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Research Article

In Vitro PlantGermination, Micro propagation and Plantlet Formation of *Withania somnifera* (L.) DUNAL

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ABSTRACT

Withania somnifera (L.) Dunal known as Ashwagandha is commonly used in traditional Indian medicine system. In the present study, different explants of W. Somnifera were used for establishing cultures, including seeds, axillary and apical shoots etc. For surface sterilization the seeds were washed with water for several times and then treated with 0.1% HgCl2 (mercuric chloride) solution for 5-10 minutes and followed by sterile distilled water for four times. The seeds were then inoculated on MS nutrient basal medium of different concentration with MS vitamins, supplemented with Cytokinins like BAP, KN, with sucrose (20 g/l) and gelled with agar (4.5 g/l). To optimize the medium concentration of the medium, and Cytokinins (BAP and KN) for seed germination, the seeds were induced on MS with BAP or KN (0.1 to 1.0 mg/l) alone or in combination, with sucrose (20 g/l). In addition, Auxins like IAA or NAA, (0.1 to 1.0 mg/l) alone or in combination were also tested for seed germination and were added to MS basal medium with different concentration of sucrose (10 to 40 g/l). Results shows that germinated seedlings raised 2-3 cm in length after 20-25 days and were used for multiplication. Axillary, Apical bud and hypocotyle, part of the seedlings give better response for shoot initiation in the medium MS containing BAP (0.5-1.0 mg/l) with NAA (0.5 mg/l). One to two shoot buds initiated within 10 days. From the meristem and from the base of the shoot. hypocotyls region produced direct shoot in the medium containing NAA.

Keywords: *Withania somnifera (L.)* Dunal; MS medium; Germination; Micro propagation; Cytokinins (BAP, KN); Auxins (IAA or NAA).

INTRODUCTION

Withania somnifera (L.) Dunal (family: Solanaceae), commonly known as "Indian Ginseng", is a medicinally and industrially important plant of the Indian subcontinent and other warmer parts of the world. The plant has multi-use medicinal potential [1]. W. sominifera is a green shrub found throughout the drier parts in India, Baluchistan, Pakistan, Afganistan, Shri Lanka, Congo, South Africa, Egypt, Morocco, and Jordan. In India, it is widely grown in the provinces of Madhya Pradesh, Uttar Pradesh, plains of Punjab and northwestern parts of the India like Gujarat and Rajasthan, growing up to a height of 30cm-150 cm [2].

It is considered as important medicinal plant in the Ayurvedic and indigenous medicinal system of India. It has many medicinal properties like anti-infl ammatory, anticancer, antistress, anti-ageing, immune-modular, adaptogenic and shows the free radical scavenging activity It is used for treatment of tuberculosis, rheumatism, infl ammatory conditions and cardiac diseases. It is also useful as abortifi cient, amoebicide, anodyne, bactericide, and contraceptive and spasmolytic. The roots are also used as sedative for senile debility and for the prevention and inhibition of Alzheimer's disease [3-7].

Traditionally W. somnifera is propagated from seeds, but the

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mature and healthy seeds are not always available for germination. The viability period of seeds is very short and their germination is also poor. The provision of alternative sources of W. somnifera by encouraging its cultivation will go a long way in reducing their heavy dependence on the wild populations. Conventional propagation methods have proved to be inadequate to meet this challenge. Large scale production through plant *In Vitro* regeneration will provide a means of putting the plant onto the market at lower prices [8-23].

The present study was done to determine the effect of growth hormones on shoots initiation, multiplication, rooting and hardening of Ashwagandha to standardize the micro propagation technique in Ashwagandha.

MATERIALS AND METHODS

In Vitro **seed germination**

The effect of season, age of seeds of various medium on *In Vitro* seed germination was studied. Fully ripe red berries fruits of W. somnifera were collected from matured field grown plants. The seeds were taken out and surface sterilized. MS vitamins supplemented with Cytokinins like BAP, KN, with sucrose (20 g/l) and gelled with agar (4.5 g/l). In the preliminary experiments, the matured seeds were responded best in the medium MS 1/2. To optimize the medium concentration of the medium, and Cytokinins (BAP and KN) for seed germination, the seeds were induced on MS with BAP or KN (0.1 to 1.0 mg/l) alone or in combination, with sucrose (20 g/l). In addition, Auxins like IAA or NAA, (0.1 to 1.0 mg/l) alone or in combination were also tested with different concentration of sucrose (10 to 40 g/l). Different medium tried for *In Vitro* seed germinations are in Table 1.

