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Enhanced Production of Therapeutically Valuable Withaferin A From *In Vitro* Propagated *Withania Somnifera* (L.) Dunal

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Abstract- Withanolides from *Withania somnifera* are one amongst the most exploited secondary metabolites for their enormous therapeutical and pharmaceutical benefits. Large scale production of these herbs from different explants of *in vitro* germinated seedlings were tried in order to meet the growing market demand. A rapid and efficient indirect regeneration protocol was optimized from the leaf explants of *W.somnifera*. Compact green calli were obtained from leaf explants cultured on MS media supplemented with 2mg/L BAP showed good regeneration potential. Maximum multiple shoots were obtained from calli grown on MS media fortified with 1 mg/L BAP and 0.1 mg/L NAA. The micro shoots were excised and efficient rooting was induced in MS media supplemented with 0.2 mg/L IBA. The *in vitro* propagated plantlets were found to be high in their withaferin A content (3.21mg/g DW). The withaferin A content was around 2.12-fold higher than the control explants. Micropropagation of such a highly valuable medicinal plant is extremely necessary in meeting the growing demands of this therapeutically valuable medicinal plant.

Key words- *Withania somnifera*, callus, regeneration, micropropagation, withaferin A, HPLC

I. INTRODUCTION

W.somnifera belonging to the family Solanaceae is a medicinal plant which is a rich reservoir of pharmaceutically important triterpenoids. It is one of the most endorsed genera of medicinal plants in the Indian Ayurvedic system. Ashwagandha is also an important herb in Unani and Chinese medicinal system and is used to enhance overall strength, vitality and health. The steroidal lactones of this plant are a remedy for impotency, bone weakness, chronic fatigue, dehydration, emaciation, muscle tension and help in managing stress and depression. In Ayurveda, berries and tender leaves are used for external application of tumors, carbuncles and ulcers [1]. As a result of this continuous exploitation due to its immense medicinal properties, *W.somnifera* has become extinct from its natural habitat and is now listed one among the endangered species [2,3]. In spite of its commercial value, there is a limitation in development of improved varieties of this plant. For genetic enrichment and improved conservation of different genotypes, it is utmost important to evaluate and conserve the existing variants. Conventional propagation methods are time consuming, laborious and yields very less produce due to the slow growth rate and poor germination potential of seeds [4]. *In vitro* tissue culture is a profound alternative to traditional farming and could help in achieving increased biomass. Biotechnological interventions in plant tissue culture helps in providing improved agronomic yield with the use of different combinations and concentrations of phytohormones [5]. By means of tissue culture, soma clonal variants can be introduced by callus cultures, thus aiding in achieving improved yields. This kind of strategy involving media manipulation using phytohormones is a promising approach where we have achieved an increased biomass and higher withaferin A content.

II. MATERIALS AND METHODS

Plant material:
The seeds of *W.somnifera* were obtained from CIMAP, Hyderabad. All the chemicals used in performing the experiments were purchased from different sources – MS media (Hi media), plant growth regulators (Duchefa, Netherlands), chemicals for analysis (Hi media), HPLC grade solvents (Hi media).

Surface sterilization and seed germination:
The seeds were manually cleaned of contaminating physical impurities and then washed under running water with teepol (4%) for 10 min. The washed seeds were then disinfected with 0.1% v/v mercuric chloride for 3 min, followed by several

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rinses with distilled water. The sterilized seeds were inoculated on MS media fortified with different concentrations of $GA_{3.}$ The *in vitro* germinated plantlets were used for all our studies.

Establishment of *in vitro* cultures:
Different parts of *in vitro* germinated plantlet, viz., leaf, petiole, stem, root was excised and cultured on MS media supplemented with different concentrations and combinations of phytohormones. The plant growth media was supplemented with BAP, NAA, 2,4 D, IBA and Kn alone or in combination in the range (0.2-3mg/L) to study callus mediated regeneration. In all the media combinations, the pH of the media was maintained at 5.88.

Culture conditions:
The plant cultures were studied by incubating under standard culture conditions at 25±2°C with 16/8h light/dark regime and 40-50 mol $^{-2}s^{-1}$ light. The relative humidity was maintained at 55-60%. The explants were maintained by periodic sub culturing every 21-28 days.

Callus Induction:
Circular disc of leaf (3cm), root (1cm) and petiole (1cm) were transferred to MS media fortified with different concentrations of auxins (2,4 D, NAA) and cytokinins (BAP) to study callus induction. The callus obtained was transferred to MS media fortified with different concentrations of auxins and cytokinins to study shoot initiation.

