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Callus induction and biochemical analysis of *Gymnema sylvestre* R.Br

[1]Bishnupriya Hansdah and [2]S. L. Sahoo
[1] Assistant Professor of Botany, North Orissa University, Takatpur, Baripada, Odisha, India
[2] Professor of botany, Utkal University, Vani Vihar, Bhubaneswar, India
Corresponding Email- bpriya123_bot@yahoo.com

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ABSTRACT

Gymnema sylvestre R. Br. (Family Asclepiadaceae) commonly called as "Gudmar" is one of the well known medicinal plants recognized as a potent drug plant in Ayurveda as well as in homeopathic systems. This plant is used medicinally in throughout India and Southeast Asia for treatment against diabetes and also employed in the treatment of many other healths hazardous. This study developed a novel tissue/cell culture system for in vitro callus induction, proliferation and biochemical evaluatin of this species with improving the active principles in the plant. Nodal/leaf explants were inoculated in Murashige and Skoog's (1962) basal medium, with supplementation of different concentrations (0.5 to 3.0 mg/l) of Auxins (2, 4-D and NAA) and Cytokinins (Kinetin and BAP). The explants were allowed to grow and the callus was maintained on the same medium for weekly analysis. The maximum callusing was obtained in the medium supplemented with combinations of Kn (2.5 mg/l) along with 2,4-D (2.5 mg/l). The total carbohydrate content was obtained after 6 weeks callus induced on MS medium fortified with NAA and BAP (36.45 ± 0.93 mg/g fr.wt.) at 2.5 mg/l and 2.0 mg/l respectively. NAA+ BAP supplement medium also exhibit highest amount of reducing sugar i.e. (27.44 ± 0.09 mg/g fr.wt). Total soluble protein content was observed maximum in the callus tissues generated in MS medium with NAA+BAP (55.86 ± 0.66mg/g fresh tissue) as comparison to callus grown in other phytohormones. From the above study, it was quite conclusive that, the phytohormones play a significant role in regulation of the growth of callus and biochemical activity.

Keywords : Gymnema sylvestre R. Br., diabetes, Callus induction, proliferation, biochemical analysis.

I. INTRODUCTION

Plant tissue culture is a very important; an eco-friendly technology which includes micro-propagation leads to mass propagation of high quality planting material of nutritional as well as medicinal important plant within a limited period. A considerable majority of people of the world`s population still rely on the traditional medicine for their primary health care necessities. The use of plants as medicine due to the presence of various chemical substances is ever increasing day by day [1].

Currently demand of herbal medicines has been enhanced; and it is very difficult to fulfill the demand from field plants and also so many plants are exploited and endangered due to climatic effects, overgrazing, aforestation etc. Hence, *in vitro* propagation of plant by tissue culture can be used to fulfill the requirement of medicinal plants [2,3]. *In vitro* culture of plant cells and tissue has attracted considerable interest over recent years because it provides the means to study plant physiological and genetic processes in addition to offering the potential to assist in the breeding of improved cultivars by increasing genetic variability [4,5]. *Gymnema sylvestre R. Br.* (Asclepiadaceae), a vulnerable species is a slow growing, perennial medicinal woody climber found in South China, India and Vietnam. It is widely used in indigenous system of medicine for treatment of Diabetes [6,7]. Besides, the plant species is also used in the treatment of asthma, obesith, eye complaints, imflammations and snakebite [8,9,10,11]. In addition, it possesses anti-microbial, anti-hypercholesterolemic and sweet suppressing activities [12,13,14]

To fulfill the increasing demand of *G. sylvestre*, its large scale propagation is essential and plant tissue culture technique acts as alternative methods for the development and multiplication of this medicinal plant species. Callus induction is a powerful tool to regenerate plants. Callus is a disorganized mass of undifferentiated tissue comprised of actively dividing cells. The cells of callus dedifferentiate and thus regain their meristematic properties, including rapid proliferation. Our main objective of this study was to investigate the optimal cultivating conditions for this *G. sylvestre* by means of callus induction and proliferation and biochemical characterization.

