INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-7, Issue-2, April-June-2016 Coden IJABFP-CAS-USA

Received: 7th Feb 2016 *Revised: 27th Feb-2016* *Accepted: 29th Feb 2016*

Research article

MASS PROPAGATION AND GC-MS ANALYSIS OF CRITICALLY ENDANGERED PLANT *WITHANIA COAGULANS*

Nishesh Sharma[1], Indra Rautela[2] and Manish Dev Sharma[3]*

[1]Department of Biotechnology, Chinmaya Degree College, Haridwar, Uttarakhand. [2]Department of Cellular and Tissue Engineering, SHIATS, Allahabad, [3]Department of Life Sciences, Shri Guru Ram Rai Institute of Technology and Science (SGRRITS), Dehradun.

ABSTRACT: *Withania coagulans* is an important medicinal plant possessing several biological activities and has been immensely utilized in traditional as well as modern system of medicine. Due to various reasons (over utilization, poor germination and survival rate, habitat destruction) the plant has become critically endangered. The present study was conducted to develop an efficient protocol effective for mass propagation and conservation of the species. Nodal segments obtained from young plant were cultured *in vitro* onto MS medium fortified with different plant growth regulators such as 2,4-D, Kn and BAP either alone or in combination. Shoot bud regeneration was achieved onto all the three hormones analyzed, Kn was found to be most suitable for multiple shoot bud induction from cultured nodal segment, with a maximum of 15 shoots and an average of 11.0±0.4 shoots/explant onto MS+10µM Kn. All the cultures exhibited regeneration of shoots onto this concentration of Kn. Beside this MS +2,4-D+BAP was also found significantly effective for multiple shooting.½ MS+10µM IAA media composition was significantly effective for development of *in vitro* roots. About 78.4% of regenerated plants survived during acclimatization and transplantation. GC-MS analysis of methanolic extract of leaves of mother as well as micropropagated plant revealed presence of several biologically important metabolites which account for the medicinal potential of the plant.

Key words: *Withania coagulans*, conservation, *in vitro* study

*Corresponding author: Manish Dev Sharma, Department of Life Sciences, Shri Guru Ram Rai Institute of Technology and Science, Patel Nagar, Dehradun, India E-mail: sharma.manishdev@gmail.com

INTRODUCTION

Withania coagulans is an important medicinal plant better known as Indian rennet or Doda paneer since the plant harbours an enzyme responsible for formation of cheese from milk. Plant is found to be naturally distributed in drier regions of Iran, Afghanistan, Pakistan, India and Nepal (Jain *et al.*, 2009; Valizadeh and Valizadeh, 2009). Like all other medicinal plants *W. coagulans* also possesses several biological and pharmaceutical properties such as anti-inflammatory, anticancer, hepatoprotective, anti-hyperglycemic, cardiovascular, immunoregulatory, free radical scavenging CNS depressant activities, antibacterial and antifungal activities. The plant finds its application in treatment of tooth ache, intestinal disorders, nervous exhaustion, diabetes, disability, insomnia, impotence, dyspepsia and also acts as blood purifier (Jaiswal *et al.*,2009; Mathur *et al.*,2011; Pezeshki *et al.*,2011; Khodaei *et al.*, 2012). *W. coagulans* is generally propagated through seeds but seeds of *W. coagulans* are known to bear extremely poor rate of germination alongwith an equally low survival rate of germinated seeds. There has been an ever increasing demand of the herb for traditional as well as medicinal purposes. Unrestricted collection from wild stand to meet these demands and immensely poor rate of propagation in nature alongwith habitat destruction has eventually resulted in present endangered state of the plant. The plant has rapidly vanished from wild stands of India. In an earlier tissue culture study conducted by Jain *et al.*,(2009), they could spot only two plants in whole Ajmer district of Rajasthan (India).

Hence, immediate concern is required towards conservation and mass propagation of the plant. In recent past plant tissue culture technique has been established as an extremely successful and effective technique for conservation of rare and endangered plants. There are very few reports (Jain *et al* 2009; Valizadeh and Valizadeh 2009, 2011, Sharma *et al* 2015) available pertaining to *invitro* propagation of *W. coagulans*, hence the present study was conducted with an objective to standardize a simple and rapid propagation protocol for mass propagation and conservation of *W. coagulans*.

MATERIAL AND METHODS

Selection and sterilization of explant

Nodal segments obtained from young plant of *W. coagulans* (Fig.2A) maintained maintained in Department of Biotechnology, Chinmaya Degree College, Haridwar were utilized as explants in the present study. Nodal segments were surface sterilized by initially washing with tapwater and tween 20 (Detergent) to remove dust particles and oily impurities. Explants were then treated with fungicide Bavastin followed by rinsing the explants with 70% ethanol for not more than 60 seconds. Explants were then surface sterilized with 0.1% $HgCl_2$ for 3-4 minutes under aseptic conditions. After every treatment explants were washed with sterile distilled water. Explants were finally dried using sterile filter paper, excised and inoculated.

Establishment of cultures

MS (Muashigue T and Skoog K, 1962) medium containing different concentrations (2-12µM) of 2,4-D, Kn, BAP either alone or in combination were utilized for induction and proliferation of shootbuds from cultured nodal segments. In seperate and independent experiments different concentrations (2-12µM) of each of these hormones were utilized to find out optimum concentration of each PGR for multiple shoot induction from cultured nodal segments.Each experiment comprise of a mininu of 20 cultures and each experiment of repeated atleast twice. Cultures were incubated at 25±2^{o}C under 18 hours photoperiod of 15µE/m^{2}/s irradiance in the culture room.

Invitro rooting

Well developed and elongated shoots were transferred to freshly prepared rooting medium. Full as well as ½ strength MS medium fortified with varying concentration of IAA (2-20µM) were utilized as rooting medium.

