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INDIRECT REGENERATION FROM LEAF SEGMENTS FOR MASS PROPAGATION OF *WITHANIA SOMNIFERA* : AN ENDANGERED MEDICINAL PLANT

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ABSTRACT

Withania somnifera, is an important medicinal plant known for its numerous traditional and medicinal uses. Low rate of germination in nature, poor seed viability and unrestricted collection from natural habitat have rendered the plant endangered. Hence, efforts are needed for conservation of this important medicinal plant. Present study has been carried out with an objective of developing a rapid and efficient protocol for mass propagation and conservation of the species. Callus development from leaf segments was achieved onto MS medium containing (2-10µM) NAA and (2-4µM)Kn or (2-10µM)IAA and (2-10µM)BAP. However, only the callus obtained from MS+IAA+BAP exhibited regeneration of shoot buds. Regenerated shoots were proliferated onto MS+BAP, with 11.04±0.20 average and 16 number of maximum shoots onto MS+10µM BAP. Elongated shoots were rooted onto ½ MS+IBA. Around 86.4% cultures were successfully rooted onto ½ MS+10µM IBA. Regenerated plants were acclimatized and about 82.4% plants survived during field transfer.

KEYWORDS : Callusing, Multiple shooting, Invitro rooting, Acclimitization.

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INTORODUCTION

Withania somnifera (Linn.) commonly known as Ashwagandha or winter cheery is an important medicinal plant belonging to family Solanaceae and is found to be distributed in the region of Pakistan, Afghanistan, Palestine, Egypt, Jordan, Morocco, Spain, Canary Island, Eastern Africa Congo, Madagascar and South Africa and grows throughout drier part and subtropical India. In India it is found to inhabit Gujrat, Rajasthan, Madhya Pradesh, Uttar Pradesh, Punjab plain extending to mountains of Himachal Pradesh and Jammu.[1] Plant possess immense medicinal potential and has been extensively utilized in both traditional as well as modern system of medicine. Anti inflammatory, anti stress, anti tumor, antioxidant, antineoplastic effects, rejuvenating tonic, immunomodulatory activity, cardioprotective activity and hypothyroid activity are some of the biological properties exhibited by the plant. Such medicinal and pharmacological attributes of the plant have been well documented and reported from the findings of research carried out by various workers[2,3,4,5,6]. Beside this *W. somnifera* is also known to possess antibacterial and antifungal activities against several microorganisms[7,8,9,10,11]. The medicinal potential of the plant is attributed to presence of range of bioactive metabolites like alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc), steroidal compounds including ergostane type steroidal lactones, withaferin A, withanolides A-Y, withasomniferin-A, withasomidienone, withasomniferols A-C, withanone etc[12,13,14,15]. Because of high medicinal value the plant has been extensively utilized traditionally and also there is ever increasing demand from the pharmaceutical sector. *Withania somnifera* is either propagated vegetatively by cuttings or seeds. Since vegetative propagation is time consuming and comparatively less productive hence, this method of propagation is insufficient for conservation of the species. Moreover, seed germination is low with poor seed viability along with a great deal of variation[16,17,18,19]. As a result of slow propagation in nature and unrestricted collection of the plant from wild stands the plant has become endangered[20,21]. Hence, invitro cuture technique provides a suitable alternate for rapid and mass propagation of the plant. The present study has been conducted to develop a protocol for conservation and mass propagation of the plant.

MATERIALS AND METHODS

Young leaves obtained from mature plant of *Withania somnifera* (fig. 1) maintained in green house of Department of Tissue Engineering SHIATS, Allahabad were utilized as explants for the present study.