II. MATERIALS AND METHODS

The healthy plants were collected from apex of 2 years old genotype of *Gymnema sylvestre* R. Br. for standardization of callugenesis protocol in Department of Botany, Utkal University, Bhubanesware, Odisha. Collected explants were first washed with running tap water (10 minutes) and then surface sterilized with Teepol (5%), 70% ethanol for 1-2 minutes and were rinsed in distilled water thrice. Then, they were taken to the laminar air flow chamber where treated with 0.1% $HgCl_2$ for 2-3 minutes and washed with sterile double distilled water. It was then inoculated in the appropriate Murashige and Skoog's (1962)[15], which contained sucrose (3%), and pH (5.8). The cultures were incubated at 25±2°C under 2000 lux light intensity provided by white fluorescent lamp for 8 hours photoperiod. The basal MS medium was used with derived supplementation of phytoregulators for callus induction from leaf and nodal segment of *G. sylvestre*. The MS basal media were supplemented with different auxins: 2,4D 0.5-3 mg/l, and Naphthalene Acetic Acid (NAA) 0.5-3 mg/l and cytokinin: Benzyl Amino Purine (BAP) and Kinetin 0.5-3 mg/l. after callus induction periodically sub culture was done better callus developments and proliferation. The fresh and dry biomass was also calculated for appropriate estimation of callus production. The total carbohydrate content was measured by Anthrone reagent method [16]. The Arsenomolybdate method of Nelson [17] was followed to determine the reducing sugar content of callus tissue of *G. sylvestre*. Quantitative of soluble protein estimation was done according to Lowry *et al.* [18] methods.

III. RESULT AND DISCUSSION

The nodal explants were inoculated in MS medium supplemented with various concentrations and combinations of auxins and cytokinins (Table 1). The remarkable callus proliferation was observed when MS medium was prepared with cytokinins i.e. kinetin (0.5 mg/l), BAP (0.5 mg/l) and 2, 4-D, NAA (0.5 mg/l) among the auxins. According to our experiment, among the various treatments applied, best response (95 ± 1.20) towards the callus induction was obtained in MS medium fortified with 2,4-D, kinetin (2.5mg/l) each. The single supplementation of 2,4-D at 2.5 mg/l (Fig. 1 A), Kinetin at 2mg/l (Fig 1, B), NAA at 2.5 mg/l (Fig 1, D), and BAP at (2 mg/l) (Fig 1, E), caused 90±1.20%, 82.3±0.83%, 79.6±0.92% and 75.2±1.16% of callus induction, respectively showing that 2,4-D and kinetin was marginally more effective in causing induction of callus than of the other two plant growth regulators. However, the differences in the efficiency in induction of callus was marginal but significant (F=

8.63; p≥0.05). In all the media tested, 2, 4-D single or proliferation.
Kinetin found to be more effective for the callus

Table 1. Effect of MS basal medium supplemented with different concentration of plant growth regulators (PGRs) on induction of callus and characteristic of callus.

Phytohormone concentration (mg/l)				Percentage of callus response (%)	Texture and Color
2,4-D	**Kinetin**	**NAA**	**BAP**	**(Mean ± S.E.M)***	
0.5				40±1.46	White and friable
1.0				49.9±1.50	White and friable
1.5				60 ± 1.58	White and friable
2.0				73 ± 1.05	White and friable
2.5				**90 ± 1.20**	**Light brown and friable**
3.0				75 ± 1.02	Light brown and friable
	0.5			36 ± 0.99	White and friable
	1.0			60 ± 0.89	White and friable
	1.5			81 ± 0.91	White and friable
	2.0			**82 ± 0.83**	**White and less hard**
	2.5			79 ± 1.50	Light yellow and less hard
	3.0			72 ± 1.20	Light yellow and less hard
0.5	0.5			42 ± 1.03	White and friable
1.0	1.0			64 ± 0.95	White and friable
2.0	2.0			78 ± 1.05	White and friable
2.5	**2.5**			**95 ± 1.11**	**White and friable**
3.0	3.0			67 ± 0.98	Light brown and friable
		0.5		43 ± 1.04	White and friable
		1.0		62 ± 1.08	White and friable
		1.5		70 ± 1.40	White and friable
		2.0		75 ± 0.89	Fluorescent green and friable
		2.5		**79 ± 0.92**	**Fluorescent green and granular**
		3.0		76 ± 1.30	Fluorescent green and granular
			0.5	50.9 ± 1.20	White and friable
			1.0	65 ± 1.41	White and friable
			1.5	72 ± 0.87	White and friable
			2.0	**75 ± 1.16**	**Light green and compact**
			3.0	70 ± 1.92	Light green and compact
		0.5	0.5	47 ± 1.54	Light green and friable
		1.0	1.0	56 ± 0.78	Light green and friable
		2.0	2.0	70 ± 1.22	Light green and compact
		2.5	**2.0**	**82 ± 1.01**	**Green and compact**
		3.0	3.0	66 ± 0.89	Light green and compact

