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In-Vitro Propagation of *Withania Somnífera* (L.) Dunal (Ashwagandha) an Endangered Medicinal Plant

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ABSTRACT: *Withania somnifera* is an important medicinal plant and used worldwide in pharmaceutical industry. Although withania propagates vegetatively in its natural state, but propagation rate is too slow to meet demand of high quality planting material for commercial cultivation. A rapid and highly effective method for the Micro propagation method for elite selection of withania by auxiliary branching method using shoot tip as explants was standardized. Shoot cultures were initiated on MS medium containing BA (0.5 – 2.0 mg/L) with (NAA 0.2-0.5 mg/L) containing 4.5 gms/l agar, and 3% commercial sugar.

Keywords: *Withania somnifera*, propagation, MS medium and proliferation.

I. INTRODUCTION

Due to the toxic and adverse reactions of synthetic and chemical medicines being observed round the globe herbal medicine has made come back to improving the fulfillment of our present and future health needs. Religious-cultural faith, weak economy in accessibility and consequently lack of modem medicinal facilities in these villages seems to be the cause of dependence on these medicinal plant species in addition to their proven ameliorative effects. *Withania somnifera* L. Dunal commonly known as Ashwagandha, as recognized today, as potentially one of the most valuable plant because of its widely used medicinal value its ability to grow in even the most arid and nutrient deficient soils, as well as its many commercially exploitable by products and environmental , and medicinal attributes. This medicinally potent plant is a native to the dry arid areas of India, Pakistan, China Bangladesh, where it has been grown wildly for ages. A multipurpose medicinal plant with a unique properties, while being an energy tonic like ginseng or Codonopsis for instance *Withania Somnifera* one of the best known and most researched Ayurvedic herbs and holds an Ayurvedic traditions similar to Ginseng in Chinese therapies. For that reasons Somnifera has been often referred to as the 'Indian Ginseng' in Ayurvedic world. *Withania somifera*, better known in India as ashwagandha, is destined to rise significantly and take its place with all the other better known tonics. Unlike many tonics, Ashwagandha is also anti-inflammatory, anti-arthritic,anti-anxiety calmative and aphrodisiac. *Withania somnifera* commonly known as ashwagandha belongs to family Solanaceae also called ashwagandha (Sanskrit). The major biochemical constituents of ashwagandha from which its primary medicinal properties emanate are based upon the actions of certains steroids alkaloids & steroidal lactones in a class of constituents called withanolide**s**.

II. METHODOLOGY

The technique involves the isolation, inoculation and regeneration of plant cells, tissues, organs under controlled conditions in culture vials, containing synthetic nutrient medium. Both the chemical compositions of the medium and the controlled environmental conditions (light, temperature, humidity, aeration etc.) effectively control the expression of any genotype or phenotype potential in the explants.

PREPARATION OF MEDIA

The preparation of 1 liter MS media involves following steps :

- 500 ml of distilled water was taken in sterile flask.
- 20 or 30 g of sucrose (w/v) was taken and shaken till it dissolve.
- 100 ml of macrosalts, 5 ml of microsalts, 5 ml of vitamins, 5 ml of myo-inositol were added to stock solution.
- Required amount of growth regulators were added.
- The volume of the medium was made to 1000 ml by adding distilled water.
- pH of the medium was adjusted to 5.4-5.8 by adding 1N HCL or 1N NaOH.
- 4.5 g/l agar were added.
- The medium was boiled to dissolve the agar and then dispense in culture vials.

- Finally the culture vials with medium were capped and autoclaved at 15 1bs, 121°c for 15 to 20 minutes.
- For sterilization of media the minimum time required depends upon the volume of the media in the vessel. Prolonged autoclaving may result in breaking and denaturation of media ingradients.

Autoclaving

The nutrient was generally sterilized by autoclaving at 121°c for 20 minutes. The minimum time required for sterilization depend upon the volume of the medium in the vessel. Prolonged autoclaving may result in breaking and denaturation of media in small liquid form.

Collection of Explant Material.

Axillary and apical buds were collected from plants of Withania sominifera grown in the botanical garden of the institute during sprouting and non sprouting season at different developmental stages.

1. Mature flowering plant. .
2. Mature non flowering plant.

Preperation And Sterilization Of Explant.

Collected axillary and apical buds were excised into one and half inches and were washed with liquid soap for ten to fifteen minutes followed by distilled water for 4 to 6 times. Washed explants were treated with 0.1% of mercuric chloride solution for 8 to 10 minutes after sterilization the explants were washed with sterile distilled water.

