and then in the field.

STATISTICAL ANALYSIS

Each tube or flask with three to five explants or petri plate with approximate 20 seeds were taken as single set (replicate) of experiment. Each experiment performed with either ten (in case of tubes), five (in case of flasks) or three (in case of petri plate) replicates for each treatment; and all experiments were repeated at least twice. Only the shoots with distinctly visible apical meristems were counted and the precociously sprouting axillary buds (observed in some treatments) were not taken into account for counting the number of shoots. . The values of data for regenerated shoot buds and height of proliferated shoots are presented as mean ± standard deviation of five replicate cultures. Results of seed germination were subjected to analysis of variance and significance test.

III. RESULTS AND DISCUSSION

Comparative seed germination observed in semisolid half strength MS media and liquid Hoagland's medium. MS media was preferred for further experiments as only 2-4% germination was recorded on liquid medium over thin foam sheet (Fig. 1). Previous reports also showed that half strength MS medium devoid of any growth hormone was better for seed germination [13]. Studies of Kambizi et al. [8] showed enhanced germination at 16/8 h photoperiod which was also found more conductive for germination during present study.

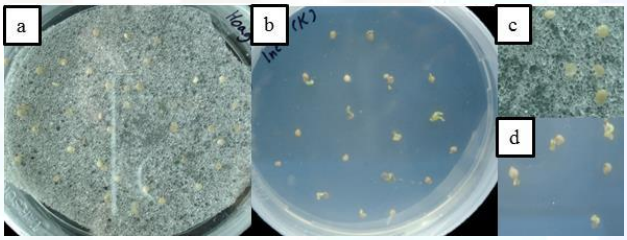

Fig. 1 Effect of media composition on *In vitro* seed germination of *Withania somnifera* seeds (a,c) Hoagland liquid media (b,d) Half strength MS media

Incision given to the seeds enhanced the germination percentage drastically almost double to those without incision (Fig. 2).

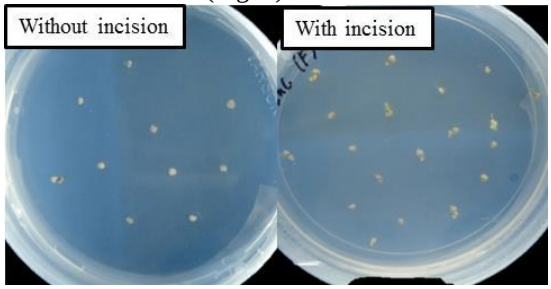

Fig. 2 Effect of incision on seed germination efficiency shows enhanced germination after incision.

The incision was made for proper interaction of seed embryo with the medium; it does not hamper the germination process in anyway.

The analysis of variance shows that the influences of all these treatments were significant within the parameters as well as among the different chemotypes (Table 2). Results of seed germination have been found significant within the treatment and also in the replicates. Seed germination took place within 15 d of culture incubation.

Table 1. *In vitro* seed germination in different chemotypes of *W. somnifera*

Exp. No.	Different parameters	% Germination in different chemotypes		
		NIMITLI-108	NIMITLI-101	NIMITLI-130
1.	½ MS semisolid media	21.3±2.3	23.7±2.0	31.7±2.6
2.	Hoagland's liquid medium on filter paper	2.7±1.4	5.0±0.6	4.3±2.3
3.	Seeds kept continuous dark	0	0	0
4.	Seeds kept in 16 h light and 8 h dark	28.7±2.3	34.3±1.2	39.0±2.0
5.	Seeds inoculated with incision	59.7±2.8	70.3±3.2	74.0±2.4
6.	Seeds inoculated without incision	27.6±2.1	31.0±3.0	34.7±2.3
7.	Freshly picked seeds	69.0±2.0	73.3±2.0	79.3±2.3
8.	Fresh but dried seeds of the same season	71.0±2.0	78.7±1.8	84.3±2.1
9.	One year old dried seeds	4.0±2.0	7.3±1.4	3.6±2.0

Note: Each value represents mean percentage germination ± S.E.