Note: Data represents the mean ± SEM of 5 replicates for each treatment.

Similar results were found in many plant including *G. entiana* spp. [19] , Hansdah and Sahoo [20] and other dicot plants [21]. Komalavalli and Rao, [22] reported that there could be good positive effect of coconut and malt extract with 2, 4-D on callusing of *G. sylvestre,* when these are supplemented to MS medium. The callus response was increased with increasing concentration of auxins and cytokinins but at higher concentration the percentage of callus decreased. The friable and white/brown callus were obtained in MS medium fortified with

2,4-D and Kinetin but the callus became green and compact when MS medium supplemented with BAP and Kinetin, singly or in combination.

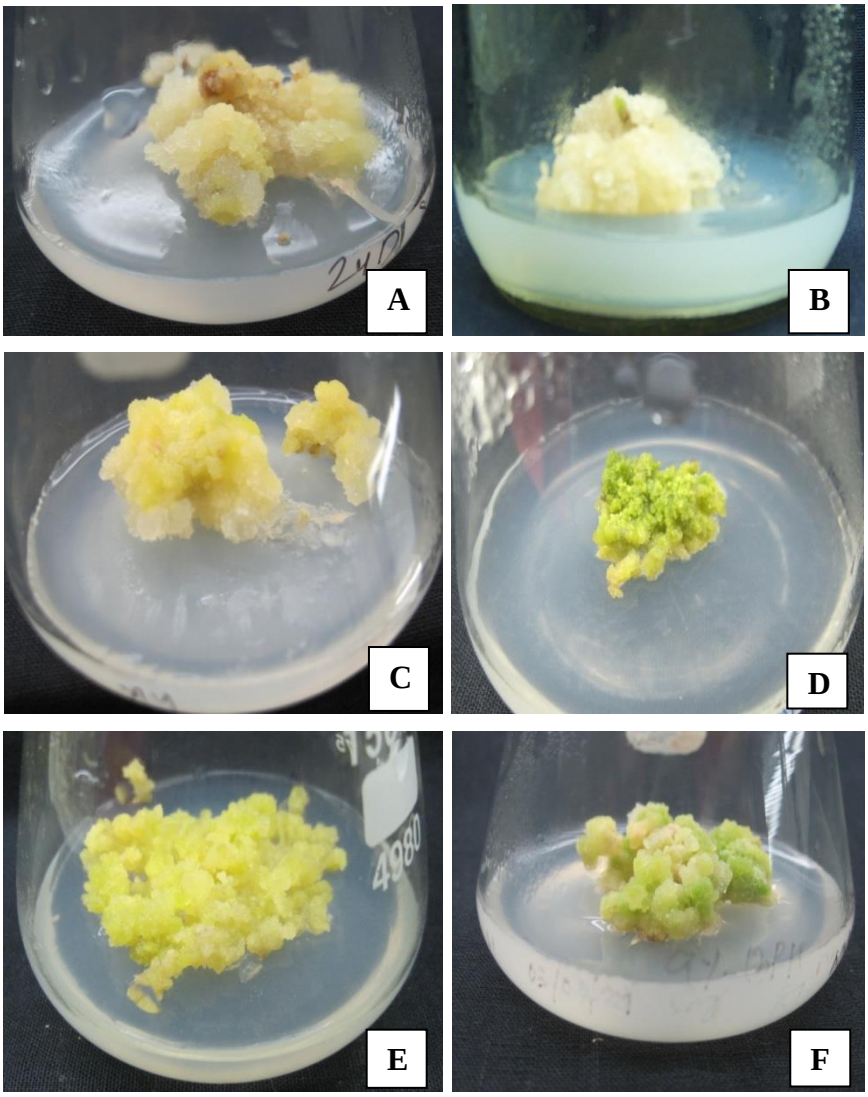

Fig. 1. Callogenesis in *G.sylvestre*: (A) friable callus in 2,4-D (2.5 mg/l), (B) White and less hard callus in Kinetin (2.0 mg/l), (C) White and friable callus at 2,4-D & Kinetin (2.5 mg/l) each, (D) Fluorescent green and granular at NAA (2.5 mg/l), (E) Light green and compact at BAP (2.0 mg/l), (F) Green and compact at NAA (2.5 mg/l) & BAP (2.0 mg/l).

This part of study present the percentage of callus response increases with increasing concentration of phytohormone i.e. auxin and cytokinin. But auxins are most preferred for callus growth and development [23]. In all the media tested, 2, 4-D single or with Kinetin found to be more effective for the callus proliferation. The maximum biomass was obtained with auxin and cytokinins combination at 40 days of culture.

Biochemical Analysis of Callus Tissue

The biochemical analysis of the callus generated from *G. sylvestre* was done on the basis of callus biomass yield (Data not shown here). From the observation it was evident that the supplementation of phytohomones exhibited maximum growth at definite concentration. So the callus tissue generated and harvested were