Table 1: Effect of different medium for seed germination

Sr. No.	MS-A	MS-B	MS-C	MS-D
1	A1-MS full+10 gmssucrose	B1-MS full+0.1 mg/l BAP+20 gmssucrose	C1-MS full+0.1 mg/l BAP+0.1 mg/l KN+20 gmssucrose	D1-MS full+ 0.1 mg/l IAA+20 gmssucrose
2	A2-MS full+20 gmssucrose	B2-MS full+0.1 mg/l BAP+30 gmssucrose	C2-MS full+0.3 mg/l BAP+0.1 mg/l KN+20 gmssucrose	D2-MS ½+ 0.1 mg/l IAA+20 gm ssucrose
3	A3-MS full+30 gmssucrose	B3-MS full+0.3 mg/l BAP+20 gmssucrose	C3-MS full+0.1 mg/l KN+20 gmssucrose	D3-MS full +0.5 mg/l IAA+20 gmssucrose
4	A4-MS ½+20 gmssucrose	B4-MS full+0.5 mg/l BAP+30 gmssucrose	C4-MS full+0.3 mg/lKN+20 gmssucrose	D4-MS ½+0.1 mg/l NAA+ 20 gmssucrose
5	A5-MS ½+30 gmssucrose	B5-MS full+1.0 mg/l BAP+30 gmssucrose	C5-MS full+0.5 mg/l KN+20 gmssucrose	D5-MS full+0.1 mg/l NAA+ 20 gmssucrose
6	A6-MS ½+40 gmssucrose	B6-MS full+1.0 mg/l BAP+20 gmssucrose	C6-MS ½+0.1 mg/l BAP+0.1 mg/l KN+20 gmsSucrose	D6-MS full+0.5 mg/l NAA+ 20 gmssucrose
7			C7-MS ½+0.5 mg/l BAP+0.1 mg/l KN+20 gms sucrose	D7-MS ½+0.5 mg/l NAA+ 20 gmssucrose

The measurement of growth was done.

Initiation

After 20-25 days of seed germination, to initiate the shoots from different explants MS basal medium supplemented with different concentration. To optimize the concentration of Cytokinin, BAP and KN (0.1-5.0 mg/l) alone and with combination of Auxins NAA or IAA (0.1-3.0 mg/l) were tried. Number of experiments was carried out to maximize the produce shoots. This includes:

1. Use of low concentrations of cytokinins (BAP and KN 0.1-1.0 mg/l)
2. Use of dilutions of MS macro and micro elements keeping MS vitamins constant i.e. full strength.3. Use of low concentration of auxins (NAA 0.1-0.5 mg/l)
3. Finally the effect of growth regulators on initiation of shoots was studied in Table 2.

Table 2: Effect of growth regulators on initiation of shoots

Sr. No.	MS-A (BAP)	MS-B (KN)	MS-C (NAA)
1	AI MS+0.1 mg/l BAP	B1 MS+0.1 mg/l BAP+0.1 mg/l KN	C1 MS+0.1 mg/l BAP+0.1 mg/l NAA
2	A2 MS+0.2 mg/l BAP	B2 MS+0.5 mg/l BAP+0.1 mg/l KN	C2 MS+0.3 mg/l BAP+0.1 mg/l NAA
3	A3 MS+0.5 mg/l BAP	B3 MS+0.8 mg/l BAP+0.5 mg/l KN	C3 MS+0.5 mg/l BAP+0.1 mg/l NAA
4	A4 MS+0.8 mg/l BAP	B4 MS+1.0 mg/l BAP+0.5 mg/l KN	C4 MS+1.0 mg/l BAP+0.1 mg/l NAA
5	A5 MS+1.0 mg/l BAP	B5-MS+1.0 mg/l BAP+1.0 mg/l KN	C5-MS+0.5 mg/l BAP+0.5 mg/l NAA
6			C6-MS+1.0 mg/l BAP+0.5 mg/l NAA

