ISSN: 2321-3132

Research Article

International Journal of Chemistry and Pharmaceutical Sciences

www.pharmaresearchlibrary.com/ijcps

In Vitro Propagation of *Withania coagulance* through seedling segment (epicotyledonary node)

Bhanwar Lal Jat[1], Girraj Prasad Meena[2], CR Choudhary[3], Raaz K Maheshwari[4], Garima Jeswani[5]

[1]*Department of Botany, SBRM Govt PG College, Nagaur, Rajasthan*
[2]*PhD Scholar, Mewar University, Gangrar, Chittorgarh, Rajasthan*
[3]*Pro-President of Mewar University, Gangrar, Chittorgarh, Rajasthan*
[4]*Department of Chemistry, SBRM Govt PG College, Nagaur, Rajasthan*
[5]*Department of Physiology, SN Medical College, Jodhpur, Rajasthan, India*

Received: 10 May 2014, Accepted: 18 June 2014, Published Online: 27 July 2014

Abstract

The seeds of *Withania coaguulance* . were germinated which elongated after 4-5 weeks. The seedlings were used as explants. The epicotyledonary nodes of seedling were used to regenerate multiple shoots. The explants were inoculated aseptically on MS medium containing various combination and concentration of plant growth regulators, viz BAP (2.21µM/lit to 22.19µM/lit), kinetin (2.32µM/lit to 23.23µM/lit), IAA (0.57µM/lit to 11.41µM/lit) and NAA (0.53µM/lit to 6.71µM/lit). In a combination of two Cytokinins (BAP and kinetin) with one auxin (IAA) a mean of 50% epicotiledonary explants showed shoot regeneration on BAP (2.21µM/lit), kinetin (2.32µM/lit) and IAA (0.57µM/lit) on this medium a mean of 3 shoots were produced while attained a length of 14mm in 4-5 weeks. The shoot buds achieved from epicotiledonary node explants cultured on MS medium containing BAP (13.31µM/lit), kinetin (13.93µM/lit) and IAA (7.13µM/lit) were further subcultured on the MS medium with same concentration BAP, kinetin and reduced IAA (5.70µM/lit). The excised shoots produced on MS medium containing BAP (13.31µM/lit), kinetin (13.93µM/lit) and IAA (2.85µM/lit) alone were transferred on rooting medium. The medium on which the multiple shoots were produced showed marked influence on subsequent rooting behavior of such shoots originally grown on MS + cytokinins and auxins. The present investigation describes the development of efficient *in vitro* propagation protocol by seedling segment (epicotyledonary node).

Keywords: *Withania coagulance, media, in vitro, field evaluation, epicotyledonary node*

List of Abbreviations:

Benzylamino-purine (BAP) Kinetin (KIN) Indole 3-acetic acid (IAA) ά Naphthalene acetic acid (NAA) and 2, 4-Diclorophenoxy acetic acid (2, 4-D)

Contents

***Corresponding author**
Bhanwar Lal Jat
Department of Botany, SBRM Govt. PG College, Nagaur, Rajasthan
Manuscript ID: IJCPS2116

PAPER-QR CODE

1. Introduction

Withania coaguulance, a perennial plant, is widely distributed along the shores of the Mediterranean *sea*, as well as in India, South Africa, Pakistan and other countries They are also used in dyspepsia, flatulent colic and other intestinal infections. The fruit of the plant is commonly used in Sind (Pakistan), North India, and Afghanistan instead of rennet to coagulate milk. It is also used as an emetic and in smaller doses as a remedy for dyspepsia arising from chronic liver diseases .. Various therapeutic properties have been attributed to this plant and it has been used in the indigenous system of medicine for the treatment of ulcers, rheumatism, cough, dropsy, consumption, and sensile debility. A number of withanolides have been isolated previously from *Withania* spp. Some of these compounds have shown interesting biological activities. For instance, with a ferin A has shown antibacterial properties against acid-fast bacilli and Gram-positive microorganisms. 3P-Hydroxy-2, 3-dihydrowithanolide F has hepato protective activity against CC1,-induced hepatotoxicity in adult albino rats. We now describe the isolation and structure elucidation of two new withanolides, coagulin 111 and with as omidienone 121. Indigenous herbs are used as remedies against various diseases in the traditional system of medicine or in ethno medicinal practices. In folk/tribal medical practice many plants are used to treat diabetes mellitus in India. Most of these medicinal plants are not scientifically validated for their therapeutic efficacy and safety. Scientific study on these plants is likely to provide invaluable anti-diabetic drugs.