Acclimatization

Completely developed plants were acclimatized and transplanted to natural soil. Plants were aseptically taken out from culture tubes and all the media attached to the roots was removed by washing with sterile water. Plants were then transferred to plastic pots containing sterile soil and sand (1:1). Pots were covered with transparent polybags and kept in a plastic chamber. Pots were irrigated (as per requirement) with dilute solution of major nutrients utilized in MS medium. Poly bags were cut from one end after new leaves started emerging followed by transfer of pots to green house where slowly polypags were removed after 20-25 days plants were transferred to natural soil.

Statistical analysis

All the cultures were regularly monitored for growth and development. Results were expressed as percent cultures response alongwith average and maximum number of shoota and roots obtained. Data recorded was subject to DMRT analysis for evaluation of statistical significance of each treatment.

GC-MS analysis

GC-MS analysis was performed using a regular Perkin Elmer Auto System XL GC-MS analyzer. For GC-MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51 ml/min and an injection volume of 2µl was employed. Software adopted to handle mass spectra and chromatograms were Turbo Mass. Identification of compounds was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC-MS was conducted using the database of NIST (National Institute Standard and Technology) having more than 62,000 patterns and Wiley library. The name, molecular weight and structure of the components of the test material were ascertained by correlating with the library. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas (Sharma *et al* 2015).

RESULTS AND DISCUSSION

Multiple shoot regeneration

Nodal segments of *W. coagulans* were found to be an effective explant with respect to *in vitro* culture studies as multiple shoot induction was achieved onto all media combinations utilized in the present study. Media supplemented with different concentrations (2-12 µM) of 2,4-D, Kn and BAP all resulted in regeneration of shoots. However, morphogenic response obtained onto Kn supplemented media was far better as compared to the response obtained on 2,4-D and BAP supplemented medium.

Onto lower concentration of 2,4-D (2-5 µM) about 90-94.2 % cultures exhibited shoot bud induction with a maximum of 3 shoots onto MS+5µM 2,4-D. However, when the concentration of 2,4-D was enhanced upto 10µM, percentage culture developing shoot buds was 65.5 with a maximum of 2 shoots per explants. Overall, *invitro* cultured nodal segments of *W. coagulans* exhibited relatively low organogenic response onto 2,4-D supplemented medium irrespective of concentration of 2,4-D in terms of % culture exhibiting shoot bud development, average and maximum number of shoots. Not only was the number of shoots obtained low, but regenerated shoots showed slow growth and did not elongated until excised and transferred to another medium (Fig.3A,Table 1). On contrary, when nodal segments were cultured onto medium enriched with 2-5 µM Kn about 64.2 to 78.6 % cultures exhibited shootbud regeneration within one week of inoculation (Fig. 4A-B). On all higher concentrations of Kn (8-12µM) 100% cultures exhibited regeneration of multiple shoots. A maximum of 15 shoots with an average of 11.0±0.4 shoots/explant were obtained onto MS+10µM Kn (Fig.4C). Induction of shoots was followed by elongation of shoots onto Kn supplemented medium. These shoots formed an excellent material for further subculture experiments to produce large number of plants and also for *invitro* induction of roots.

Multiple shoot induction was also achieved onto MS+BAP, when MS medium was fortified with 2-5µM BAP, about 48 to 56.4 % cultures exhibited shoot bud regeneration. On gradually increasing the concentration of BAP to 8,10 and 12µM shoot bud regeneration response was accordingly enhanced upto 75.5 ,82.0 and 92.4 respectively (Table 1). Among different concentration of BAP analyzed, a maximum of 8 shoots alonwith 5.6±1.2 average number of shoots/explant was obtained on MS+12µM BAP (Fig.5A-C). In separate and independent experiments conducted to evaluate the response of nodal segments containing different hormonal combinations, MS media supplemented with 2,4-D and BAP was found to be most suitable media composition for induction and proliferation of multiple shoots from cultured nodal segments. According to the observations made medium supplemented with high concentration of 2,4-D and low BAP concentration, callus formation was the predominant morphogeneic response obtained. Callus obtained was green and fragile but didnot exhibited regeneration of shoots and eventually dried out on prolonged culture untill excised and subcultured onto fresh medium. However, media fortified with higher concentration of BAP (8-10µM) with different concentrations of 2,4-D (lower or higher) resulted in multiple shoot regeneration (Table 2) from cultured nodal segments. MS+5µM 2,4-D+10µM BAP (Fig. 6A-B); MS+10µM 2,4-D+10µM BAP (Fig. 6C-D) were among the most productive media compositions. A maximum of 12 shoots/explant with an average of 8.8±0.6 shoots/explants were obtained onto MS+10µM 2,4-D+10µM BAP. About 88.4% cultures developed shoots onto this media composition. In earlier studies conducted by Rohit *et al* (2009) and Valizadeh and Valizadeh (2011) also media enriched with combination of hormones was reported to be significantly effective for multiple shoot induction. Valizadeh and Valizadeh (2011) obtained 7.2±1.0 shoots/explant onto MS+2mg/l BA+0.5mg/l IBA. Study conducted by Rohit *et al* (2009) revealed MS+Kn+BA to be effective enough for multiple shoot regeneration from nodal segments. Effectiveness of these hormones has also been reported in a more commonly known species of Withania i.e, *Withania somnifera*. Siddique *et al* 2004 obtained callus from nodal segments of *W. somnifera* onto MS supplemented with either 2.4-D, BAP or Kn. In their independent studies Shukla *et al* 2010 and Kumar *et al* 2013 achieved multiple shoot formation onto medium supplemented with Kn, BAP respectively.