collected from the best responded medium and allowed to sub-culture for weekly analysis. The most efficient concentration of hormones like 2,4-D at 2.5 mg/l, NAA at 2.5 mg/l among the auxins and BAP followed by Kinetin at 2.0 mg/l each. In phytohormones combination the synergistic effect of 2,4-D (2.5 mg/l) with Kinetin (2.5 mg/l) or BAP (2.0 mg/l) with NAA (2.5 mg/l) shows better response for growth the callus tissue so, we select these tissue for our experiment. For the biochemical analysis (carbohydrate, reducing sugar, and total protein content) of cell mass, starting from 2 weeks to 8 weeks, callus was collected at 2 weeks interval for further study.

Total soluble carbohydrate content of callus tissue

The total carbohydrate content was measured by Anthrone reagent method [16]. The total sugar content of *G. sylvestre* callus was calculated and represented in (Table 2). The total soluble sugar content was found to be more in callus grown in MS medium supplemented with NAA in comparison to other treated phytohormones. At every two weeks interval the total carbohydrate content was measured. The highest total carbohydrate content was obtained after 6 weeks callus induced on MS medium fortified with NAA and BAP (36.45 ± 0.93 mg/g fr.wt.) at 2.5 mg/l and 2.0 mg/l respectively. Further it was also observed that NAA (31.21 ± 0.92 mg/g fr.wt.) at 2.5mg/l, BAP (26.09 ± 0.79 mg/g fr.wt.) at 2.0 mg/l, Kinetin (26.09 ± 0.79 mg/g fr.wt.) at 2.0 mg/l and 2,4-D (18.13 ± 0.73 mg/g fr.wt.) at 2.5 mg/l generated callus tissue but less than that of above recorded data. After sixth weeks, there was decrease in total sugar content in almost all the medium with different phytohormones. So the total sugar content was increase up to a certain period and gradually declines with the depletion of the nutrients in the medium.

Table 2. Effect of MS basal medium supplemented with different concentration of plant growth regulators (PGRs) on total sugar contents of callus tissue of *G. sylvestre*.