Ethno-medical clues are the basis of search of herbal drugs for the treatment of various ailments and an ethnomedical survey was conducted by the author revealed that aqueous extract of *Withania coagulance*, commonly known as Panir, Family-Solanaceae its fruits are used as a remedy for diabetes in folk medical practice in Chhattigarh region of India. Atta – ur –Rahman et al., 2003 isolated two withanolides namely 20-L-hydroxy-1-oxo-(22 R)-witha-2, 5, 24-trienolide and withacoagulin, Budhiraja et al., 1983, reported cardiovascular effects of a with anolides and Choudhary et al., 1995, reported antifungal steroid from *Withania* coagulance. Literature reveal that no systematic anti-diabetic evaluation on this plant was found therefore work was concentrated to investigate and corroborate the anti-diabetic activity of *Withania coagulance* with folk claim. It was abundant until few decades ago, but ruthless collection for medicinal purposes, habitat destruction and climate changes makes the species to become endangered in their natural conditions.

The erosion of plant biodiversity is a matter of global concern. Therefore, it is important to propagate and conserve them to future demand. The conventional propagation of this species is performed through seeds and cuttings of stem since root is too slow and labourious. *In vitro* regeneration technique may be best solution for rapid multiplication and reestablishment in field. *In vitro* propagation techniques have been found to be useful for large propagation of endangered and threatened plants (Sarasan et al., 2006). Micropropagation technique has many advantages over conventional methods of vegetative propagation. *In vitro* propagation protocol in *Withania coagulance* by nodal segments (Jafar Valizadeh and Moharm Valizadeh, 2011) but present study describes the developed of efficient micropropagation by seedling expants.

2. Materials and Methods

Preparation of plant materials:

The germplasm (seeds) was collected from various sites. The collected seeds were first washed with a solution of Tween-80 which followed by several washes with distilled water. Surface sterilized seeds of *Withania coagulance* were cultured on half strength MS basal medium with activated charcoal (100mg/lit) and 3% sucrose, incubated at $25\pm2^{\circ}$ C and under 1500-2000 lux intensity of yellow and white light. The seeds were germinated and elongated after 4-5 weeks. The seedlings were used as explants. The explants (epicotyledonary node) showed swelling at nodal region which was followed by the emergence of shoot buds.

Chemicals:

Pure or analytical grade chemicals of E. / C. Merck, British Drug Houses (BDH), Sigma chemical company, USA, Koch-light Laboratories, England, C.H. Boerhringer Sohn Ingelheim Am Rhien, Germany, Reachim, U.S.S.R., Hi-media Laboratories, India, Qualigens Fine chemicals, India, and Loba chemical company, India were used throughout the period of study.

Preparation of Culture media:

The composition of plant tissue culture media is inorganic salts, vitamins, amino acids, plant growth regulators, carbohydrates and the medium matrix. All the components were soluble in distilled water. Only Ms-basal media **(Murashige and Skoog-1962)** were used in present study. For preparation of the medium, after mixing the salts in distilled water the Agar-Agar was added to the medium. The amount of Agar-Agar varied from 0.5 – 0.8% and that of sucrose 3 to 4% which was used as a source of carbohydrates and Plant growth hormones like Benzylamino-purine (BAP) Kinetin (KIN) Indole 3-acetic acid (IAA) ά Naphthalene acetic acid (NAA) and 2,4-Diclorophenoxy acetic acid (2,4-D) were used. The pH was adjusted to 5.8 $\pm$0.2 with 0.5 N KOH and 0.1N Hcl solutions before sterilization. 15-20 ml and 35-40 ml medium was poured in tubes, conical flasks and 40-50 ml medium was poured

in culture bottles respectively finally the medium was sterilized by autoclaving it at 15 pound per square inch (Psi) pressure for 15 minutes.

Inoculation:

The inoculation of plant materials were carried out in a laminar air flow cabinet under sterile conditions. All the appliances were sterilization and the explants after surface sterilization were inoculated vertically or horizontally on the surface of media. The inoculated culture room under controlled conditions.

Culture conditions: The cultures were maintained at the temperature of 28±2˚C with 14 hrs illumination of light of intensity 2000 to 2500 lux and relative humidity (RH) 60-70%. The source of light was cool fluorescent tubes and incandescent bulbs. The temperature, light and humidity were varied according to the experiments.

Sub-culture and multiplication of propagules: After establishment of explants aseptically in culture media. The sub-culturing was carried out in some cultures for 5-6 times to multiply them for further experiments. The tissues were subcultured regularly after an interval of 4-5 weeks. The growth hormones were used either individually or in combinations of different concentrations.