All the chemotypes were germinating in semisolid half strength MS medium, although percentage germination differed in selected three chemotypes of *W. somnifera*. It was the highest with 31% in NIMITLI-130, followed by 23% in NIMITLI-101 and 21% in NIMITLI-108 (Table 1; Fig. 3). Freshly isolated seeds had good germination percentage of 71%, 78.7% and 84.3% in NIMITLI-130, NIMITLI-101 and NIMITLI-108 respectively as compared to 4%, 7.3% and 3.6% of one year old seeds. Similar results for variation in seed germination of different chemotypes of *Thymus vulgaris* were reported by Tarayre et al. [23] and monoterpenes were found responsible for this variation in seed germination.

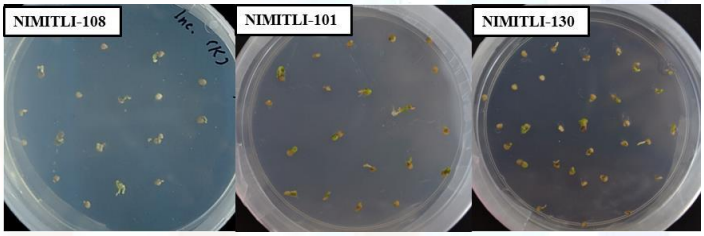

Fig. 3 *In vitro* seed germination in three different chemotypes (NIMITLI-101, NIMITLI-108, NIMITLI-130) of *Withania somnifera*

Table 2. Variation in shoot organogenesis in response to BA (1.0 mg L^{-1} IAA was common to all the treatments).

BA (Conc. mg/l)	[1]Responding cultures (%) ± SE	[2]Time taken (days)	[3]Avg. number of shoot buds/explant ±SD	[4]Avg. height of shoots ±SD (cm)	General condition of the explants / regenerated shoots after 5 weeks of culture incubation
0.5	72.0±0.58	-	-	-	Swelling and expansion of explants
1.0	81.67±1.22	30	5.86±0.06	5.63±0.08	Shoots were fresh, green and healthy
1.5	89.33±0.67	30	6.46±0.03	5.30±0.05	Shoots were fresh, green and healthy
2.0	90.0±1.15	-	-	-	Large callus was formed

[1]Response in the form of swelling, expansion, callus, or shoot organogenesis in leaf explant; [2]Visibility of shoot with naked eye; [3]Average of five explants; [4]Average height of proliferating shoots after 60 d of culture incubation of leaf explants.

The seeds which were fresh but dried, showed better germination percentage than those of freshly picked seeds (Table 1). There are reports on seed germination which were focused on the influence of growth regulators [25], effect of temperature, pre-chilling and light [8]. On the other hand, results of this study clearly showed that nutrient medium, light conditions and freshness of the seeds affecting the germination rate of *W. somnifera* seeds.

The germinated seedlings had been transferred in the test tubes in MS medium supplemented for further growth (Fig. 4). As NIMITLI-130 chemotype possess maximum percentage seed germination, different seedling parts of the same chemotype were further analyzed for their comparative regeneration efficiency.