Multiplication

After 15 to 20 days of initiation the apical and axillary meristems were showing response. Experiments were carried out to maximize the production. This includes:

1. Use of high concentrations of cytokinins (BAP and KN, 0.5 to 3 mg/l)
2. Use of additives like coconut water 10 to 50 percent.
3. Use of dilutions of MS macro and micro elements keeping MS vitamins constant
4. Use of low concentration of Auxins (NAA 0.1 mg/l to 1.0 mg/l

Finally the effect of multiplication was studied using different growth regulators with MS basal nutrients and additional coconut water shows in Table 3.

Table 3: The effect of multiplication using MS media with using 30 gmssucrose

Sr. No.	MS-A	MS-B	MS-C	MS-D
1	A1 MS+0.5 mg/1BAP+0.5 mg/l KN	B1-MS +0.5 mg/l BAP+0.1 mg/l NAA	C1-MS+0.5 mg/l BAP+10% CW	D1MS+0.5 mg/lBAP+0.1 mg/l NAA+20%CW
2	A2 MS+1.0 mg/l BAP+0.5 mg/l KN	B2-MS+1.0 mg/lBAP+0.1 mg/lNAA	C2-MS+0.5 mg/l BAP+20% CW	D2MS+0.5 mg/lBAP+0.5 mg/lNAA+40% CW
3	A3MS+1.5 mg/lBAP+0.5 mg/l KN	B3MS+1.0 mg/lBAP+0.5 mg/l NAA	C3-MS+0.5 mg/l BAP+40% CW	D3-MS+1.0 mg/lBAP+0.5 mg/lNAA+20%CW
4	A4MS+2.0 mg/lBAP+0.5 mg/l KN	B4-MS+2.0 mg/l BAP+0.5 mg/l NAA	C4-MS+1.0 mg/l BAP+20% CWv	D4MS+1.0 mg/lBAP+0.5 mg/lNAA+40%CW
5	A5MS+3.0 mg/lBAP+0.5 mg/l KN	B5-MS+2.0 mg/l BAP+1.0 mg/l NAA	C5-M+1.0 mg/l BAP +40% CW	D5MS+2.0 mg/lBAP+0.5 mg/lNAA+20%CW
6		B6-MS+3.0 mg/l BAP+0.5 mg/l NAA	C6-MS+2.0 mg/l BAP+20% CW	D6MS+2.0 mg/lBAP+0.5 mg/lNAA+40%CW
7		B7-MS+3.0 mg/l BAP+1.0 mg/l NAA	C7-MS+2.0 mg/l BAP+40% CW	D7MS+3.0 mg/lBAP+0.1 mg/lNAA+20% CW
8			C8-MS+2.0 mg/l BAP+50% CW	D8MS+3.0 mg/lBAP+0.1 mg/lNAA+40% CW

9			C9-MS+3.0 mg/l BAP+20% CW	D9MS+3.0 mg/lBAP+0.5 mg/lNAA+20% CW
10			C10-MS+3.0 mg/l BAP+40% CW	D10MS+3.0 mg/lBAP+0.5 mg/lNAA+40%CW
11			C11MS+3.0 mg/ lBAP+50%CW	D11MS+3.0 mg/lBAP+1.0 mg/lNAA+40%CW

Effect was observed after 15 to 20 days intervals.

Root induction

After multiple shoots development the healthy, strong, elongated shoots of more than 2.5 cm in length were separated and *ex vitro* as well as *In Vitro* rooting experiments were carried out.

Ex vitro **rooting**

For *ex vitro* rooting the individual shoots were dipped into Auxins NAA or IAA (1 mg/l) as a rooting solution for half to one hour and then directly transferred to sand: soil (1:1) mixture.