Callus Development: Epicotyledonary node explants (length 26.5mm) from sterile *in vitro* seedling were cultured on MS medium containing different concentration of 2,4-D and IAA + NAA for 4 weeks.

Root induction: When the aseptic shoots were raised in sufficient numbers, these were transferred to root inducing media like White's MS basal, MS ½ salt strength ¾ and ¼ strength of MS Salts. *In vitro* produced shoots were also transferred to the other media like B5 basal medium, WP medium and WS medium supplemented with vitamin, amino acids, and various root inducing hormones like NAA, IAA, and IBA. The physical conditions, concentrations and combination of growth regulators, vitamins, amino acids and inorganic salts of media were changed according to the need of culture.

Establishment of plantlets in the soil

Before the establishment of *in vitro* raised plantlets these were kept in the culture vessel until the nutrients of the medium completely exhausted. This was done to strengthen the plants. The strengthened plants before transfer to the earthen pots were washed thoroughly with tap water to remove adhered agar medium. Final wash was given with distilled water. These plantlets were transferred to pots containing a mixture of vermiculite + sand + gravelli soil in the ratio (1:1:1) or to the pot containing only sand dune soil for hardening and acclimatizing. The potted plantlets were covered by polythene bags for first few days to prevent immediate desiccation due to the high transpiration rate. The polythene bags were removed after, 4-5 days and the plants were exposed to the environment. Finally after one month the hardened and acclimatized plantlets were transferred to the field.

Hardening and Acclimatization

Once the reproducible procedure for large scale plant regeneration *in vitro* is achieved and sufficient number of plants is obtained, the next steps to be taken are: (1) Hardening of the plants *in vitro*. (2) Acclimatization and finally (3) Transfer to the field where the performance of plants is to be tested. These steps are critical and vital as behavior of aseptically grown plants is very different from plants grown *in vivo* and also the plants are transferred from aseptic conditions to free living conditions. Hardening of the plants *in vitro* is done by gradual reduction of nutrient levels in the medium with root promoting auxins and culture conditions (Ziv, 1986). Acclimatization *in vitro* involves the expose of plants to reduced relative humidity and external environment without disturbing or injuring the delicate root and shoot systems (Brainerd and Fuschigami, 1981, Wardle et al, 1983, Ziv et al, 1983). Several authors have indicated that the waxy cuticle and stomata on leaves of *in vitro* grown plants are inadequate or inoperative (Grout and Aston, 1977, Wetzstein and Sommer, 1982). Such leaves are incapable of preventing or reducing the water loss that can occur in variable humidity of the *in vivo* conditions. If sterilized soil mixture is used to transfer the plants, it eliminates the changes of infection but in some cases the unsterilized soil can be used to transfer the plants. Some plants produce a little callus *in vitro* at the base on which the Microorganisms start growing and move into the shoots causing infection, in this situation the sterilized soil must be used. Agar- agar adhered to roots and plant must be washed gently before transfer. Once roots are prepared for further growth, acclimatization, and reestablishment for autotrophic adaptation before transfer to the field conditions. The plants under investigation are well adapted to arid environment but in the cozy environment of culture vessels they are different in nature. On transfer to the field these show general deterioration of growth.

Hardening: The plantlets produced *in vitro* were hardened under aseptic conditions. Plantlets developed root system within 4-6 weeks on rooting media. These plants were not transferred to fresh medium for hardening but allowed to grow on the same medium under low humidity (RH 50%) and slightly higher temperature (30˚C ± 2˚C) and light of intensity (2000-2500 lux) under these cultural conditions the plantlets were allowed to grow for two weeks. In this period entire medium was exhausted in the culture vessels.

Acclimatization and Field Transfer:

The regenerated plants were taken out from culture vessel and running tap water to remove adhered medium. The agar-agar adhered to the roots and shoots was removed carefully and gently. These plants were kept in distilled water for 15-20 minutes and washed again with distilled water for 5-6 times. The plants were then treated with systemic fungicide, carbendazim (Bavistin) 0.1% for 5 minutes and finally washed with distilled water to remove fungicide. These plantlets were transferred to earthen pots containing soil mixture. The different soil mixtures in

various ratios were tried for pot transfer of plantlets. For *Withania coagulance* the soil of natural habitat, vermiculate and gravels were used in the pots. The plants were transferred in the pots. The pots were put in a growth chamber (Haraeus HPS-1500) at 28 ˚C ±2 ˚C temperatures, 16 hours photoperiod of light of intensity 1500-2000 lux and RH 65% for two weeks. The relative humidity decreased to 45% and light intensity and temperature were increased. During acclimatization initially the plantlets were kept covered with glass cover and watered with Hoagland nutrient solution. After two weeks in the pots these were transferred to green-house where the plants were watered daily.