Indirect Organogenesis:
The compact green mass of calli obtained was studied for shoot induction and root initiation by transferring them to MS media supplemented with different combinations of cytokinins (BAP, Kn) and auxins (NAA, IBA).

Phytochemical analysis:
The quantitation of withaferin A content from different explants of *in vitro* regenerated plantlets was carried out according to the protocol described by Sivanandhan et al., 2012a [6]. The explants were air dried to obtain constant weight by getting rid of excess moisture. One gram of each of the explants were completely crushed in 30ml HPLC grade methanol and were evaporated completely to concentrate the sample. This procedure of extraction was repeated thrice and the final sample was dissolved in 1ml of HPLC grade methanol. The sample was filtered using 0.22µm filter and used for analysis. Quantitative analysis for withaferin A in all the samples were carried out using Shimadzu HPLC and LCAD system (Japan) using C18 column at 230nm. The analysis was done using isocratic elution with methanol: water (65:35) as the mobile phase, with a flowrate of 2ml/min. Pure withaferin A from Natural Laboratories, Bengaluru was used for the comparative study.

Statistical analysis:
Each experiment was repeated thrice and the triplicate data were expressed as mean± standard deviations. The data were compared by least significant difference ($P \leq 0.05$) test using Statistical Analysis System (SAS, ver. 9.1)

III. RESULTS

In vitro seed germination:
W. somnifera seeds germinated with 82% germination frequency (Table 1) in MS media supplemented with 0.5% GA_3. The germinated seedlings were found healthy and were successfully propagated *in vitro* in different combinations of plant growth media used.

Indirect regeneration:
Establishment of in vitro callus cultures:
Among all the explants tested for regeneration, the leaf explant yielded the yielded best results in minimum number of days. Irrespective of the type of the explant used, callus induction was observed after a period of 4-5weeks. Upon 2-3 weeks after transfer to callus inducing media, emergence of cell aggregates was observed (fig a-e). Maximum induction of friable calli was observed in MS media fortified with 1mg/L 2,4D and 0.5mg/L Kn, whereas compact mass of green calli were observed in basal media that was fortified with 2mg/L BAP (Table 2).

Effect of phytohormones in combination (cytokinin + auxin):
Regeneration was studied both from friable and compact green calli. Upon employing various concentrations and combinations of BAP and NAA, it was observed that friable calli initiated maximum shooting when MS basal media was supplemented with 2 mg/l BAP and 0.5mg/l NAA, where as a much lesser concentration of the phytohormone (1 mg/l BAP and 0.1mg/l NAA) was found sufficient in inducing maximum shoot growth from compact green calli.

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Rooting:
Rooting was observed from the *in vitro* propagated plantlets in MS basal media. Though basal media was sufficient in inducing rooting, increased frequency was achieved in MS media fortified with 0.2 mg/L IBA.

Withaferin A quantification:
Withaferin A content was quantified from *in vitro* propagated callus cultures and multiple shoots. The withaferin A content in callus cultures was found to be very low when compared to the differentiated tissues. The secondary metabolite content from the shoot samples was 3.21mg/g DW which was around 7.8-fold higher than that obtained from callus and 2.12-fold higher than the control field grown explants. (fig.2)

IV. DISCUSSION

Nature is a boundless source of medicinal plants that are been constantly exploited for therapeutic purposes. Because of over exploitation and poor agricultural produce, *in vitro* propagation is a promising approach to meet the increasing demands of such commercially valuable medicinal plants. In order to produce increased yield, we optimized an efficient *in vitro* propagation protocol using which numerous clones could be produced from a single explant.

W.somnifera has been predominantly propagated on MS medium [7] though other media like B5 [8], SH [9] and NN [10] were also tried by different researchers. Among the various combination of nutrient media and different concentrations of phytohormones used, the maximum frequency of seed germination was achieved using GA_3 at 0.5mg/L. Similar results were obtained by Khanna et al., 2013 [11]. Sucrose concentration in all our studies was maintained at 3% based on reports from previous researchers [11, 12]. Increasing the sucrose concentration from 4-10% has decreased the shoot number and size. Moreover, use of other carbon sources like fructose or glucose too had a negative effect in shoot proliferation [13]. Among the different explants like leaf, petiole, node, roots employed in the study, the leaf explants responded best to regeneration emphasizing the importance of actively dividing meristematic tissue in in vitro propagation. Leaves had the ability to give rise to whole new shoot in 3-6 weeks' time. It has been reported that very young or too old leaves of *W.somnifera* fail to regenerate due to development stage specific auxin requirement [14]. In concurrence with our report, the regeneration potential of petiole explants was also reported to be far less than the leaf explants by Ghimire et al., 2010 [15] and Rout et al., 2011[16]. This distinctive response of different explants is determined by their physiological status and the combination of different phytohormones used in vitro, that is said to govern the cellular behaviour of the in vitro germinated plantlets [17].