Invitro rooting

MS media supplemented with IAA was found to be suitable rooting medium as *invitro* root induction was achieved on all concentrations of IAA. In the present study *invitro* induction of roots was achieved onto full as well as half strength MS medium. However, rooting response in terms of % culture exhibiting root development, average number of shoots per explants and maximum number of shoots per media composition was greatly enhanced when the strength of medium was reduced to ½. Valizadeh and Valizadeh (2011) also supported ½ MS to be effective for *invitro* rooting from regenerated shoots of *W. coagulans*. However, contrary to results obtained in the present study they found IBA to be better root inducing PGR as compared to IAA. Work carried out by Jain etal 2009 have also supported IBA to be effective enoughfor invitrorooting of regenerated plants. A maximum of 62.6% cultures responded to rooting media with an average of 6.8±0.8 roots/explant onto full strength MS medium containing 20 µM IAA (Fig.7A, Table 3). When the strength of medium was reduced to ½ with same concentration of IAA about 88.6% cultures exhibited rooting, with an average of 12.0±0.6 roots/explants. A maximum of 14 roots with a root length of 8.0 cm was attained onto this medium combination.

Acclimatization

Well rooted plants were acclimatized and finally transplanted to natural soil. About 74.6% plants survived during transplantation (Fig.8A). The transplanted plants exhibited normal growth and development (Fig.8B-D). Valizadeh and Valizadeh (2011) also reported about 75% survival rate of tissue culture raised plants.

GC-MS Analysis

As aresult of GC-MS chromatogram obtained from methanolic extract of leaves of mother and tissue cultured plant of W. Coagulans about 44 and 56 phytochemical compounds were found to be present in leaves of mother plant and *invitro* regenerated plant respectively (Table 4,5 ; Fig. 9A-B).

Phytochemical compounds such as Hexadecanoic acid, 2-hydroxy-1,3-propanediyl; Octadecanoic acid; Benzoic acid, 4-ethoxy-ethyl ester; Phenol 2,4-Bis (1,1-Dimethyl ethyl); methyl ester 1-Pentadecanamine, N,N-dimethyl; Tetradecanoic acid and Cholesterol were found to be present in leaves of mother as well as micropropagated plant. On the other hand presence of compounds such as 2-dodecenal; Lycopene Decanoic acid and Octanoic acid was confined only to leaves of mother plant and compounds like Naphthalene; Betulin; Stigmasterol and Methyl tetra decanoate were found to be present only in tissue culture raised plants in th epresent study. Major compounds identified in mother plant were1-Penta decanamine, N,N-dimethyl; Fucosterol; Betulin; Palmitic acid, Methyl ester; Phthalic acid; butyltridecyl ester; 9-octadecenoic acid methyl ester (E); Hexadecanoic acid, 2-hydroxy-1-(Hydroxymethyl) ethyl ester; Stigmasterol; Benzyl Benzoate Anthracane and Tetradecanoic acid. Major compounds found to be present in leaves of invitro cultured plant were; Stigmasta-5, 23-Dien-3-ol (3 Beta); Cholesterol; Hexadecanoic Acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester; Lycopene; Tetradecanoic acid; 1-Pentadecanamine, N,N-dimethyl; Benzoic acid, 4-ethoxy-ethyl ester; octanoic acid; gamma-Tocopherol; and 9,12 octadecadienoic acid (Z,Z). Most of these compounds have been associated with several biological or pharmaceutical properties which are responsible for medicinal potential of the plant. Lakshmi *et al* 2011,Konovalova *et al* 2013 have reported anticancous property of tetradecanoic acid. Octadecanoic acid possesses anti inflammatory, hepatoprotective, hypercholesterolemic, anticancer and many other properties (Gunstone *et al* 2007, Gunesekaran *et al* 2013). Naphthalene is well known for its antimicrobial activity (Rokkade and Sayeed 2009: Sethi et al 2013). Similarly, alomost all the compounds identified exhibits some or the other medicinal value.

Table 1: Response of nodal segments of *W. coagulans* to different plant growth regulators

Concentration (µM)	2,4-D		Kn		BAP	
	Avg. no. of shoots	Max. no. of shoots	Avg. no. of shoots	Max. no. of shoots	Avg. no. of shoots	Max. no. of shoots
2	1.7±0.2 [a]	3	4.2±0.8 [e]	06	3.3±0.2 [de]	5
5	1.8±0.4 [a]	3	6.1±0.4 [d]	08	3.1±0.4 [de]	4
8	1.0±0.0 [b]	2	10.9±0.2 [bc]	12	4.6±0.2 [b]	6
10	1.0±0.0 [b]	2	11.0±0.4 [a]	15	4.0±0.6 [bc]	6
12	1.4±0.6 [ab]	2	11.2±0.6 [a]	13	5.6±1.2 [a]	8

Values are mean of seven replicates. Mean values followed by same letters are not significantly different at p≥0.05 DMRT.

Table 2: Effect of 2,4-D and BAP onto nodal segment culture of *W. coagulans*.

Hormone combination		% culture developing multiple shoots	Average No. of shoots	Maximum No. of shoots
2,4-D (µM)	BAP (µM)			
2	**8**	68.4	4.2±0.2 [f]	6
2	**10**	75.3	6.6±0.8 [c]	9
5	**8**	72.4	3.8±0.4 [g]	6
5	**10**	80.8	6.4±1.2 [c]	10
8	**8**	73.6	5.4±0.1 [d]	8
8	**10**	76.0	5.1±0.4 [de]	7
10	**8**	80.2	7.1±0.2 [b]	8
10	**10**	88.4	8.8±0.6 [a]	12

Values are mean of seven replicates. Mean values followed by same letters are not significantly different at p≥0.05 DMRT.