Induction Experiment

The effect of season, age of the explant and the effect of various cytokinins on initiation of shoot was studied simultaneously in the preliminary studies. For these study the sterile buds were inoculated on MS basal medium with vitamins, supplement with cytokinins like BAP, KN, 2iP (0.5 - 3.0 mg / l) alone or in combination with other cytokinins of each, sucrose 30 grams and gelled with agar 4 grams per liter. In addition Auxins like IAA or NAA (0.1 –1.0 mg / l) were used with cytokinins for promoting the shoot initiation .

Different Medium Used For Shoot Initiation

MEDIUM I .1- MS + 0.5 mg/l BAP
MEDIUM I. 2- MS + 1.0 mg/l BAP +0.5 KN mg/l
MEDIUM I .3 - MS + 2.0 mg/l BAP +0.5 KN mg/l
MEDIUM I .4- MS + 3.0 mg/l BAP
MEDIUM I .5 – MS + 0.5 BAP + O.1 NAA mg/l
MEDIUM I .6 – MS + 0.5 BAP + 0.1 KN + NAA mg/l
MEDIUM I .7 - MS + 1.0 BAP + 0.5 NAA mg/l
MEDIUM I .8- MS + 1.0 BAP + 0.5 NAA mg/l
MEDIUM I .9 - MS + 1.0 BAP + 1.0 NAA +0.5 KN mg/l
MEDIUM I . 10 – MS + 2.0 BAP + 0.5 NAA mg/l
MEDIUM I. 11 - MS + 2.0 BAP + 1.0 NAA mg/l

Number of experiments were carried out to maximize the initiation of shoot from axillary and apical meristem. The measurement of growth was taken by the percentage of buds showing response, number of shoots initiated per explant, shoot length and callus formation according to the method described.

Experiments For Shoot Multiplication

After eight to ten days of initiating experiment the buds started responding by bud break and sprouting. After 20 days of culture the initiated grown shoots were separated and subculture onto shoot proliferation medium, number of experiments were carried out to maximize the rapid multiplication of shoots from one axillary bud. These includes : Use of high concentration of cytokinins , BAP, KN, (0.5 –3.0 mg/l) as compare to induction medium. Use of additional , coconut water at the concentration of 10 to 40 percent of the medium. Use of dilution of macro and micro elements and vitamins of MS keeping constant, i.e. full strength.

Different Medium Used For Multiplication

MEDIUM. M1- MS + 0.5 mg/l BAP
MEDIUM. M2 – MS + 0.5 BAP + 0.5 KN mg/l
MEDIUM M3 – MS + 1.0 BAP mg/l
MEDIUM M4 – MS + 1.0 BAP + 0.5 NAA mg/l
MEDIUM M5 – MS + 2.0 BAP mg/l

MEDIUM M6 – MS + 2.0 BAP + 0.5 KN mg/l
MEDIUM M7 – MS + 3.0 BAP mg/l
MEDIUM M8 – MS + 3.0 BAP + 0.5 NAA +0.5 KN mg/l
MEDIUM M9 – MS + 2.0 BAP + 1.0 NAA mg/l
MEDIUM M10 – MS + 3.0 BAP + 1.0 NAA +0.5 KN mg/l

The measurement was taken on the basis of percentage if shoot response, number of multiple shoots developed, shoot length and callus formed from each ten replicates.

Experiments for Root Induction
Regenerated multiple shoots of Withania sominifera were separated, each strong and elongated shoots were treated with root initiating growth regulators
MS + 10 g/l sucrose
MS + 1 mg/l NAA + 20g sucrose
MS + 200 mg activated charcoal
MS + 20g/l sucrose

III. RESULTS AND OBSERVATIONS

Effect Of Age Of Explant

The effect of season, age of the explants and the effect of various cytokinins on induction of sprouting was studied simultaneously. The sterilized nodal explants were inoculated on MS medium supplemented with vitamins and cytokinins alone or in combination of two. Explants from non flowering plants showed 85% sprouting with in 10 days. The part of the explants which was in contact with medium swelled as an activity of meristmatic activity. This was accompanied by abscission of subtending leaf. The portion of the explants above the medium did not show any callusing.

Bud growth from nodal explants of the mature flowering plants was negligible; most of the axillary buds turned light brown and died. However only 10to 20 % apical tips and first three nodal explants from matured flowering plants showed initiation of single sprout after 20 to25 days of incubation on initiation medium. Continued incubation did not show nay improvement in the formation of shoots. However, the explants either died or the entire explants formed non fragile callus. The newly sprouted branches from non flowering plants were more responsive than those from mature, flowering plant of *Withania sominifera*.