Times interval	Callus tissue grown with different concentration and combination of phytohormone (Mean ± S.E.M)*					
	2,4-D	Kinetin	2,4-D+Kn	NAA	BAP	NAA+BAP
2 weeks	4.55 ± 0.29	7.54 ± 0.44	8.64 ± 0.54	6.64 ± 0.64	7.86 ± 0.91	9.34 ± 0.55
4 weeks	6.19 ± 0.61	13.26 ± 0.63	14.83 ± 0.68	10.56 ± 0.93	15.73 ± 0.40	18.71 ± 0.68
6 weeks	18.13 ± 0.73	26.09 ± 0.79	29.33 ± 0.73	31.21 ± 0.92	28.13 ± 0.75	36.45 ± 0.93
8 weeks	8.53 ± 0.58	14.35 ± 0.66	16.13 ± 0.91	16.22 ± 0.35	25.36 ± 0.69	26.21 ± 0.75

Note: Data represents the mean ± SEM of 5 replicates for each treatment.

Reducing sugar content of callus tissue

The Arsenomolybdate method of Nelson [17] was followed to determine the reducing sugar content of callus tissue of *G. sylvestre* and data were representing in (Table 3). The callus developed on MS medium supplemented with NAA along with BAP shows highest reducing sugar content at 2.5mg/l and 2.0 mg/l concentration respectively. The reducing sugar content was calculated spectrophotometrically at an interval of 2 weeks and it was observed that the reducing sugar content was more at six weeks callus. At the 8th weeks the

reducing sugar decreased. Maximum amount of reducing sugar was observed when MS medium supplemented with combination of NAA+ BAP (27.44 ± 0.09 mg/g fr.wt) followed by NAA (25.75 ± 0.51 mg/g fr.wt) at 2.5mg/l concentration, 2,4-D+Kinetin (21.33 ± 0.43) at 2.5 mg/l each, BAP (20.23± 0.06 mg/g fr.wt.) at 2.0 mg/l, Kinetin (19.35 ± 0.10 mg/g fr.wt.) at 2.0 mg/l and 2,4-D (11.78 ± 0.68 mg/g fr.wt.) at 2.5 mg/l. The reducing sugar content was slightly enhanced from 2nd week callus to 4th week but at six week the metabolite was doubled when compared with the previous weeks.

Table 3. Effect of MS basal medium supplemented with different concentration of plant growth regulators (PGRs) on reducing sugar contents of callus tissue of *G. sylvestre*.

Times interval	Callus tissue grown with different concentration and combination of phytohormones (Mean ± S.E.M)*					
	2,4-D	Kinetin	2,4-D+Kn	NAA	BAP	NAA+BAP
2 weeks	2.44 ± 0.32	5.89 ± 0.02	6.78 ± 0.14	4.87 ± 0.91	5.30 ± 0.03	7.22 ± 0.31
4 weeks	4.84 ± 0.68	9.35 ± 0.03	10.34 ± 0.11	8.35 ± 0.64	11.02 ± 0.07	14.66 ± 0.24
6 weeks	11.77 ± 0.68	19.35 ± 0.10	21.33 ± 0.43	25.75 ± 0.51	20.23 ± 0.06	27.44 ± 0.09
8 weeks	6.35 ± 0.84	9.03 ± 0.05	15.12 ± 0.14	12.21 ± 0.50	11.21 ± 0.14	13.78 ± 0.33

Note: Data represents the mean ± SEM of 5 replicates for each treatment.

Comparison of total sugar and reducing sugar

This graph shows the comparison of total sugar and reducing sugar obtained on MS basal medium supplemented with different concentration of plant growth regulators (PGRs) in (mg/g fresh weight) of callus tissue (Fig. 2). The histograms represent the total sugar and the line graph represents the reducing sugar. From the graph it is clear that the total sugar and reducing sugar greatly influence by the phytohormones i.e auxins and cytokinins