Different medium used for root initiation are

A1-MS full+agar+10 gms sucrose

A2-MS full+agar+20 gmssucrose

A3-MS full+0.5 mg/l NAA liquid+30 gmssucrose

A4-MS ½+0.5 mg/l NAA solid+30 gmssucrose

A5-MS full+0.5 mg/l IAA liquid+30 gmssucrose

A6-MS full+0.5 mg/l IAA solid+30 gmssucrose

A7-MS ½+1.0 mg/l NAA liquid+30 gmssucrose

A8-MS full+1.0 mg/l NAA solid+30 gmssucrose

B1-MS full+100 mg/l activated charcoal+30 gmssucrose

B2-MS½+100 mg/l activated charcoal+30 gmssucrose

B3-MS full+200 mg/l activated charcoal+30 gmssucrose

B4-MS½+200 mg/l activated charcoal+30 gmssucrose

The cultures incubated in dark for 48 hours and transferred to hormone free MS ½ strength in light conditions with 16-18 hours photoperiod till the roots produced.

RESULTS AND DISCUSSION

Effect of season and age of the explant

The effect of season and age of the seeds and also the effect of various growth regulators on the seed germination, initiation and multiplication of shoots were studied.

Seeds collected from the field grown plants in every month of flowering season were used for *In Vitro* seed germination. From immatured (green and yellow) fruits the seeds showed no response and fail to germinate. Seeds isolated from matured fruits (orange and red) germinated fairly. Seeds of W. somnifera germinate six to seven month of harvesting or only fresh seeds of the season show *In Vitro* germination.

In Vitro **seed germination**

Seeds sterilized with 0.1% mercuric chloride solution for 5 minutes gives 90 percent sterile culture. It is observed that after inoculation of 5-6 days the seeds coats starts breaking and germination took place within 10 days.

Effect of medium concentration on seed germination

Seeds germinated in the medium MS full and half concentration without growth hormone. The maximum seed germination was recorded in the medium A4, A5 and A6, about 75 to 85 percent of seed germination was recorded in the medium A5 i.e. MS medium half concentration with 3% sucrose. Germinated seeds raised into 2-3 cm length seedlings within 20 to 25 days.

MS medium containing BAP and Kinetin does not give satisfactory results. Only low concentration of BAP (0.1 mg/l) shows 40%-50% of seeds germination and developed one or two stunded shoots while higher concentration of BAP produces brown non fragile callus [24-25]. MS medium containing auxins (IAA or NAA) fails to germinate the seeds (Table 4).

Table 4: Effect of media and growth regulators on *In Vitro* seed germination of Withania somnifera

S. No.	Medium	Percentage of Seed Germination (10 Days)	Seedlings Growth (20 Days)	Callussing
1	A1	10-15	1-2	-
2	A2	20-25	1-2	-
3	A3	30-34	1-2	-
4	A4	70-71	2-3	-
5	A5	75-85	2-3	-
6	A6	75-80	2-3	-
7	B1	40-45	2-3	+

8	B2	10-12	1-2	+
9	B3	2	1	++
10	B4	NIL	NIL	++
11	B5	NIL	NIL	NIL
12	B6	NIL	NIL	NIL
6	A6	75-80	2-3	-
7	B1	40-45	2-3	+
8	B2	10-12	1-2	+
9	B3	2	1	++
10	B4	NIL	NIL	++
11	B5	NIL	NIL	NIL
12	B6	NIL	NIL	NIL

Initiation of shoots

Effect of cytokinins: Parts of *In Vitro* raised seedlings were taken for initiation of shoots. The medium MS containing different concentration of BAP, KN and NAA tried, initiated shoots from axillary, apicals and hypocotyle portions. Maximum percentage of bud break and shoots were initiated from axillary bud in the medium A3 to A5. About 3 to 4 shoots were produced achieving the length of 1-3 cm. The medium containing BAP (1.5-3.0 mg/l) with combination of KN (0.1 and 0.5 mg/l) showed about 40 to 45% of shoot initiation at the lower end of the explant shoot. In this medium maximum shoot length was reported 1 cm in length which was not very healthy.

Effect of auxins: MS medium containing auxin NAA (0.1 mg/l) with BAP (B1-B5) for initiating the shoots did not favour more. Only one shoot initiated from axillary and apical bud of one cm in length with lots of callus development (Table 5).