Prior to inoculation explants were treated with 0.2% v/v solution of Tween 20 and kept under running tap water for about 15 minutes. Explants were them rinsed with 90% ethyl alcohol for 10-15 seconds and finally surface sterilized with 0.1% $HgCl_2$ for 2-3 minutes and then repeatedly washed with sterile distilled water to remove all traces of $HgCl_2$. Sterilized leaf segment were dried with sterile tissue paper and excised to appropriate size and aseptically inoculated onto MS medium[22] containing auxin (NAA, IAA) and cytokinin (Kn, BAP) in varying combination. For each treatment, a minimum of 20 cultures was raised and each experiment was repeated atleast twice. All the observations made were subjected to calculation of average mean ± standard deviation. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. Callus obtained was aseptically excised and transferred to medium enriched with higher concentration of cytokinin and lower concentration of auxin for regeneration of shoots. Regenerated shoot buds from callus were further proliferated onto medium containing BAP. Elongated shoots (5-6cm) were excised and transferred to freshly prepared rooting medium, ½ MS with IBA(5-20 µM).Plants with well developed roots were

taken out and thoroughly washed with sterile water to remove all traces of media after which plants were transferred to pots containing autoclaved coarse sand and garden soil (1:1). Pots were covered with transparent polybags and kept under similar cultural conditions utilized for earlier cultures for 20-25 days. Plants were irrigated with low concentration of inorganic nutrients according to the requirement.

RESULTS

In the present study, no morphogenic response was obtained when leaf segments were cultured onto MS medium deprived of plant growth regulators. However, MS medium containing NAA (2-10µM) and Kn (2-4µM) (in different combinations) was found to be suitable enough for callus induction from leaf segments of *Withania somnifera*. Within 1-2 week afrer,the establishment of cultures an initial crumbling of leaves was observed and colour of the leaves changed from green to pale yellow or brown. After an interval of 4-5 weeks, callus induction was initiated in the cultures. Formation of callus was observed in all the cultures when MS medium was supplemented with NAA ≥ 5µM with different concentration of Kn (see table I). Growth of callus initially occurred at the edges of innoculated leaf segments which were in direct contact with media (fig. 2), also along midrib and later spread over the entire surface of leaves. Callus obtained was compact and creamish white in colour which turned brown on sustained growth. Extent of callusing was enhanced onto higher concentration of NAA and lower concentration of Kinetin, with maximum callusing onto MS medium containing 10µM NAA and 2µM Kn (Table I). Although callusing was achieved with almost all combinations of NAA and Kn but the callus obtained failed to regenerate even when transferred to MS medium containing high concentration of Kn. In an independent experiment, development of callus from leaf segments was achieved onto MS medium containing (2-10µM) IAA & (2-10µM) BAP (in different combination). Leaf segments initially turned yellowish brown and after an interval of 4 weeks callus initiation occurred (Fig. 3). Callus obtained was fragile and whitish green which on prolonged growth turned green (Fig. 4). Development of callus was obtained onto all combinations of IAA & BAP, with maximum degree of callusing occurring onto MS containing 4µM IAA and 10µM BAP. After an interval of 5-6 weeks of callus induction, regeneration of shoot buds from callus occurred (Fig. 5). However, callus obtained onto medium containing higher concentration BAP(8-10µM) exhibited better regeneration potential (Fig. 6, Table II). Shoots regenerated indirectly through callus were of variable length and showed slow growth onto the induction medium. Regenerated shoots were aseptically excised and transferred onto MS medium containing BAP (2-12µm) for further proliferation. Multiple shoot regeneration along with elongation of shoots occurred on all concentration of BAP (Fig.7, Table III). Onto lower concentration of BAP (2-5µM), 48.4 – 85.2 % cultures exhibited multiple shoot induction. On further increasing the concentration of BAP (8-12µM) multiple shooting occurred in 100% cultures. Maximum number of shoots (16) was obtained onto MS fortified with 10µM Kn with an average number of 11.4±0.20 shoots per culture onto same medium. Shoots attained a length of 8-9cm within 2-3 weeks of subculture. Along with multiple shoot regeneration sparse callusing also occurred in the cultures (Fig. 8).