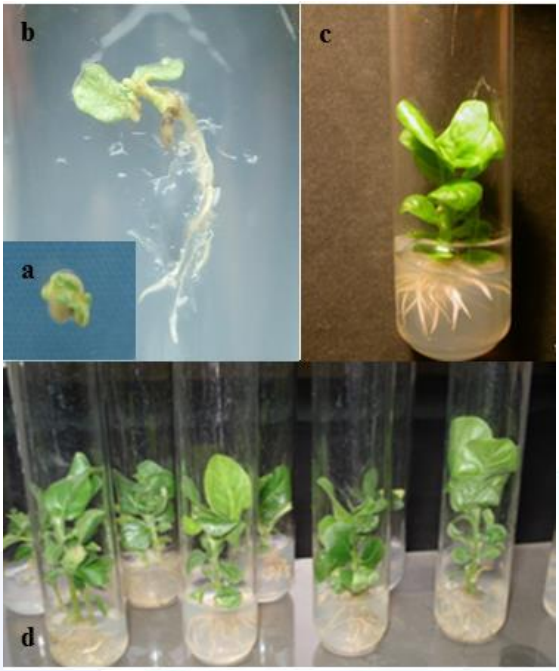

Fig. 4 *In-vitro* seed germination of *W. somnifera* (NIMITLI-130) (a) Germinated seedling, (b) Two week old seedling, (c) Proliferation of seedling into mature plantlet, (d) Raised plantlets.

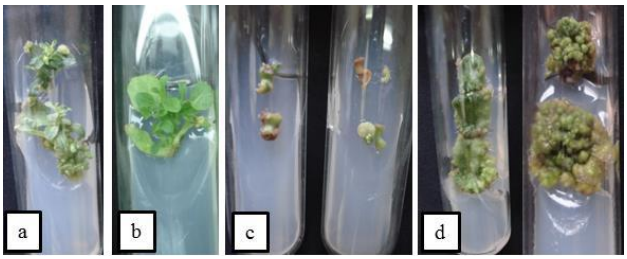

Fig. 5 Comparative regeneration efficiency of different parts of (NIMITLI-130 plantlet) *W. somnifera* (a) Nodal explant (b) Shoot tip (c) Internodal explant (d) Leaf explants

Nodal segments proved as the better explants as compared to shoot tips (Fig. 5a, 5b). It has been observed that regenerative potentiality of shoot tips and nodal segments, regarding shoot formation as well as the height of the regenerated shoots was the highest (Fig. 5).

It was found that BA was more conducive to shoot regeneration as compared to kinetin; whereas the height of the regenerated shoots was little more in Kn supplemented explants as compared to BA supplemented explants (Table 2, Fig. 5). Almost same results were obtained for regenerated shoots from callus culture obtained from leaf segments [3, 18].

Table 3. Efficacy of BAP and Kinetin on regeneration of shoots from different explants of *W. somnifera* NIMITLI-130 chemotypes.

Treatments (Conc. mg/l)	Data for Regenerated Shoots	Different explants			Remarks (General condition of regenerating shoots)
		NS/ST	L	INT	
1.0 BAP	Avg. no. of shoots	7.9±0.4	7.9±0.43	10±0.57	Shoots were fresh, green and healthy.
	Avg. height of shoots (cm)	1.6±0.04	0.5±0.04	0.4±0.05	
1.0 Kinetin	Avg. no. of shoots	5.1±0.33	5.9±0.46	8.3±0.42	Regenerating shoots were fresh, green and healthy
	Avg. height of shoots (cm)	2.1±0.09	0.4±0.05	0.2±.05	

* Average of 10 replicate cultures ± S.E.; NS/ST – Nodal segments/Shoot tips; L – Leaf segments; INT – Internodes

These studies [3, 18] also suggested that 1mg/l to 2mg/l of BAP in combination with 0.5mg/l to 1.0mg/l auxin are best for regeneration of shoots from leaf to callus.

Regenerated shoots of NIMITLI-130 remained fresh, green and healthy for a longer period of 6 weeks (Fig. 6). Internodal explants are less likely to regenerate into shoots. Kulkarni et al. [13] also reported no differentiation of shoots from internodes of var. WS-20. It has been observed that leaf segments were having highest regeneration of shoots (11 shoots) with lesser i.e. 10-20% of callusing. Callusing was observed in all the three segments of leaf, but regeneration was observed maximum in the lower segments of leaf. The regenerated shoots along with the leaf explants were subcultured in the proliferation medium, where all the shoots grew along with the formation of new shoots (Figure 6a). Shoot bud took almost 8-10 weeks to proliferate in to multiple shoots (Figure 6b). All the transferred plants survived with 100% transplant success in the field (Figure 6c).