Statistical Design: Experiments were set up in Randomized Block Design (RBD) and 10 replicates for each treatment were tested for shooting medium and 10 replicates for each treatment were tested for *in vitro* regeneration. Data from each experimental stage were analyzed separately and mean and SD (Standard Deviation) of each experimental stage were calculated.

3. Results and Discussion

In vitro* Regeneration, Multiplication, Rooting, Hardening and Acclimatization of *Withania coagulance.

***In vitro* regeneration (shoot initiation): (Table-1& Figure-1)**

The plant materials (epicotyledonary node) were obtained from *in vitro* seedling. The seeds were surface sterilized by successive treatments of sodium hypochlorite (5% activated chlorine, 20 minutes) and 0.1% $HgCl_2$ solution for 5 minutes and thoroughly wash with autoclaved distilled water 3-4 times and germinated on half strength MS medium supplemented with 3.0% sucrose, shoots were collected 5-6 weeks old seedling. The epicotyledonary nodes of seedling were used to regenerate multiple shoots. The explants were inoculated aseptically on MS medium containing various combination and concentration of plant growth regulators, viz BAP (2.21µM/lit to 22.19µM/lit), kinetin (2.32µM/lit to 23.23µM/lit), IAA (0.57µM/lit to 11.41µM/lit) and NAA (0.53µM/lit to 6.71µM/lit). In a combination of two Cytokinins (BAP and kinetin) with one auxin (IAA) a mean of 50% epicotiledonary explants showed shoot regeneration on BAP (2.21µM/lit), kinetin (2.32µM/lit) and IAA (0.57µM/lit) on this medium a mean of 3 shoots were produced while attained a length of 14mm in 4-5 weeks. As the concentration of BAP, kinetin and IAA increased the percentage of shoot induction from explants also increased with number of shoots and their length under aseptic conditions. The maximum number of explants regeneration (98.0%) was found in the combination of BAP (13.31µM/lit), kinetin (13.96µM/lit) and IAA (7.13µM/lit) on this medium a mean of 18 shoots were produced with 36.5mm length after 4-5 weeks. As the concentration of BAP, kinetin and IAA increased the percentage response of explant and shoot induction rate was decreased to 71.4± 1.85% (Table-1).

Multiplication of propagules in *Withania coagulance*: Once the proliferation of shoots is achieved from *in vitro* cultured epicotyledonary node. The next step is to multiply the *in vitro* grown shoots. In *Withania coagulance* during present investigation experiments were conducted to further multiply the shoot buds regenerated from epicotyledonary node explants. These shoot buds after 4 weeks of culture, when attained a length of 36.5mm were excised (remove) epicotyledonary node and carefully subcultured to the fresh MS medium containing various growth regulators. Once the optimum conditions for induction and growth of shoots from subcultured shoot buds were defined the cultures were maintained by sub culturing them every 4 weeks. The data were recorded periodically. Suitable control was also taken to compare with treatment. The following growth regulators in their combinations were used.

Effect of Cytokinins and auxins on shoot multiplication: (Table-2 & Figure-2)

The shoot buds achieved from epicotiledonary node explants cultured on MS medium containing BAP (13.31µM/lit), kinetin (13.93µM/lit) and IAA (7.13µM/lit) were further subcultured on the MS medium with same concentration BAP, kinetin and reduced IAA (5.70µM/lit). Shoots were subcultured on the other combination and concentration of PGR.

Effect of BAP + kinetin + IAA: The results obtained are presented in Table-2. As the concentration of BAP (2.21µM/lit to 13.31µM/lit), kinetin (2.32µM/lit to 13.93µM/lit l) and IAA (0.57 µM/lit to 2.85µM/lit) increased, the number of shoots produced was also increased. On MS containing BAP (13.31µM/lit), kinetin (13.93µM/lit) and IAA (2.85µM/lit) 25 shoots with 29.5 mm length shoots were regenerated from a single shoot bud. On this medium the maximum number (25 shoots) were produced. The shoot number and length was decreased with the increase in the concentration of BAP (17.75µM/lit to 22.19µM/lit), kinetin (18.58µM/lit to 23.23µM/lit) and IAA (5.70µM/lit to 11.41µM/lit). The length (23.5mm) and number of shoots (18) obtained on increased cytokinins and auxins with little callusing on the shoot base.