Effect Of Position Of Explant

The axillary buds of the newly sprouted branches, which were nearer to the apical bud, were more responsive. After about three or four axillary bud the stems becomes woody and such buds showed no response in culture. In the preliminary experiments, effect of cytokinins BAP, KN, 2iP alone or in combination were tested. Maximum (70-80%) number of bud break and initiation of shoot was reported in BAP alone (1.0 to 2.0 mg/l), about one to two shoots were developed in the cultures contained BAP and followed by KN. With 2iP there is no any initiation of shoot has been reported. The combination of BAP and KN shows 40 to 45% of initiation was found to the formation of only one shoot with the formation of callus. The maximum length of shoot was observed (one to two cm) in the medium containing BAP alone in comparison to the medium supplemented with BAP with KN. Since the preliminary experiments indicated the synergistic effect of BAP KN and on sprouting of shoot further experiments were conducted to evaluate the optimum concentration of cytokinins. Only the combination of lower concentration of BAP and KN produced best results while higher concentration of BAP favored less, short and weak shoot with the formation of more callusing from the explants base.

Effect of Auxins

Addition of auxin like NAA (0.5 to 1.0 mg/l) to the induction medium did not favours in the induction of direct shoot formation. The cultures containing NAA observed the formation of callus, after 20 to 25 days weak shoot initiated from the callus.

Multiplication

In order to optimize a suitable medium for mass multiplication of shoots from a single initiated nodal region, the effect of various media on number of shoots and their length was assessed. The highest number of shoots were observed in the medium containing high concentration of BAP (2.0 to 3.0 mg/l).These medium shows about 15 to 20 number of shoots per culture when sub cultured in the same fresh medium after 15 days duration. The length of the shoots elongates to 4 to 5 cms. In various combinations with KN low concentration

of BAP was not effective. However, rapid multiplication and elongation of shoots was observed in higher concentration of BAP with KN. MS basal medium supplemented with lower concentration of BAP (0.5and 1.0 mg/l) and KN i.e. medium M1, M2, M3, M4 produced 5to 9 shoots with shoot length 2 to 3 cm, while MS with BAP (2.0 to 3.0 mg/l) with (0.5 mg/l) KN produced maximum number of shoots i.e. 15 to 20 with 4 to 5 cm in length. Addition of auxin like NAA in the concentration of (0.5 mg/l) promotes the multiplication of shoots. The cultures containing BAP with NAA in low concentration produced direct shoots with a little callus development.

Effect Of Additional Vitamins

For further improvement in proliferation, shoots were sub cultured in the medium containing high BAP with coconut water in the concentration of 10 to 40 percent as additive which show cytokinin effect. Cultures containing CW in 40 percent gave better response showed increase in shoot number and shoot length.

ROOTING

Experiments were carried out for the induction of ex-vitro as well as invitro rooting. Shoots of 2 to 3 cm in length, healthy, strong were used for rooting.

EX -VITRO ROOTING

The individual shoots were directly transferred to polybags containing sand : soil mixture rooted with in 20 days. The plantlets were grown in greenhouse under 70% humidity, where 80% survival was observed after two weeks. Some shoots started wilting after one month and the survival rate drastically reduced to 50 percent.

IN VITRO ROOTING

For invitro rooting induction types of media were used. 85 percent rooting was observed in the medium containing Activated charcoal with in 15 to 20 days, 4 to 7 thin long roots were developed which increases with the age of cultures. Medium containing NAA developed 50 percent 2 to 4 thick tap roots in comparison to the previous. These plantlets when transferred to soil showed 90 percent survival under green house conditions.