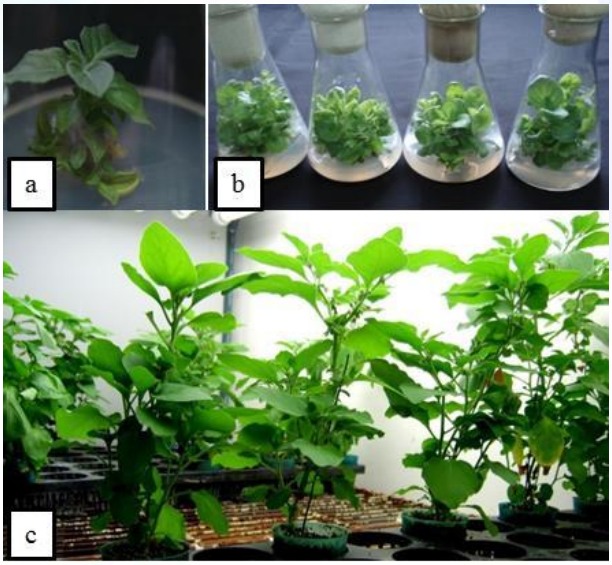

Fig. 6 Proliferation of regenerated shoots (a) proliferated shoot, (b) shoot multiplication, (c) individual shoot transferred to glass house.

In vitro seed germination in *W. somnifera* is very difficult but optimization of culture conditions improved the germination percentage. Incision in the overnight soaked seeds increased the germination percentage on MS media of half strength. Regenerative potentiality was chemotype dependent. NIMITLI-130 chemotype was showing more regeneration of shoots from shoot tips as well as from nodal segments.This study would help in selecting a chemotype while starting any work related transformation in *Withania*.

IV. ACKNOWLEDGEMENTS

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Table. 4 Analysis of variance of seed germination in different chemotypes of *W. somnifera*

Source of Variation	*SS*	*Df*	*MS*	*F*
Rows	22918.7	8	2864.838	305.23**
Columns	251.4467	2	125.7233	13.39501**
Error	150.1733	16	9.385833	
Total	23320.32	26		

**Significant at 5% level

REFERENCES

[1] Bhattachrya SK, Bhattacharya D, Sairam K, Ghosal S (2002) Effect of *Withania somnifera* glycowithanolides on a rat model of tardive dyskinesia. Phytomedicine 9:167-170

[2] Chopra R N, Chopra I C, Handa KL, Kapur, LD (1958) Indigenous drugs of India UN Dhur and Sons, Calcutta, pp. 436-437

[3] Dewir YH, Chakrabarty D, Lee SH, Hahn EJ, Paek KY (2010). Indirect regeneration of *Withania somnifera* and comparative analysis of withanolides in *in vitro* and greenhouse grown plants. Biologia Plantarum 54:357-360

[4] Dhuley JN (2000) Adaptogenic and cardioprotective action of ashwagandha in rats and frogs. J Ethnopharm. 70:57-63

[5] Furmanowa M, Gajdzis-Kuls D, Ruszkowska J, Czarnocki Z, Obidoska G, Sadowska A, Rani R, Upadhyay SN (2001) In vitro propagation of *Withania somnifera* and isolation of withanolides with immunosuppressive activity. Planta Medica 67:146-149

[6] Hoagland DR, Arnon DI (1950) The Water-culture method for growing plants without soil. Calif Agric Exp Station Circ (Berkley) 347:1-32

[7] Ihsan-ul-Haq, Youn UJ, Xingyun Chai, Park EJ, Kondratyuk TP, Simmons CJ, Borris RP, Mirza B, Pezzuto JM, Chang LC (2013) Biologically active withanolides from *Withania coagulans*. J Nat Prod 76:22-28

[8] Kambizi L, Adebola PO, Afolayan AJ (2006) Effects of temperature, pre-chilling and light on seed germination of *Withania somnifera*; a high value medicinal plant. S African J Botany 72:11-14