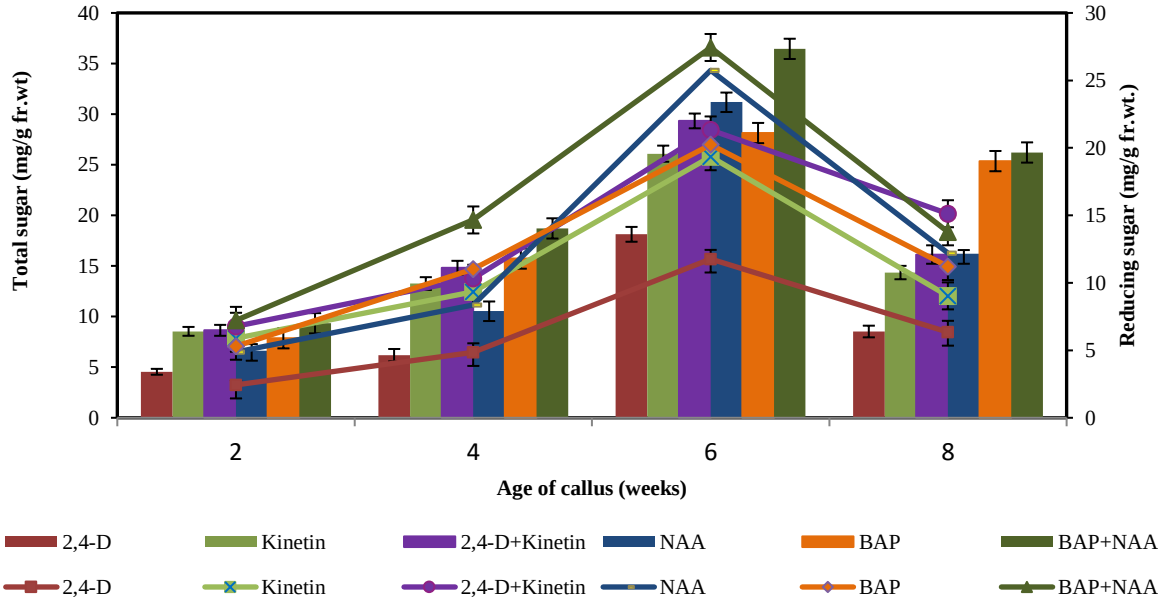

Fig. 2. Comparison of total sugar and reducing sugar obtained on MS basal medium supplemented with different concentration of plant growth regulators (PGRs) in form of (mg/g fresh weight) of callus tissue.

Total soluble protein content of callus tissue

Quantitative of protein estimation was done according to Lowry *et al.,* [18] methods. The spectrophotometric measurement of protein was carried out in 2 weeks interval from 2nd to 8th weeks. Maximum protein content was observed in the callus tissues generated in MS medium with NAA+BAP (55.86 ± 0.66mg/g fresh tissue) followed by NAA (53.90 ± 0.84 mg/g fresh tissue), followed by BAP (50.48 ± 0.68 mg/g fresh tissue), 2,4-D+Kinetin (45.47 ± 0.37 mg/g fresh tissue) Kinetin (40.38 ± 0.68 mg/g fresh tissue) and 2,4-D (36.67 ± 0.32 mg/g fresh tissue). Data are representing in (Table 4). The highest protein content was observed in NAA along with BAP supplemented medium then that of other phytohormones.

Table 4. Effect of MS basal medium supplemented with different concentration of plant growth regulators (PGRs) on total protein contents (mg/g fresh weight) of callus tissue of *G. sylvestre*.

Type of callus tissue	2 weeks (Mean ± S.E.M)*	4 weeks (Mean ± S.E.M)*	6 weeks (Mean ± S.E.M)*	8 weeks (Mean ± S.E.M)*
2, 4-D	33.23 ± 0.52	36.67 ± 0.32	34.12 ± 0.91	12.022 ± 0.75
Kinetin	36.09 ± 0.97	40.38 ± 0.68	36.30 ± 0.51	16.21 ± 0.44
2,4-D+Kinetin	37.21 ± 0.57	45.47 ± 0.37	38.12 ± 0.87	17.77 ± 0.92
NAA	48.41 ± 0.84	53.90 ± 0.84	34.53 ± 0.64	15.94 ± 0.79
BAP	47.12 ± 0.75	50.48 ± 0.68	27.64 ± 0.50	23.06 ± 0.84
BAP+NAA	48.33 ± 0.58	55.86 ± 0.66	37.79 ± 0.23	25.53 ± 0.37

Note: Data represents the mean ± SEM of 5 replicates for each treatment

Comparison of total sugar and protein content of callus tissue

Total carbohydrate and total protein content of callus tissue was compared in this graphical representation (Fig. 3). Histogram shows the total protein content and the line graph represents the total carbohydrate. In this graph it was reveals that the protein content was high at 4 weeks callus tissue but in case of total sugar content increase up to 6 weeks then declined.