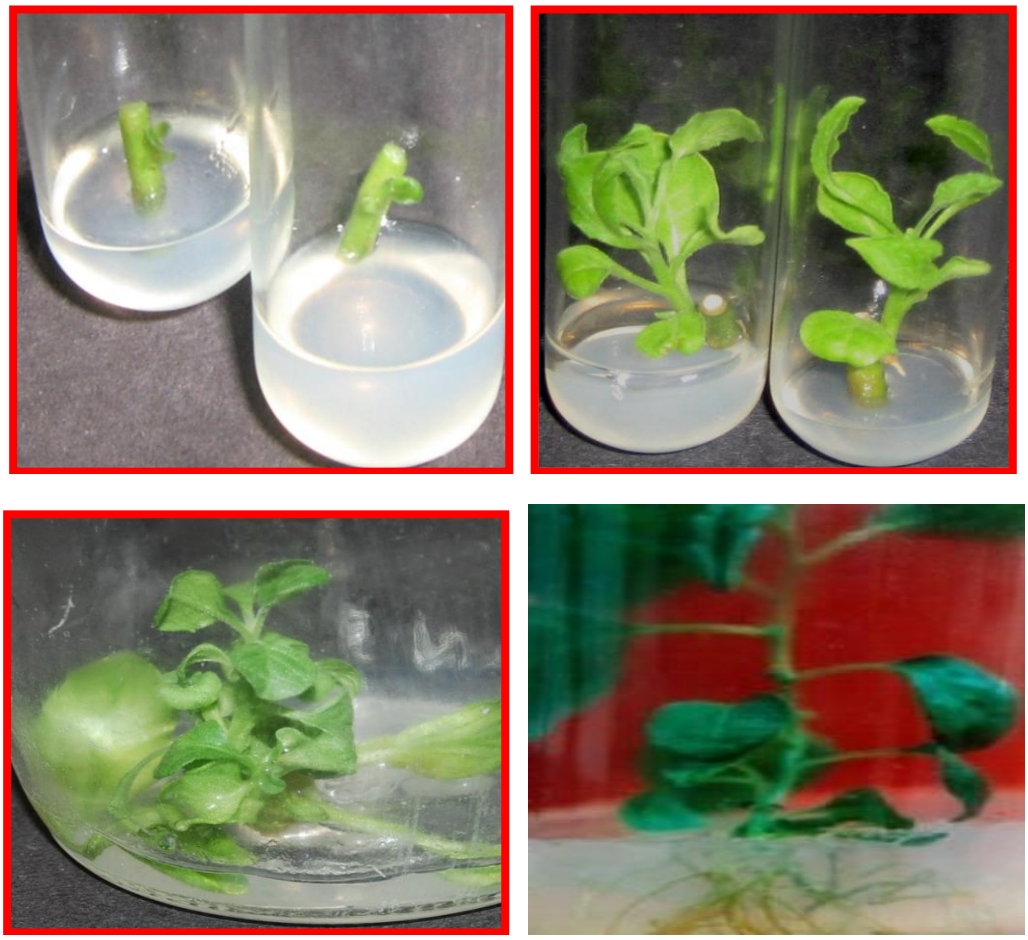

Fig: Rooted shoot of *Withania Somnifera*.

Since Ashwagandha is propagated mainly by seeds and the success rate of vegetative propagation being very low, this rapid and efficient regeneration protocol could be used for large scale production of selected cultivated varieties. This direct regeneration method which minimizes genetic instability that is normally encountered during callus mediated regeneration will help in producing large number of selected superior chemo types ashwagandha which has good demand in the present Indian market.

Table-1 EFFECT OF GROWTH REGULATORS ON SHOOT INDUCTION IN *WITHANIA SOMNIFERA.*

S.NO	Medium + Growth hormones mg/l	%age of shoot induction	No. of shoots per culture	Average shoot length in cm.	Callussing
1	MS+0.5 BAP	70%	1-2	1-2	-
2	MS+1.0 BAP	75%	1-3	2 cm	-
3	MS+1.5 BAP	70%	1-2	2cm	-
4	MS+2.0 BAP	60%	1-2	1-2	-
5	MS+0.5 KN	40%	1	1-2	+
6	MS+1.0 KN	42%	1	1-2	-++
7	MS+0.5 BAP+0.5 KN	45%	1	1	++
8	MS+1.0 BAP+0.5 KN	45%	1-2	1	+

Table-2 EFFECT OF GROWTH REGULATORS ON MULTIPLICATION OF SHOOTS IN *WITHANIA SOMNIFERA*

S.NO	Medium + Growth hormones mg/l	%age of response	Average no. of shoots	Average shoot length in cm.	Callussing
1	MS+1.0 BAP	60	15	2-3	-
2	MS+2.0 BAP	85	15-20	2 -3	-
3	MS+2.0BAP+0.5KN	50	8-10	2-5	+
4	MS+3.0 BAP+40% CW	95	20-25	3-5	-
5	MS+3.0 BAP+1.0KN	80	10-18	1-2	+
6	MS+1.0 BAP+2.0 KN	45	4-9	1-2	++

Table-3 EFFECT OF DIFFERENT MEDIA ON ROOTS INDUCTION IN WITHANIA SOMNIFERA.

S.NO	Medium	% age of shoots rooting	Root length in cm	Root Morphology
1	MS+ 10g/l sucrose	50-60	2-3	Thin, short
2	MS + 1.0 mg/l NAA + 20g Sucrose	70-75	2-5	Thin, short
3	MS + 200mg activated charcoal	85-90	3-5	Thin, long

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