Table 3: *In vitro* rooting of regenerated shoots of *W. coagulans*

Media	% culture developing roots	Average Number of roots	Maximum root length (cm)	Maximum Number of shoots
MS+ 5 µM IAA	44.4	4.2±0.4 [e]	2.8	8
MS+ 10 µM IAA	56.2	6.4±0.5 [cd]	4.2	6
MS+ 20 µM IAA	62.6	6.8±0.8 [c]	4.6	6
½ MS+ 5µM IAA	76.4	10.4±0.4 [ab]	8.4	10
½MS+10µM IAA	82.2	12.2±0.2 [a]	8.2	11
½MS+20µM IAA	88.6	12.0±0.6 [a]	8.0	09

Values are mean of seven replicates. Mean values followed by same letters are not significantly different at p≥0.05 DMRT.

Table 4: Phytochemical compounds identified in methanolic extract of leaves of wild *W. coagulans*

Peak	R. Time	Area%	Name	Mol. formula	Mol. weight
1	5.247	0.72	Naphthalene	$C_{10}H_8$	128
2	9.415	2.20	2,4- Ditert butyl phenol	$C_{14}H_{22}O$	206
3	9.591	2.26	p-Carbethoxy phenol	$C_9H_{10}O_3$	166
4	10.268	1.33	1-Pentadecene	$C_{15}H_{30}$	210
5	10.754	0.84	1-Nitro- Naphthalene	$C_{10}H_7NO_2$	173
6	11.224	1.25	8-Pentadecanone	$C_{15}H_{30}O$	226
7	11.550	0.48	Dimethyl heptylamine	$C_9H_{21}N$	143
8	11.787	0.70	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242
9	12.298	1.25	Tetradecanoic acid	$C_{14}H_{12}O_2$	228
10	12.461	0.93	Benzyl Benzoate	$C_{14}H_{12}O_2$	212
11	12.761	0.62	Phenanthrene	$C_{14}H_{10}$	178
12	13.014	1.25	Neo Phytadiene	$C_{20}H_{38}$	278
13	13.271	0.27			
14	13.392	0.53	8-Octadecanone	$C_8H_{36}O$	268
15	13.469	1.17	1,7 pentatriacontene	$C_{13}H_{64}O$	452
16	13.676	20.31	Cetyldimethylamine	$C_{18}H_{39}N$	269
17	13.897	8.15	Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270
18	14.166	0.65	2-(4-tert butyl benzyl) butanol	$C_{15}H_{22}O$	218
19	14.373	3.12	Butyl octyl phthalate	$C_{20}H_{30}O_4$	334
20	14.605	0.31	9(10H)-Anthracenone	$C_{14}H_{10}O$	194
21	15.597	4.94	9-Octadecenoic acid, methyl ester	$C_{19}H_{38}O_2$	296
22	15.733	2.46	Mentha camphor	$C_{10}H_{20}O$	156
23	15.813	3.20	Octadecanoic acid, methyl ester	$C_{19}H_{36}O_2$	298
24	16.150	0.58	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	$C_{31}H_{52}O$	440
25	16.330	0.52	Betulin	$C_{30}H_{50}O_2$	442
26	17.219	0.43	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C12H_{23}NO_2$	213
27	17.364	1.31	Glycerol 1,3 disterate	$C_{39}H_{76}O_5$	624
28	17.611	0.33	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326
29	17.977	0.29	Octadecanoic acid, 3-hydroxypropyl ester	$C_{21}H_{42}O_3$	342
30	19.008	0.32	Undecanal, 2-methyl-	$C_{12}H_{24}O$	184
31	19.129	0.64	Benzediex	$C_{10}H_{21}N$	155
32	19.292	0.47	9-octadecenal	$C_{18}H_{34}O$	266
33	19.361	0.29	2-methyl-Hexadecanal	$C_{19}H_{38}O$	282
34	19.540	0.96	Diethylene glycol dibenzoate	$C_{18}H_{18}O_5$	314
35	19.826	10.02	Glycerol 1- myristate	$C_{17}H_{34}O_4$	302
36	20.326	0.86	1,2-Benzenedicarboxylic acid	$C_{24}H_{38}O_4$	390
37	22.918	2.21	Methyl hexadecadienoate	$C_{17}H_{32}O_2$	266
38	23.104	3.41	Glycerine 1,3 diesterate	$C_{39}H_{76}O_5$	624
39	28.763	0.69	.alpha.-Tocopherol-.beta.-D-mannoside	$C_{35}H_{60}O_7$	592
40	30.799	2.58	Cholesterol	$C_{27}H_{46}O$	386
41	31.494	2.83	Stigmasterol	$C_{29}H_{48}O$	412
42	32.885	2.61	Stigmast-5-EN-3-OL, (3.BETA.)-	$C_{29}H_{50}O$	414
43	33.354	1.61	Fucosterol	$C_{29}H_{48}O$	412
44	4.986	2.10	Anthracene, 1,4,4A,9,9A,10 Hexahydro	$C_{19}H_{26}$	254

Table 5: Phytochemical compounds identified in methanolic extract of *in vitro* regenerated leaves of *W. coagulans*