[9] Kattimani KN, Reddy NY (1999) Effect of pre-sowing seed treatment on germination, seedling emergence, seedling vigourand root yield of Ashwagandha (*Withania somnifera* Dunal.). Seed Sci Technol 27:483-488

[10] Khedgikar V, Kushwaha P, Gautam J, Verma A, Changkija B, Kumar A, Sharma S, Nagar G, Singh D, Trivedi PK, Sangwan NS, Mishra PK, Trivedi R (2013) Withaferin A: a proteasomal inhibitor promotes healing after injury and exerts anabolic effect on osteoporotic bone. Cell Death Dis 4:e778

[11] Kothari SK, Singh CP, Kumar YV, Singh K (2003) Morphology, yield and quality of Ashwagandha (*Withania somnifera* Dunal.) roots and its cultivation economics as influenced by tillage depth and plant population density. J Horticult Sci Biotechnol 78:422-425

[12] Kulkarni AA, Thengane SR, Krishnamurthy KV (1996) Direct in vitro regeneration of leaf explants of *Withania somnifera* (L.) Dunal. Plant Sci. 119:163-168

[13] Kulkarni AA, Thengane SR, Krishnamurthy KV (2000) Direct shoot regeneration from node, internode, hypocotyls and embryo explants of *Withania somnifera*. Plant Cell Tiss Org Cult 62:181-185

[14] Misra L, Lal P, Sangwan RS, Sangwan NS, Uniyal GC, Tuli R (2005) Unusually sulphated and oxygenated steroids from *Withania somnifera*. Phytochem 66:2702-2705

[15] Mishra LC, Singh BB, Dagenias S (2000) Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review. Alt Med Rev 5:334–346

[16] Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

[17] Rani G, Virk GS, Nagpal A (2003) Callus induction and plantlet regeneration in *Withania somnifera* (L.) Dunal. In Vitro Cell Dev Biol Plant 39:468-474

[18] Rout JR, Sahoo SL, Das R (2011) An attempt to conserve *Withania somnifera* (L.) Dunal -A highly essential medicinal plant, through in vitro callus culture. Pak J Bot 43:1837-1842

[19] Sangwan RS, Chaurasia ND, Lal P, Misra L, Uniyal GC, Sharma R, Sangwan NS, Suri KA, Qazi G N, Tuli R (2004) Phytochemical variability in commercial herbal products and preparations of *Withania somnifera* (Ashwagandha). Curr Sci 8:6461-465

[20] Sangwan RS, Chaurasia ND, Lal P, Misra L, Uniyal GC, Tuli R, Sangwan NS (2007) Withanolide A biogeneration in in vitro shoot cultures of Ashwagandha (*Withania somnifera* DUNAL), a main medicinal plant in Ayurveda. Chem Pharma Bull 55:1371-1375

[21] Sharda M, Ahuja A, Suri K A, Vij SP, Khajuria RK, Verma V, Kumar A (2007) Withanolide production by in vitro cultures of *Withania somnifera* and its association with differentiation. Biol Plant 51:161-164

[22] Siddiqui NA, Bari MA, Shahnewaz S, Rahman MH, Hasan MR, Khan MSI, Islam MS (2004) Plant regeneration of *Withania somnifera* (L.) Dunal (Ashwagandha) from nodal segments derived callus an endangered medicinal plant in Bangladesh. J Biol Sci 4:219-223

[23] Tyler VE, Brady LR, Robbers JE (1981) Pharmacognosy. Lea and Febiger, Philadelphia p. 520

[24] Uddin Q, Samiulla L, Singh VK, Jamil SS (2012) Phytochemical and pharmacological profile of *Withania somnifera* Dunal: A Review. J Appl Pharma Sci 2:170-175

[25] Vakeswaran V, Krishnasamy V (2003) Influence of plant growth regulators in germination of *Withania somnifera* Dunal seeds. Seed Technol 25:207