Elongated shoots were again excised aseptically and transferred to rooting media (MS+IBA). Different concentration of IBA (5-20µM) with full and half strength MS medium were utilized for invitro regeneration of roots. In the present study, half strength MS was found to be more effective in induction and development of in vitro roots as compared to full strength MS medium. ½ MS containing 10µM IBA was found to be the most effective medium for invitro root formation, with 85.4% cultures exhibiting root formation onto this medium, with an average root number of 7.2±0.5 and a maximum root length of around 9.2cm (Fig. 9, Table IV).Whereas onto full

strength MS medium a maximum of 34.6% cultures responded to invitro root formation onto MS medium containing 20µM IBA. Alongwith rooting, elongated shoots also exhibited further regeneration of shoot buds from nodes onto 5-20µM IBA. However, there was a gradual decrease in percentage of cultures showing shoot bud regeneration from 80-33.33% when IBA concentration was increased from 5 to 20µM. Moreover, callus induction was also found to occur onto full strength MS medium containing IBA (Fig. 10, Table IV) *Invitro* rooted plantlets were transferred to plastic pots for hardening and acclimatization. Around 82.4% plants survived during field transplantation.

Table I
Response of leaf explants to NAA + Kinetin

NAA (µM)	Kinetin (µM)	% Cultures exhibiting callusing	Degree of Callusing	Texture / colour of callus
2	2	75.5	+	Compact / whitish brown
4	2	94.2	+	Compact / whitish brown
5	2	98.8	++	Compact / whitish brown
8	2	100	+++	Compact / whitish brown
10	2	100	++++	Compact / whitish brown
2	4	80.4	++	Compact / whitish brown
4	4	88.4	+++	Compact / whitish brown
5	4	100	+++	Compact / whitish brown
8	4	100	+++	Compact / whitish brown
10	4	100	+++	Compact / whitish brown

+ = poor, ++ = moderate, +++ = good, ++++ = excellent

Table II
Response of leaf explant to BAP +IAA

IAA (µM)	BAP (µM)	% Cultures exhibiting callusing	Degree of callusing	Texture/ colour of callus	Cultures exhibiting regeneration (%)
2	2	40.0	++	Fragile/ green	25.5
2	4	58.2	++	Fragile/ green	44.8
2	8	64.4	++	Fragile/ green	75.6
2	10	85.0	+++	Fragile/ green	100
4	2	44.4	++	Fragile/ green	33.3
4	4	68.4	++	Fragile/ green	65.5
4	8	70.2	+++	Fragile/ green	100
4	10	96.0	++++	Fragile/ green	100
8	2	45.0	++	Fragile/ green	45.0
8	4	68.8	++	Fragile/ green	65.4
8	8	80.2	+++	Fragile/ green	100
8	10	100	+++	Fragile/ green	100
10	2	45.6	++	Fragile/ green	43.0
10	4	55.5	++	Fragile/ green	54.0
10	8	88.0	+++	Fragile/ green	100
10	10	100	+++	Fragile/ green	100

+ = poor, ++ = moderate, +++ = good, ++++ = excellent

Table III
Proliferation of shoot buds from callus onto MS+ BAP

BAP (µM)	% culture showing multiple shooting	Avg. No. of shoots	Max. No. of shoots
2	48.4	3.0±0.50	04
5	85.0	6.5±0.15	05
8	100	8.8±0.15	09
10	100	11.4±0.20	16
12	100	9.0±0.30	14