Peak	R. Time	Area%	Name of compound	Mol. formula	Mol. mass
1	4.899	0.73	Octanoic Acid	$C_8H_{16}O_2$	144
2	5.148	0.91	3,4-dimethyl-2-cyclohexen-1-one	$C_8H_{12}O$	124
3	6.089	0.12	2-Dodecenal	$C_{12}H_{22}O$	182
4	6.250	0.11	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	198
5	6.417	0.12	Phenol, 4-ethyl-2-methoxy-	$C_9H_{12}O_2$	152
6	6.544	0.32	2,4-Decadienal, (E,E)-	$C_{10}H_{16}O$	152
7	6.865	0.30	2,4-Decadienal, (E,E)-	$C_{10}H_{16}O$	152
8	7.467	0.08	Benzeneacetaldehyde, .alpha.,2,5-trimethyl-	$C_{11}H_{14}O$	162
9	7.527	0.17	Decanoic acid	$C_{10}H_{20}O_2$	172
10	8.971	0.20	2-(1,1,3,3-Tetramethylbutyl)-1,4benzenediol	$C_{14}H_{22}O_2$	222
11	9.074	0.19	Hexadecane, 1-iodo-	$C_{16}H_{33}I$	352
12	9.204	0.29	Benzene, 1,3-bis(1-formylethyl)-	$C_{12}H_{14}O_2$	190
13	9.421	1.32	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	$C_{14}H_{22}O$	206
14	9.594	2.84	Benzoic acid, 4-ethoxy-, ethyl ester	$C_{11}H_{14}O_3$	194
15	10.010	0.46	Dodecanoic acid	$C_{12}H_{24}O_2$	200
16	10.764	0.14	Caryophyllene oxide	$C_{15}H_{24}O$	220
17	10.907	0.15	1,1,4,7-tetramethyldecahydro-1h-cycloprop	$C_{15}H_{26}O$	222
18	11.172	0.44	benzeneacetic acid, 4-hydroxy-3-methoxy-	$C_9H_{10}O_4$	182
19	11.303	0.54	3-buten-2-ol, 4-(2,6,6-trimethyl-2-cyclohexen-	$C_{13}H_{22}O_3$	194
20	11.483	0.31	Cycloisolongifolen, 9,10-Dehydro-	$C_{15}H_{22}$	202
21	11.782	0.15	Cyclopropanebutanoic acid,	$C_{25}H_{42}O_2$	374
22	11.997	0.15	2,6-dimethyl-8-(tetrahydro-2h-pyran-2-yloxy	$C_{15}H_{26}O_3$	254
23	12.206	0.39	Nerolidol Z and E	$C_{15}H_{26}O_2$	238
24	12.289	1.87	Tetradecanoic acid	$C_{14}H_{28}O_2$	228
25	13.089	0.27	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268
26	13.415	0.31	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278
27	13.757	2.08	1-Pentadecanamine, N,N-dimethyl-	$C_{17}H_{37}N$	255
28	13.892	0.34	Hexadecanoic Acid, Methyl Ester	$C_{17}H_{34}O_2$	270
29	14.002	0.56	7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
30	14.167	0.10	Lanosterol	$C_{32}H_{52}O_4$	500
31	14.391	5.31	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
32	14.850	0.12	p-Benzoquinone, 2,5-di-tert-pentyl	$C_{15}H_{22}O_2$	234
33	15.551	1.37	9,12-Octadecadienoic acid (Z,Z)-,methyl ester	$C_{19}H_{34}O_2$	294
34	15.811	0.23	Octadecanoic Acid, Methyl Ester	$C_{35}H_{68}O_5$	298
35	16.160	48.46	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280
36	17.370	0.17	hexadecanoic acid, 2-hydroxy-1,3-propanedi	$C_{35}H_{68}O_5$	568
37	17.991	0.52	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324
38	19.278	0.72	trans-9-Octadecenoic acid, trimethylsilyl ester	$C_{21}H_{42}O_2Si$	354
39	19.594	0.56	Trimethylsilyl tetracosanoate	$C_{27}H_{56}O_2Si$	440
40	19.854	8.78	Hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330
41	22.362	1.16	Pregna-5,14-diene-3,20-diol-18-carboxylic acid, 3-acetate-, la	$C_{23}H_{30}O_2$	370
42	22.742	2.71	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{38}O_4$	354
43	23.142	5.77	Octadecanoic acid, 2-hydroxy-1-(	$C_{21}H_{42}O_4$	358
44	23.677	0.10	22,23-Dibromostigmasterol acetate	$C_{31}H_{50}Br_2O_2$	612
45	24.255	0.20	Lycopene	$C_{40}H_{56}$	536
46	24.633	0.11	2,8-Dimethyl-2-(4,8,12-Trimethyltridecyl)	$C_{18}H_{36}$	252
47	24.900	0.07	Delta –tocopherol	$C_{27}H_{46}O_2$	402
48	25.035	1.26	Pregn-9(11)-en-20-one, 3,6-dihydroxy-,	$C_{21}H_{32}O_3$	332
49	25.333	0.17	Cholesta-2,8-dien-6-ol, 14-methyl-, acetate,	$C_{30}H_{48}O_2$	440
50	27.393	0.35	Gamma.-Tocopherol	$C_{28}H_{48}O_2$	416
51	28.663	0.19	Cholesterol	$C_{27}H_{46}O$	386
52	30.666	0.62	Stigmasta-5,24(28)-dien-3-ol, (3.beta.)-	$C_{29}H_{48}O$	412
53	31.489	0.36	Stigmasta-5,23-Dien-3-Ol, (3.Beta.)-	$C_{29}H_{48}O$	412
54	32.884	1.18	Stigmast-5-EN-3-OL, (3.BETA.)-	$C_{29}H_{50}O$	414
55	34.993	2.82	Pregna-4,17(20)-dien-3-one, 20,21-[(methylborylene)bis(oxy)]	$C_{22}H_{31}BO_3$	354
56	36.677	0.73	9,19-Cyclolanostan-3-ol, 24-methylene	$C_{31}H_{52}O$	440