The regeneration frequency depended largely on the phytohormones used. Cell differentiation from calli and organogenesis have been found to be regulated by the coordinated action of auxins and cytokinins. Callus mediated indirect regeneration was generally practised using various combinations of the phytohormone 2,4 D and Kn [18,19]. We have achieved successful callus induction using minimal phytohormone concentration (2mg/L BAP for compact calli and 1mg/L BAP,0.1mg/L NAA for friable calli).

Recent reports have indicated that the cytokinin phytohormone in the range of 0.5- 3mg/L has been shown to yield increased multiple shoot formation alone or in combination with the auxin IAA/IBA (0.2 to 3mg/L) [20]. MS media with BAP (0.5-5mg/L) was successfully used for shoot proliferation by many researchers. The potency of BAP in inducing shoot multiplication was also reported in *Alpinia galangal* [22], in turmeric [23], in *Chlorophytum* [24]. When BAP concentration was increased to more than 3mg/L, there was a drastic reduction in the number of shoots produced. This reduction in frequency of shoot initiation with increasing cytokinin concentrations could be attributed to the effect of hyperhydricity. Our research reports a much higher number of shoots (33.04±0.1) than reported by Ray et al., 2000 [13]. The combination of auxin and cytokinin yielded increased number of shoots and higher number of shoots in comparison to cytokinin alone. This might be attributed to their synergistic effect. Though combinations of BAP with Kn [25] and BAP with IBA [26] were used earlier, BAP along with IBA yielded maximum multiple shoot generation *in vitro*. Although rooting in *W. somnifera* was obtained in MS basal media, fortification with IBA enhanced the rooting frequency and number. In support to our findings, several researchers have also reported the positive role of IBA in inducing rooting [15,27]. Researchers also used IAA in combination with IBA for increasing rooting frequency [21, 28, 29]. In our research we achieved maximum rooting in MS media supplemented with minimal auxin concentration (18.32±0.23).

In vitro culture systems can be efficiently used to produce increased quantities of valuable secondary metabolites. This increased accumulation of secondary metabolite content might be attributed to the higher rate of metabolism and nutrient supply in *in vitro* cultures. We achieved highest yield of Withaferin A without elicitation from leaves which is about 2.12-fold higher than the field explants. This increased accumulation of secondary metabolites and biomass in *in vitro* grown plantlets might be attributed to the effect of BAP in inducing shoot proliferation as Withaferin A is predominantly found in leaves.

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V. CONCLUSIONS

In vitro propagation of therapeutically valuable medicinal plants is utmost necessary to meet the growing demand and supply in the market. From our research, we developed an efficient *in vitro* propagation system for large scale production of *W.somnifera* yielding increased withaferin A content. Our *in vitro* regenerated plantlets had increased withaferin A content when compared to the control explants, which could be commercially exploited for therapeutic purpose. Our micropropagation protocol optimized for *W.somnifera* can be advantageous in producing increased biomass in a comparatively lesser time period.

Table 1: Effect of GA_3 in influencing seed germination

S.No	GA_3 concentration in percentage	Number of seeds germinated
1	0	1.23±0.02
2	0.25	1.87±0.12
3	0.5	8.42±0.06
4	0.75	8.12±0.03
5	1	6.65±0.03

Table 2: Effect of phytohormones in combination:

Phytohormone concentration in mg/L		Number of shoots initiated from friable calli	Number of shoots initiated from compact green calli
BAP	NAA		
0.2	0.1	1.10±0.83	3.12±0.89
	0.2	1.32±0.04	4.76±0.77
	0.5	1.56±0.32	6.12±0.43
	1.0	1.55±0.43	7.02±0.51
0.5	0.1	2.34±0.21	14.11±0.66
	0.2	3.43±0.43	16.12±0.98
	0.5	4.21±0.67	16.34±0.87
	1.0	4.13±0.11	20.89±0.44
1.0	0.1	4.89±0.64	23.04±0.01
	0.2	5.76±0.11	21.89±0.86
	0.5	5.98±0.74	19.32±0.54
	1.0	6.64±0.57	19.01±0.96
2.0	0.1	8.67±0.48	17.88±0.41
	0.2	10.12±0.11	17.14±0.55
	0.3	10.09±0.28	16.87±0.52
	0.5	13.12±0.34	16.03±0.68
	1.0	12.11±0.13	14.22±0.15
3.0	0.1	8.98±0.62	11.24±0.53
	0.2	8.43±0.11	11.23±0.49
	0.5	7.78±0.24	10.16±0.32
	1.0	7.54±0.13	9.77±0.14

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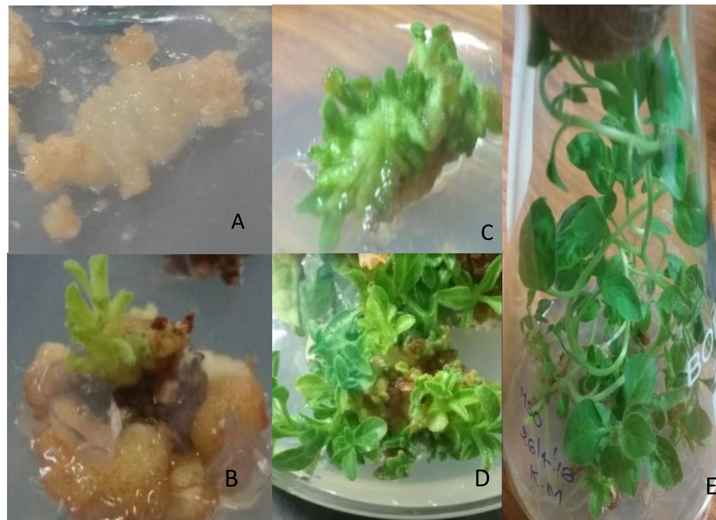

Fig. 1. *W.somnifera – In vitro* regeneration
A : friable callus on 1mg/L BAP and 0.1 mg/L NAA
B : Regeneration from friable callus
C : Compact green callus from 2mg/L BAP media, showing initiation of shoots
D : Regenerated shoots from compact callus
E : *In vitro* regenerated shoots growing on MS basal media

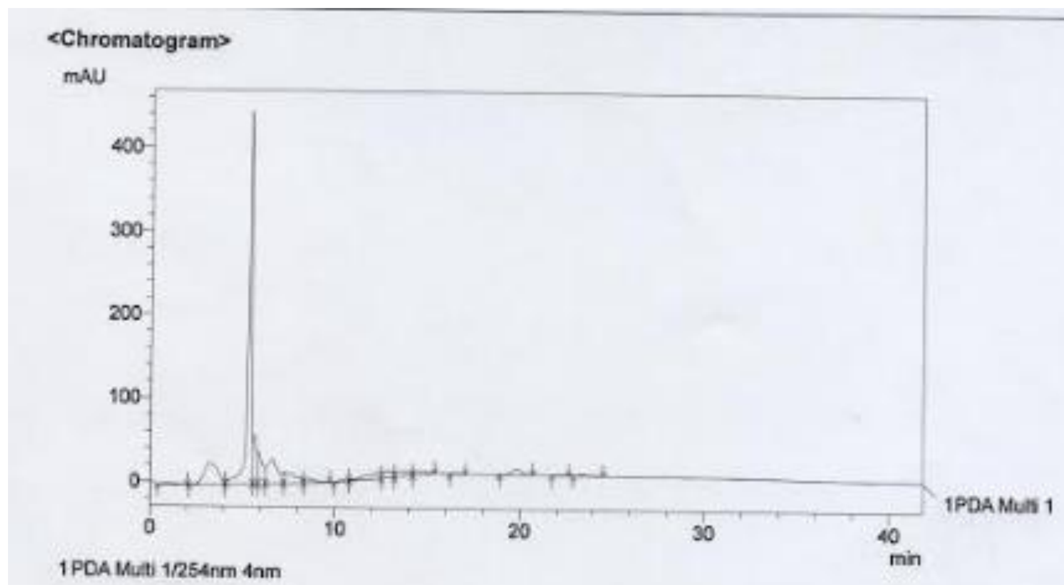

Fig.2. HPLC chromatogram showing withaferin A from invitro propagated *W.somnifera*

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