Table 5: Effect of media and growth regulators in the initiation of shoots from the seedlings of Withania somnifera

Medium	Response in Shoot Induction	No. of Shoots Per Explant	Shoot Length in cm	Callusing
A1	10	1	0.5	-
A2	20	1-2	1	-
A3	60	2-3	1-2	-
A4	60-65	3-4	1-3	-
A5	70	3-4	1-3	-
B1	10	1	1	+
B2	40	1-2	1	++
B3	40	1-2	1	++
B4	45	1-2	1	++
B5	10	1	0.5	+++
C1	5	1	0.5	++
C2	5	1	0.5	++
C2	20	1	1	++
C4	10	1	0.5	+++
C5	5	1	0.5	+++
C6	5	1	0.5	+++

Multiplication

Effect of cytokinin: To standardize a perfect media for rapid multiplication of shoots of Withania from single initiated shoot of seedlings the effect of various media on number of shoots and length was assessed. The higher number of shoots produced in the medium containing BAP in higher concentration (2.0-3.0 mg/l). The medium produces 15-20 healthy shoots of 4-5 cm length. Direct shoots production and multiplication was seen from the hypocotyle region of the seedlings in the medium containing BAP (3.0 mg/l), about 9 to 15 shoots of 2-3 cm were observed [26-27].

Effect of auxins: Addition of auxin like NAA in the concentration of (0.5 mg/l) promotes the multiplication of shoots. About 15 to 20 shoots were observed in this medium with the formation of callus, from which also shoots developed [26-27].

Effect of additional vitamin: Multiplication of shoots were enhanced in the medium containing coconut water as additive with cytokinin. Cultures containing 40% coconut water with higher concentration of BAP was reported the best medium for rapid multiplication and elongation of shoots. The maximum number of shoots achieved in this experiment was 40 to 50 per culture of 4-5 cm in length were reported. Medium contains coconut water with NAA does not affect much as compared to other media in multiplication due to formation of non-fragile callus [24-25] (Table 6).

Table 6: Effect of growth regulators on rapid multiplication of W. somnifera

Medium	Percentage of Responding	No. of Shoots per Culture	Length of Shoots in cm	Callusing
A1	25	4-5	1-2	-
A2	30	5-8	2-3	-
A3	30	5-8	2-3	-
A4	70	15-20	3-5	-
A5	80	15-20	4-5	-
B1	10	3-6	1	++
B2	20	3-6	1-2	+
B3	40	8-10	1-3	+
B4	70	15-20	2-5	+
B5	65	13-15	3-5	+
B6	70	15	1-2	+
B7	60	10-15	1-3	+
C1	20	4-5	1-2	++
C2	20	4-5	1-2	+
C3	37	4-5	1-2	+
C4	20	3-5	1-2	++
C5	30	3-5	1-2	+
C6	50	10-14	3-5	+
C7	75	15-20	3-5	+
C8	60	20-28	4-5	-
C9	70	40-45	4-5	-
C10	86	40-50	4-5	-
C11	70	40	4-5	-
D1	10	3-4	1-2	++
D2	10	3-5	1-2	+++
D3	12	3-5	1-2	+++
D4	10	2-4	1	+++
D5	10	2-4	1	+++
D6	12	2-6	1	++
D7	20	3-5	1	++
D8	20	4-6	1-3	++
D9	22	4-6	1-2	++
D10	25	4-6	1-2	++

Rooting

ex vitro rooting: Produced multiple shoots initiates root directly when transferred to polybags containing sand:soil (1:1) mixture with in 15 to 20 days. The plantlets gives 80% survival when grown in green house under 70% humidity [25].

In Vitro rooting: Root induction was observed in the entire medium tried for rooting. Maximum percentage of rooting was achieved in the medium MS containing NAA in the concentration of 0.5 to 1.0 mg/l within 10 days. About 4 to 5 thick, white tap roots were produced which increases with the age of culture (Table 7). *In Vitro* rooted plants shows 90 percent survival rate when transferred in the sand: soil mixture grown in green house [25].