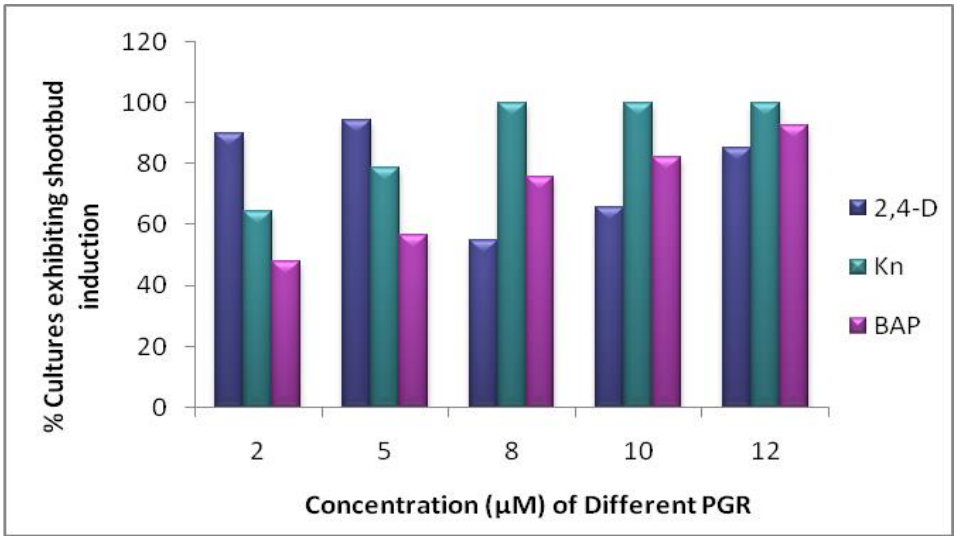

Fig 1. % Regeneration potential of nodal segments of *W. coagulans*

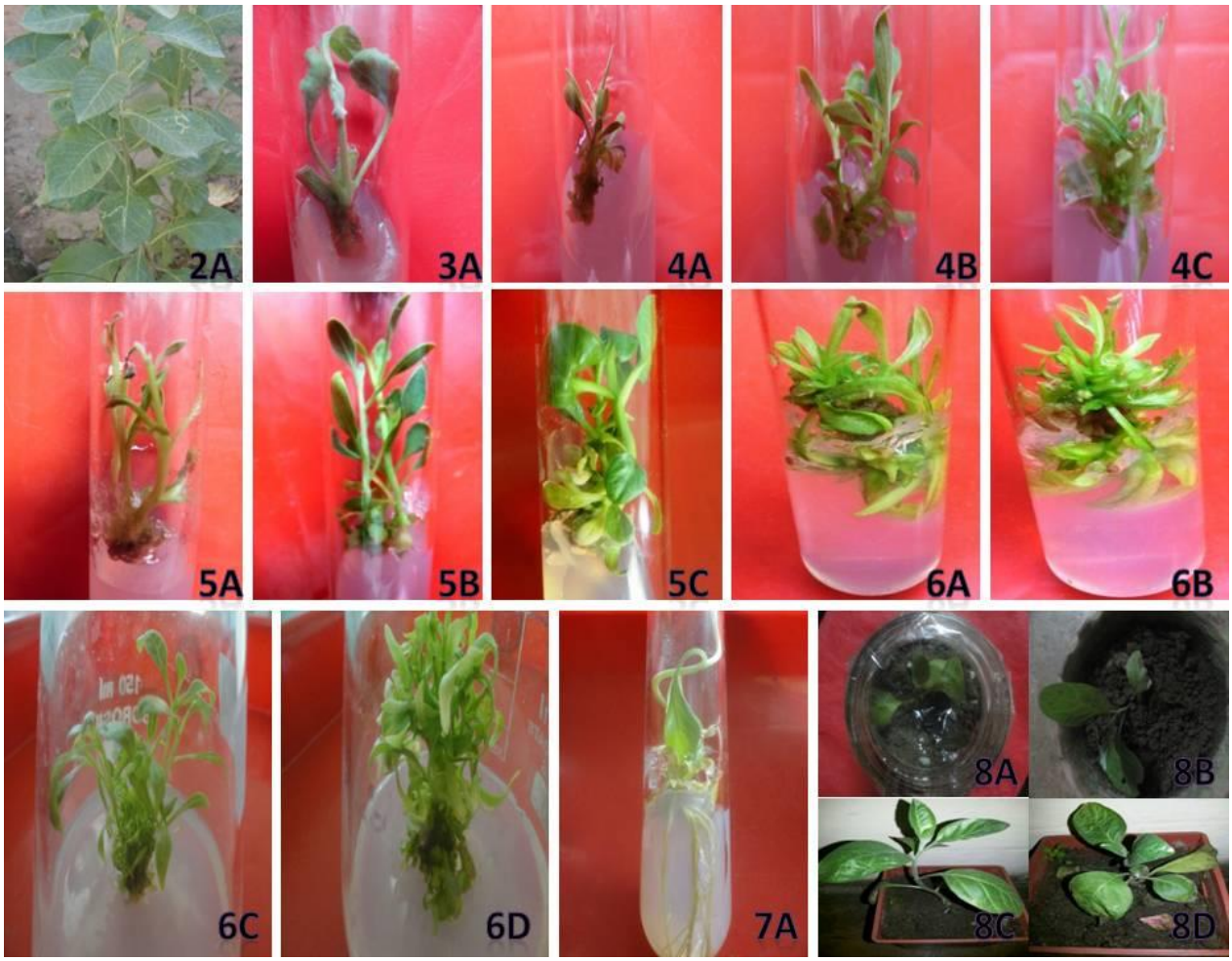

Fig. 2A- Mother Plant. Fig. 3A- Shoot regeneration onto MS+5µM 2,4-D. Fig. 4(A-B)- Shoot regeneration onto MS+5µM Kn. Fig. 4C- Shoot regeneration onto MS+5µM 10 Kn. Fig. 5(A-C)- Shoot regeneration onto MS+12µM BAP. Fig. 6(A-B)- Shoot regeneration onto MS+5µM 2,4-D+10µM BAP. Fig.6 (C-D) Shoot regeneration onto MS+10µM 2,4-D+10µM BAP. Fig. 7A- *In vitro* rooting onto MS+20µM IAA. Fig. 8A- Plant during transplantation. Fig. 8(B-D)- Transplanted plants.