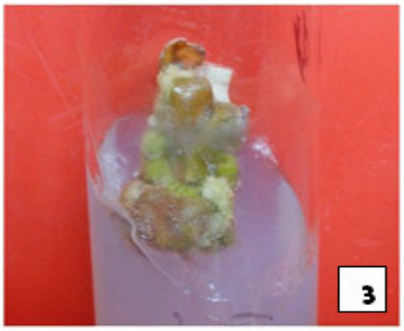
All values are mean ±standard deviation

Table IV
induction & development of roots from invitro regenerated shoots

MEDIUM	% culture showing root induction	Avg. No. of Root/explant	Root length (cm)	Callusing (%)
MS+05µM IBA	-	-	-	55.5
MS+10µM IBA	18.4	3.1±0.15	3.6	100
MS+20µM IBA	34.6	3.3±0.20	4.0	100
½ MS+05µM IBA	68.6	4.6±0.25	6.5	-
½ MS+10µM IBA	86.4	7.2±0.05	9.2	-
½ MS+20µM IBA	74.2	6.4±0.10	9.1	-

All values are mean ±standard deviation

Figure. 1 Mature plant of Withania somnifera
Figure. 2 Callus induction from leaf segments onto MS+10µM NAA+2µM Kn



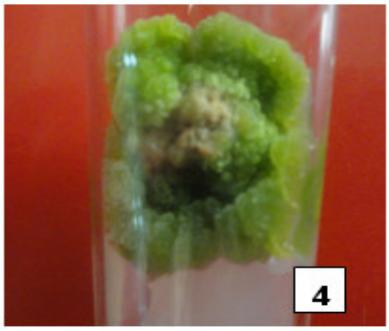


Figure. 3 Callus initiation onto MS+ IAA+BAP after 4 weeks of establishment of culture
Figure. 4 Extensive callusing onto MS+4µM IAA+10µM BAP.

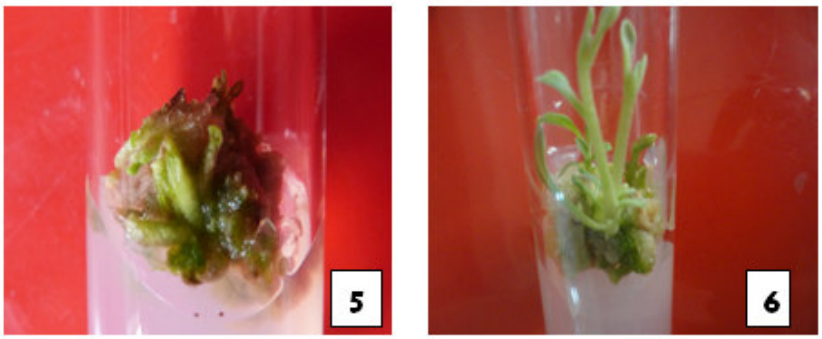


Figure. 5 Shoot bud induction from leaf derived callus, onto MS+IAA+BAP after 5-6 weeks of callus induction.
Figure. 6 Callus obtained onto medium containing higher concentration of BAP(8-10µM), exhibited better regeneration potential.

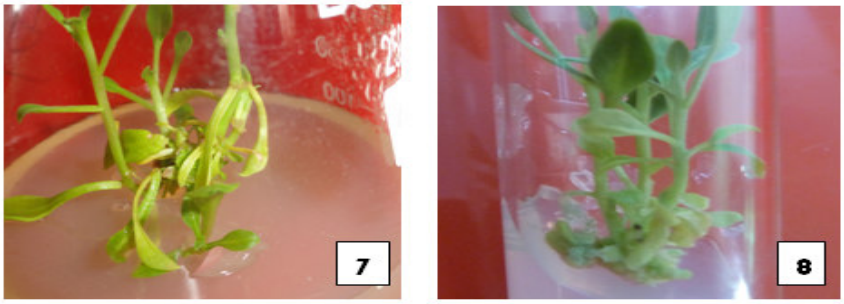


Figure. 7 Proliferation of callus derived shoots for multiple shooting onto MS+BAP(2-12µM)
Figure. 8 Sparse callusing along with multiple shoot generation onto MS + BAP