Effect of kinetin + NAA: Results obtained are presented in Table-2, in these prove that the kinetin in combination with NAA was not found as effective as BAP + kinetin + IAA for induction as well as for growth of shoots from subcultured shoot buds. The kinetin (2.32µM/lit to 23.23µM/lit) with NAA (1.34µM/lit to 13.42µM/lit) was incorporated in the medium. On lower concentration of kinetin (2.32µM/lit) with NAA (1.34µM/lit) only 3 shoots (length 9.5mm) were produced. As the concentration of kinetin increased to (13.93µM/lit) with increased concentration of NAA (8.05µM/lit) the number of shoots and length increased (21 shoots, with 26mm length). The length of shoots and number of shoots (16shoots with 20.5mm length) on higher concentration of kinetin (23.23µM/lit) + NAA (13.42µM/lit).

Effect of 2, 4-D and IAA + NAA on callus induction: (Table-3 & Figure-3)

Epicotiledonary node explants (length 36.5mm) from sterile seedling were cultured on MS salts, vitamins and 3% sucrose containing different concentration of 2,4-D (4.52µM/lit to 22.62µM/lit) and IAA + NAA (IAA 2.85µM/lit to 17.12µM/lit and NAA 2.68µM/lit to 16.11µM/lit) for 5 weeks. The 2,4-D (4.52µM/lit) NAA + IAA (IAA 2.85µM/lit and NAA 2.68µM/lit) was added in the medium the explants produce 2 shoots with 5mm length and callus induction. As the concentration of 2,4-D, increased 6.78µM/lit to 22.62 µM/lit the shoot formation was inhibited. On 2,4-D (6.78µM/lit) little callus, 2,4-D (9.04µM/lit) moderate callus and 2,4-D (13.57µM/lit to 22.62µM/lit) vigorous callus was induced by explants.

Effect of MS + IBA and MS + IAA + NAA on rooting: (Table-4 & Figure-4)

The excised shoots produced on MS medium containing BAP (13.31µM/lit), kinetin (13.93µM/lit) and IAA (2.85µM/lit) alone were transferred on rooting medium. The medium on which the multiple shoots were produced showed marked influence on subsequent rooting behavior of such shoots originally grown on MS + cytokinins and auxins. The results obtained are presented in Table-4. These reveal that the rooting of micro shoots was influenced by concentration of IBA. On lower concentration of IBA (1.23µM/lit to 3.69µM/lit) these shoots did not root and could be rooted only on medium containing 4.92µM/lit to 24.60µM/lit IBA. However even on these concentrations more time was required (25-30 days) for rooting. As the concentration of IBA increased from 4.92µM/lit to 24.60µM/lit the percentage of rooting in micro shoots enhanced from 64.7 to 98.3% on 30 days. Maximum percentage (98.3%) of rooting on medium containing IBA 24.60µM/lit. On further increase in the concentration of IBA rooting could be achieved (up to 24.60µM/lit IBA) and (up to 8.05µM/lit NAA + IAA8.56µM/lit) beyond this rooting was inhibited and excised shoots produced callus. Thus for good rooting on IBA 18.09µM/lit and NAA 8.05µM/lit + IAA 8.56µM/lit was optimum for rooting micro-shoots of *Withania cogulance*. The roots so produced attained 38.9mm length in 4-5 weeks. The plantlets so developed were however showed decline in growth.

Hardening and Acclimatization of *Withania coagulance*: (Figure-5)

When roots of plantlet were completely developed on root inducing medium (MS +IBA 1.0 $mg1^{-1}$) in 4-5 weeks. The nutrients and growth hormones levels were depleted. On this medium the plantlets were kept for two weeks. On this medium during a period of 15 days the leaves and stems of plants became broad and bright-green in color and lateral shoots were produced from main shoot. The color of stem turned green to light brown. The roots became sturdier (more than 1mm thick) and produced lateral roots. These plants were then transferred to the pots containing a mixture of soils and vermiculite. The types of soil and its composition with vermiculite were for different plant species. In *Withania coagulance* the *in vitro* regenerated plants were transferred to the pots containing different soils with vermiculite. On sand dune soil + vermiculite in the ratio of 1:1 the plants were wilted after 48 hours. On a mixture of soils of natural habitat + vermiculite + gravels (stone pieces) in ratio of 1:2:1 was found suitable for survival of plants of *Withania coagulance* in pots.