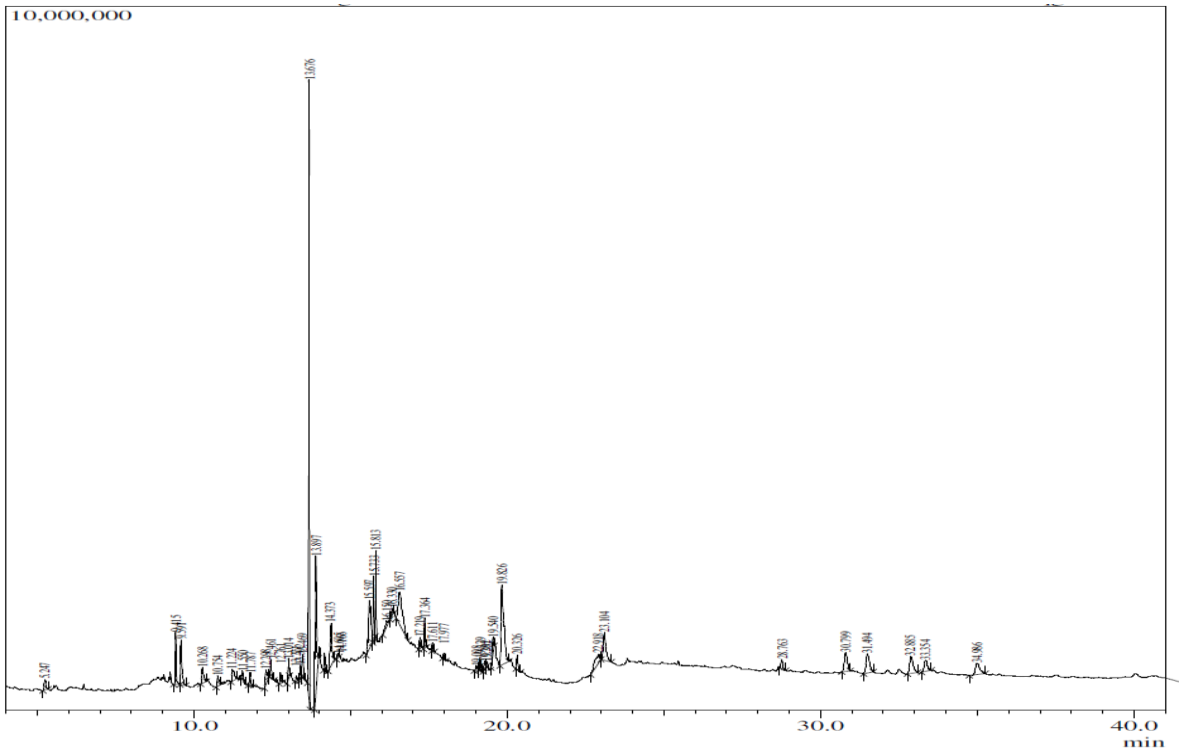

Fig 9A: GC-MS analysis of leaves of mother plant

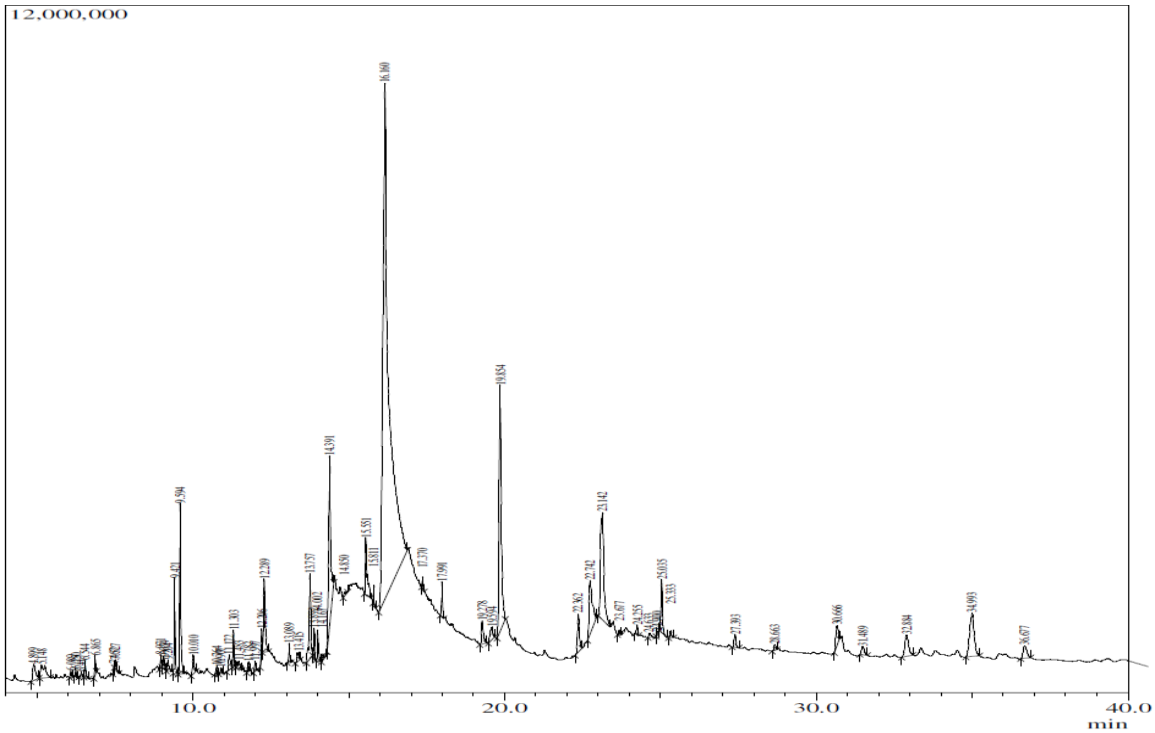

Fig. 9B: GC-MS analysis of leaves of tissue cultured plant

CONCLUSION

The present study reports a simple and effective protocol standardized for mass propagation of *W. Coagulans*. On an average more than 800 plants can be regenerated through four subsequent subcultures by following the protocol. Considering the present status of *W. coagulans* which happens to be critically endangered as the plant is rapidly vanishing from wild stands, the present report avails an efficient method not only for conservation but also for rehbilitation of the plant in natural habitat.Moreover, results obtained from GC-MS analysis provides an insight of tremendous medicinal potential of th eplant which still needs to be utilized on large scale.