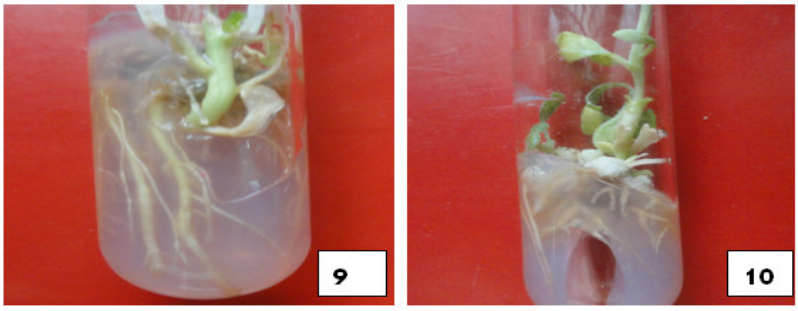


Figure. 9 Invitro rooting onto ½ MS+10µM IBA (9.2 cm root length & 7.2±0.05 avg. no. of roots)
Figure. 10 Invitro rooting onto MS +10µM IBA (3.6 cm root length & 3.1±0.02 avg. no. of roots) along with callus development.

DISCUSSION

The present study avails a rapid and efficient micropropagation protocol for mass propagation and conservation of *Withania somnifera*. In individual experiments it was found that both NAA and Kn & IAA and BAP were effective in the development of callus from a cultured leaf segment of *W. somnifera*. However, further regeneration of shoot buds could be achieved only for the callus developed from MS containing IAA and BAP. Similar results have also been obtained in previous studies done[23]. In the present study callus obtained from leaf segments onto medium MS supplemented with NAA and Kn showed no morphogenetic development and eventually driedout whereas in an earlier study

development of callus as well as shoot regeneration onto MS medium containing NAA and Kn have been reported[24]. Regenerated shoot buds from callus were further proliferated onto medium containing BAP and multiple shoot regeneration was achieved at all the concentrations of BAP (2-12µM). Previous studies also report indirect regeneration from leaf segments of *W. somnifera,* callus development from leaf segments of *W. somnifera* onto medium containing either 2,4-D alone or in combination with BAP have been reported[25]. Beside this MS medium containing BA alone or in combination with 2,4-D or NAA have also been found suitable for in vitro callusing from cultured leaf segments[26]. However, in contrast there have been research studies which have reported direct regeneration of shoot buds from invitro cultured leaf segments of *W. somnifera* onto MS medium containing IAA and BAP or NAA and BP or TDZ[27,28]. Direct regeneration from leaf segments onto MS medium containing IAA and BAP or Kn and IAA has also been reported[29].

In vitro regenerated roots were successfully rooted onto MS medium containing IBA. However percentage cultures exhibiting invitro root development onto ½ strength MS medium (74.2) outnumbered the cultures which developed roots onto full strength MS medium (34.6), keeping concentration of IBA same 20 µM. In earlier reports both ½ strength and full strength MS medium containing IBA have been reported to be effective enough to induce invitro regeneration of roots. In many previous studies[20,26] invitro rooting has been obtained onto ½ MS containing IBA. Whereas there are other studies[25,27,29,30,31] which report the effectiveness of full strength MS containing IBA suitable for induction as well as the development of invitro rooting. Around 82.4% of regenerated plants survived during the process of acclimatization and were successfully transferred to field. The transplantation rate have been found approximately similar to the survival rate (87%) of regenerated plant achieved in earlier study[20].

CONCLUSION

Hence the present study avails a protocol for mass scale propagation of *W. somnifera* with an efficient field survival rate. Regenerated plants can be utilized for rehabilitization of the plant, especially in the areas from where the plant availability had decreased drastically due to overexploitation either for industrial or traditional purposes. Moreover the protocol can be utilized for further studies to analyze various biochemical pand molecular aspects of invitro regenerated plants.

ABBREVIATION

sMuashigue and Skoog (MS), Napthalene acetic acid (NAA), Kinetin (Kn), Indole-3 acetic acid (IAA), Benzylamino purine (BAP).

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