Table 1: Effect of different Concentration of growth regulators in MS-Medium on regeneration of axillary shoots from the seedling in *Withania coagulance.*

Cytokinins		Auxins		% shoots regeneration (Mean± S.D.)	Number of axillary shoots (Mean ±S.D.)	Length of shoots (MM) (Mean± S.D.)
BAP (µM)	KIN (µM)	IAA (µM)	NAA (µM)			
2.21	2.32	0.57	----	50.0 ± 1.41	3 ± 0.43	14.0 ± 1.85
4.43	4.64	1.14	----	71.6 ± 1.85	4 ± 0.32	17.0 ± 1.02
8.87	9.29	4.28	----	80.6 ± 2.15	6 ± 0.44	19.5 ± 1.06
11.09	11.61	5.70	----	98.0 ± 1.41	10 ± 0.56	22.5 ± 1.85
13.31	13.93	7.13	----	90.8 ± 1.72	18 ± 0.70	36.5 ± 2.13
17.75	18.58	9.98	----	81.4 ± 1.39	12 ± 0.51	32.5 ± 1.41
22.19	23.23	11.41	----	71.4 ± 1.85	11 ± 0.44	28.5 ± 0.70
----	2.32	----	0.53	39.8 ± 0.92	3 ± 0.23	12.5 ± 0.71
----	4.64	----	1.07	60.6 ± 0.86	5 ± 0.23	16.5 ± 1.07
----	6.96	----	1.61	71.4 ±1.06	7 ± 0.43	18.5 ± 1.41
----	9.29	----	2.68	81.7 ± 1.53	11 ± 0.70	23.5 ± 1.53
----	13.93	----	4.83	96.3 ± 1.07	16 ± 0.83	32.5 ± 1.85
----	18.58	----	6.71	74.7 ± 1.07	14 ± 0.63	26.5 ± 1.07
2.21	----	0.57	----	42.5 ± 1.41	4 ± 0.23	11.5 ± 1.07
4.43	----	1.14	----	58.5 ± 0.86	6 ± 0.31	13.5 ± 1.53
6.65	----	1.71	----	62.5 ±1.53	12 ± 0.86	18.0 ± 1.41
8.87	----	2.85	----	71.5 ±1.07	15 ± 1.07	28.5 ± 1.85
13.31	----	3.42	----	86.5 ± 0.70	13 ± 0.44	26.5 ± 1.07
17.75	----	3.99	----	92.5 ± 0.44	10 ± 0.21	22.5 ± 0.83

Each value represents the mean ± SD of 10 replicates

Figure 1: *In vitro* regeneration (shoot initiation)

Table 2: Effect of different concentration and combination of plant growth hormones in Ms Medium on multiplication of shoots of *Withania coagulance.*

Cytokinins		Auxins		Number of multiple shoots (Mean± S.D.)	Length of shoots (MM) (Mean ± S.D.)	Callusing.
BAP (µM)	KIN (µM)	IAA (µM)	NAA (µM)			
2.21	2.32	0.57	----	6.0 ± 1.07	10.5 ± 0.92	_
4.43	4.64	1.14	----	13 ± 1.41	13.5 ± 0.83	_
8.87	9.29	2.28	----	19 ± 1.83	18.5 ± 1.07	_
13.31	13.93	2.85	----	25 ± 0.70	29.5 ± 1.70	_
17.75	18.58	5.70	----	21 ± 0.83	26.5 ± 1.70	_
22.19	23.23	11.41	----	18 ± 1.41	23.5 ± 1.43	+
----	2.32	----	1.34	3 ± 0.21	9.5 ± 0.31	+
----	4.64	----	2.68	6 ± 0.41	11.5 ± 0.43	_
----	9.29	----	5.37	13 ± 1.07	15.5 ± 0.81	_
----	13.93	----	8.05	21 ± 1.41	26.0 ± 1.31	_
----	18.58	----	10.74	19 ± 1.41	22.5 ± 1.63	+
----	23.23	----	13.42	16 ± 0.83	20.5 ± 1.41	+

− = No callusing; + = Little callusing; ++ = Moderate callusing; +++ = Vigorous callusing.
Each value represents the mean ± SD of 10 replicates