Table 7: Effect of growth regulators on root induction of W. somnifera

Medium	Percentage of Shoots Rooted	Root Length in cm	Root Morphology
A1	10	2-3	Thin, short
A2	19	2-3	Thin, short
A3	60	4-5	Thick, long
A4	67	4-5	Thick, long
A5	65	4-5	Thin, long
A6	67	4-5	Thick, long
A7	70	4-5	Thick, long
A8	80	3-4	Thick, long
B1	30	3-4	Thick, long
B2	45	2-3	Thin, short, callus at base
B3	45	2-3	Thin, short
B4	30	2-3	Thick, short, with callus

Hardening (Acllimatization)

The rooted plantlets that were transferred directly to soil, wilted during the first week of exposure to green house conditions. Therefore, plantlets were gently washed. These were then transplanted to polypots containing sand and soil (1:1) mixture and later shifted to green house where they were maintained under a very high humidity (90-100%) for 10-15 days, where survival was 100 percent. Plants acclimatized under these conditions were shifted to field conditions after two months. Data shows the field evaluation of such plants, taken after three months (Table 8).

Table 8: Field evaluation of such plants, taken after three months

Character	Control	Regenerants
Height (cm)	6.4	12.6
No. of branches	2.9	4.5
No. of leaves	10.4	18.6
Size of leaves	3.35	4.65

From the data it is clear that the growth of the *In Vitro* derived plantlets was faster than control plants.

Present study demonstrates that micropropagation techniques are of great promise for clonal propagation of a useful important medicinal plant. Maximum number of adventitious shoots ranging from 30 to 50 per explant has been induced from axillary meristem of *In Vitro* seedlings. In the present study we have succeeded in inducing rooting of *In Vitro* derived shoots *In Vitro* seeds were germinated in the medium MS with or without growth regulators; Percentage of seeds germination observed in MS 1/2 and MS full plain was 70-80%. Axillary and apical shoots, hypocotyls, and leaves of seedlings were taken for shoot differentiation in MS medium supplemented with BAP (0.5-3 mg/l) NAA (0.5-1 mg/l) and CW (10-40%). Best shoots multiplication were achieved from axillary buds and shoot base on MS with (2-3.0 mg/l) BAP and CW (30%) Regenerated shoots rooted best on MS containing NAA (0.5-1.0 mg/l) Plantlets were transferred to sand: soil (1:1) mixture, acclimatized in culture room and after words to the Glass house (Figure 1).

Figure 1: Plant regeneration from diverse explant cultures in Withania: A. Mature cotyledons after 7-10 days in culture; B. Mature embryos after 7-10 days in culture; C. Mature hypocotyls after 7-10 days in culture; D. Initiation of somatic embryos from cultured mature cotyledons; E. Germination of somatic embryos; F. Initiation of somatic embryos from cultured mature embryos; G. Germination of somatic embryos; H. Regeneration of multiple plants from cultured mature embryos; I-J. Regeneration of multiple plantlets from cultured hypocotyls; J. Elongated shootlets after 35-40 days in culture; K. Hardening of regenerant in Net House.

CONCLUSION

The effect of season, age of the seeds and the effect of various growth regulators on the initiation and multiplication of shoots was studied simultaneously. Seeds collected from immature (Green to Yellow) fruits were not responding may be due to immaturity of the embryos. Seeds from fully mature fruits (Orange-red) show 70-80% of germination within 6-8 months of maturity of plants. The storage of seeds for long last viability. Seeds show better response in the medium without growth regulators. Maximum % of seed germination was recorded in the MS ½ strength medium sucrose comparison to medium MS Full strength. Medium containing BAP in low concentration (0.1 mg/l) response lowest in seed germination with the production of one or more stunded shoot and brown fragile callus. Germinated seedlings raised 2-3 cm in length after 20-25 days and were used for multiplication. Axillary, Apical bud and hypocotyle, part of the seedlings give better response for shoot initiation in the medium MS containing BAP (0.5-1.0 mg/l) with NAA (0.5 mg/l). One to two shoot buds initiated within 10 days, from the meristem and from the base of the shoot. Hypocotyls region produced direct shoot in the medium containing NAA.

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