REFERENCES

Gunasekaran, S, Vijay, T, Sarumathyd, K, Palanie, S, Panneerselvam, B. and Srinivasan, V. (2013). Phytoconstituents evaluation by GC-MS and therapeutic efficacy of *Grewiaum bellifera* on streptozotocin (STZ)-induced diabetic rats. International Journal of Pharmacy and Lifescience. 4:2380-2386.

Gunstone, F.D., John, L. Harwood, T. and Albert, J.D. (2007). The Lipid Handbook. 3rd Ed. Boca Raton: CRC Press.

Jain R, Arunima S, kachhwaha S and Kothari SL (2009). "Micropropagation of *Withania coagulans* (Stocks) Dunal: A Critically endangered Medicinal Herb". Biotechnology 18: 1271-1275. http://dx.doi.org/10.1007/bf03263330

Jain R, Kachhwaha S and Kothari SL (2012). Phytochemistry, Pharmacology and biotechnology of *Withania somnifera* and *Withania coagulans*: A review. Journal of Medicinal Plants Research. 6 : 5388-5399.

Jaiswal D, Rai PK and Watal G (2009).Anti diabetic effect of *Withania coagulans* in experimental rats.Int. Journal of clinical biochemistry. 24 : 88-93. http://dx.doi.org/10.1007/s12291-009-0015-0

Khodaei M, Jafari M and Noori M (2012). Remedial use of Withanolides from *Withania coagulans* (stocks) Dunal. Advancements in Life Science. 2: 6-19. http://dx.doi.org/10.5923/j.als.20120201.02.

Konovalova, O., Gergel, E. and Herhel, V. (2013). GC-MS Analysis of Bioactive Components of *Shepherdia argentea* (Pursh.) Nutt. from Ukrainian Flora. The Pharma Innovation Journal. 2:7-12.

Kumar OA, Jyothirmayee G, Tata SS (2013). *In vitro* conservation of *Withania somnifera* (L.) Dunal (Ashwagandha)- A multipurpose medicinal plant. Journal of Asian Science and Research. 3:852-861.

Lakshmi, T.T.V and Rajalakshmi, P. (2011). Identification of phytocomponents and its biological activities of *Aloe vera* through the gas chromatography-Mass spectrophotometry. International Research Journal of Pharmacy. 2:247-249.

Mathur D and Agarwal RC (2011). *Withania coagulans*: A view on the morphological and pharmacological properties of shrub. World Journal of Science andTechnology. I : 30-37.

Muashigue T and Skoog K (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiology Plant. 15:473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

Pezeshki A, Hesari J, Zonoz A and Ghambarzadeh B (2011). Influence of *Withania coagulans*. Protease as a vegetable rennet on Proteolysis of Iranian UF white cheese. Journal of Agricultural Science and Technology. 13: 567-576.

Rokade, Y.B. and Sayyed, R.Z. (2009). Naphthalene derivatives: a new range of antimicrobials with high therapeutic value. Rasayan Journal of Chemistry. 2:972-980.

Sethi, A., Prakash, R., Amandeep, S.D., Bhatia, A. and Singh, R.P. (2013). Identification of phytochemical constituents from biologically active ether and chloroform extracts of the flowers of *Allamanda violacea* A.DC (Apocynaceae). Pelagia Research Library. 3:95-108.

Sharma N, Sachdeva P, Dhiman M and Koshy EP (2015). Comparative evaluation of *in vitro* regeneration potential of seeds of *W. somnifera* and *W. coagulans*. Biotechnology International 8 (1): 21-33

Sharma, M.D., Rautela, I., Gahlot, M., Sharma, N and Koshy, E. P. (2015). GC-MS analysis of Phytocomponents in juice sample of Indian cane: Saccharum barberi. International journal of pharmaceutical science and research. 6(12): 5147-5153.

Shukla D, Bhattarai N and Pant B (2010). *Invitro* mass propogation of *Withania somnifera* (L)Dunal. Nepal Journal of Science and Technology. 11:101-106. http://dx.doi.org/10.3126/njst.v11i0.4131

Siddique NA, Bari mA, Shahnewaz S, Rahman MH, Hasan MR; Khan MSI and Islam MS (2004). Plant regeneration of *Withania somnifera* (L.) Dunal (Ashwagandha) from nodal segments derived Callus an Endangered medicinal Plant in Bangladesh. Journal of Biological Science. 4: 219-223. http://dx.doi.org/10.3923/jbs.2004.219.223

Valizadeh J and Valizadeh M (2009), "*In Vitro* Callus and Plant Regeneration from *Withania coagulans*: A Valuable Medicinal plant" Pakistan Journal of Biological Science. 12: 1415-1419. http://dx.doi.org/10.3923/pjbs.2009.1415.1419

Valizadeh J and Valizadeh M (2011). Development of efficient micropropogation protocol for *Withania coagulans* (Stocks) Dunal. Applied Journal of Biotechnology. 10: 7611-7616.

ISSN : 0976-4550

INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Email : editor.ijabpt@gmail.com

Website: www.ijabpt.com