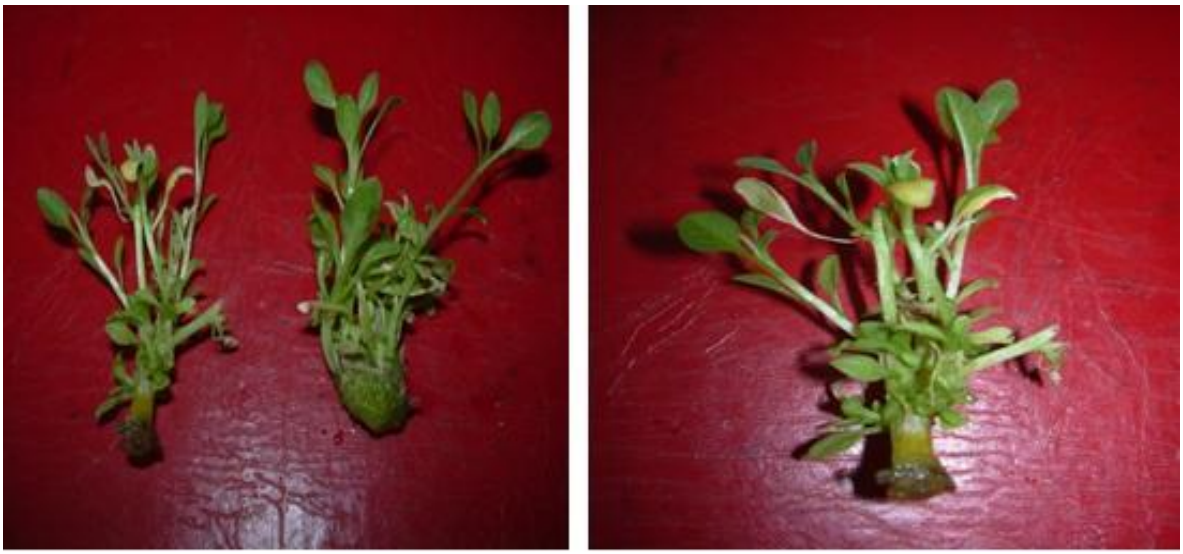

Figure 2: Effect of Cytokinins and auxins on shoot multiplication

Table 3: Effect of auxins on callus formation in *Withania coagulance.*

2,4-D (µM)	IAA (µM)	NAA (µM)	Response of shoots for callusing
4.52	----	----	−
6.78	----	----	+
9.04	----	----	++
13.57	----	----	+++
18.09	----	----	+++
22.62	----	----	++
----	2.85	2.68	−
----	5.70	5.37	+
----	11.41	10.74	++
----	17.12	16.11	+++

− = No callusing, + = Little callusing, ++ = Moderate callusing, +++ = Vigorous callusing.
Each value represents the mean ± SD of 10 replicates

Figure 3: Effect of 2, 4-D and IAA + NAA on callus induction

Table 4: Effect of auxins (IBA, NAA and IAA) on rooting behavior of cultured shoots of *Withania coagulance.*

Auxins			Percentage of Rooting in micro shoots (Mean ± S.D)	Root Length of (MM) (Mean ± S.D.)	Callusing
IBA (µM)	NAA (µM)	IAA (µM)			
4.92	----	----	64.7 ± 1.07	17.5 ± 0.70	−
9.84	----	----	75.0 ± 1.22	19.0 ± 0.70	−
14.76	----	----	84.2 ± 0.92	25.8 ± 0.44	−
19.68	----	----	94.7 ± 0.92	34.0 ± 0.70	−
24.60	----	----	90.3 ± 0.92	30.9 ± 0.86	−
----	2.68	2.85	44.0 ± 1.41	11.5 ± 0.70	−
----	5.37	5.70	61.4 ± 1.85	19.5 ± 0.70	−
----	8.05	8.56	71.2 ± 1.20	24.7 ± 0.92	−
----	10.74	11.41	76.6 ± 1.85	28.5 ± 0.70	+
----	16.11	17.12	70.3 ± 143	22.0 ± 0.70	++

− = No callusing,+ = Little callusing, ++ = Moderate callusing, +++ = Vigorous callusing.
Each value represents the mean ± SD of 10 replicates

Figure 4: Effect of MS + IBA and MS + IAA + NAA on rooting

Figure 5: Hardening and Acclimatization of *Withania coagulance*

Discussion:

The objective of present investigation has been to devise the method of mass propagation by developing efficient, for *Withania coagulance*. This plant has also been appreciated and recognized for their aesthetic and ornamental value. The natural propagation of these plants is very poor and their populations are declining with an alarming rate in the fragile ecosystems of Indian desert and Aravali areas. Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies. Moreover, indeed

the market and public demand has been so great that there is a great risk that many medicinal plants today face either extinction or loss of genetic diversity. *Withania coagulance* is also used as an emetic and in smaller doses as a remedy for dyspepsia arising from chronic liver diseases. There might be several factors including genetic and environmental for poor seed viabilities of these three medicinal plants species. Thus the application.

Multiplication of propagules and repeated sub culturing of explants:

In *Withania cogulance* of shoots were produced by repeatedly sub culturing of the original explant which remained after excision of developed shoots each time. The same explants could be subcultured on fresh medium for five times and after each subcultured it produced 30-40 shoots in the original cotyledonary node explant produced (18-19 shoots in *Withania cogulance*) and the same explant after excision of regenerated shoots was subcultured to fresh MS medium containing cytokinin and auxins. This explant could be subcultured four times after 30-35 days, on fresh medium. The formation of new shoots from subcultured explant and development of older shoots took place simultaneously. In the present investigations too, it has been possible to subculture the better yield of crop of shoots was achieved. These results will greatly facilitate progress towards automation of shoot plants.

Pre-requisites for rooting in *Withania Cogulance*:

It is well established fact that growth regulators exert carry-over effect on the tissues when these are transferred From one medium to the other or form higher concentration of growth regulators to lower ones. During *in vitro* studies of *Withania Cogulance* it has recorded that if shoot differentiation was obtained on medium with cytokinin alone, rooting of such shoots was difficult to achieve and if a few shoots could be rooted, the subsequent growth of root was very poor which did not support the growth of plantlet. In fact such plantlets could not survive for more than 1-2 weeks *in vitro*. The growth rate of such shoot was higher after rooting. About 80 percent of shoots, differentiated on NAA containing medium, were rooted while only 20 percent shoots were rooted if these shoots differentiating on cytokinin alone or with IAA containing medium. Similar observations were recorded by Bhati (1989) in *Aegle marmelos*.

Rooting of cultured shoots: The shoots obtained from various sources were successfully rooted. This was possible with extensive experimentation. The role of auxins in root development is well established and has been reviewed by Torry (1965, 1976), Scott (1972) and Gaspar and Coumans (1987). Rooting of the shoots can be promoted by low concentrations of auxins (Mott, 1981; Amerson and Mott, 1982; Minocha and Robie, 1985**).** The callus formation with roots may also reduce the survival rate of *in vitro* raised plantlets (Nemeth, 1986). Minocha (1987) also reviewed that the synthetic auxins IBA and NAA are generally more effective than IAA and 2,4-D in this regard. This is true in case of *Withania cogulance* species usually there is sufficient residual cytokinin in shoots, thus little or no cytokinin is need in rooting medium (Hu and Wang, 1983). Higher concentration of cytokinins was shown to be deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants (Pennazio, 1975; Nemeth, 1979). The other factors known to affect rooting are carbohydrates abscisic acid. For obtaining uniformity in the result the size range of the shoots for rooting should be constant (Nemeth, 1986). In *Withania cogulance* rooting was obtained in the shoots of 10.5mm to 33.5mm length on MS medium without growth hormones as well as in hormones containing medium. The strength of inorganic and organic salts of MS medium also play significant role in the rooting behavior of shoots *in vitro* of *Withania cogulance* species. In earlier publications concerning rooting of different plants species, MS micro and macro-elements at full strength were used. Similarly root could be induced in the shoots of *Withania cogulance* on MS full strength salts. Various media tried for rooting of shoots of these plants were White's, B5, WP, and MS dilution of ¾, ½, ¼ salts. Although rooting was observed in all the media without growth regulators in *Withania cogulance* rooting was obtained only on MS medium. In *Withania cogulance* 5-6 sturdy fast growing roots per shoots obtained on MS medium supplemented with IBA 24.60µM/lit (38.9 + 0.86mm length).

4. Conclusion

As suggested in the published literature, very less significant micropropagation work has been done on this plant species. With regard to the tissue culture aspect, already some of work has been done. This experiments were carried out to propagate the selected explants of cotyledon, hypocotyls and epicotyls segment were used for different growth regulators on the *in vitro* direct shoot and root initiation using MS medium.In our study the result shows *Withania cogulance* micropropagation were enabled the mass propagation of *Withania cogulance* plant and an efficient protocol was developed for direct regeneration through tissue culture method by using the cotyledon, hypocotyls and epicotyls segment.

5. Acknowledgement

The authors express deep sense of gratitude & heartfelt thanks to Dr Ramesh Joshi Lecturer in Dept of Botany, Govt PG College Ajmer, for providing lab facility. Sincere thanks is extended to Prof Ashok Gadiya, Chairman of Mewar University, Chittorgarh, Prof. C.B. Gena, Former Vice-chancellor of Maharaja Ganga Singh University Bikaner and Prof. Bhagirath Singh , Former Vice-chancellor of MDS University, Ajmer, Rajasthan, whose continuous encouragement and constant help to carry out this research work.

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