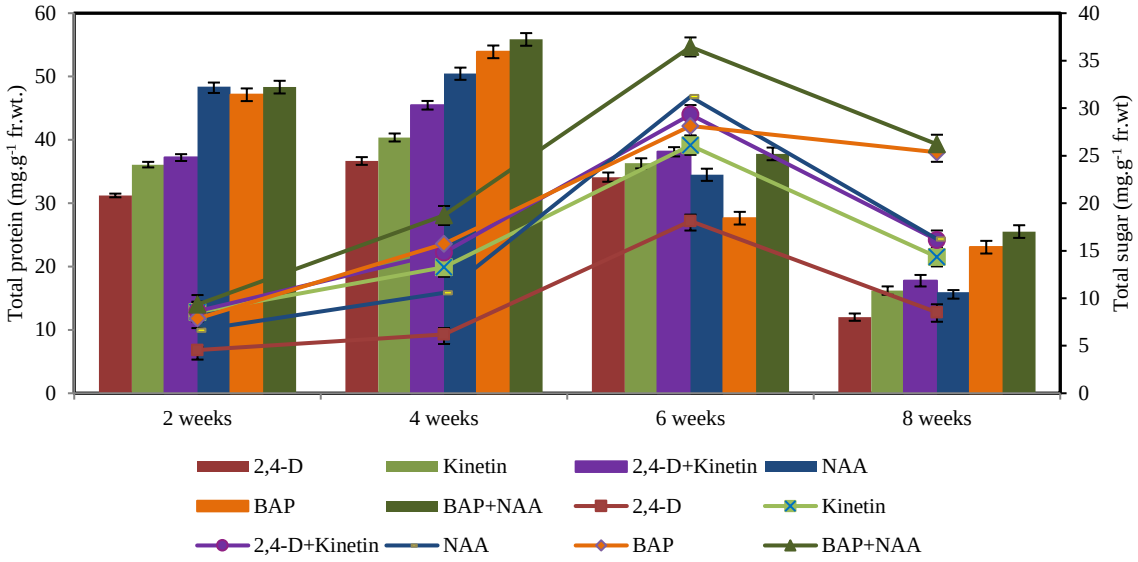

Fig. 3. Comparison of total sugar and reducing sugar obtained on MS basal mediumsupplemented with different concentration of plant growth regulators (PGRs) on total protein contents (mg/g fresh weight) of callus tissue of *G. sylvestre*.

After 8 weeks the protein synthesis was retarded due to negligible nutritional factor in the medium which induces a negative growth factor or synthesis of phenolic secretion by the callus. From the above data it was cleared that the protein content gradually increased up to 4th or 6th weeks according to the type of tissue where it was found to be maximum. After 8 weeks the amount of protein was almost constant. The increase amount of protein (*in vitro*) was found to be 1.17 times more than that of *in vivo* leaf.

IV. CONCLUSION

The present investigation revealed that the induction of regenerative callus from cultured explants is mostly induced on the exogenous application of phytohormones i.e. auxins and cytokinins. But auxins are the most preferred phytohormones for growth and development of callus. The maximum percentage of callus was achieved when MS medium was fortified with 2,4-D+Kinetin, which gave whitish and friable callus as compared to other hormones. In NAA supplemented medium the calli were found to be green fluorescent in color, highly compact and granular in nature whereas with kinetin in the medium calli became yellowish white and friable. Fresh biomass yield was found to be maximum in the medium containing 2,4-D along with Kinetin followed by Kinetin and then BAP independently. The biochemical estimation show that the maximum ttal carbohydrate, reducing sugar content was fund when callus grown in MS media fortified with NAA alng with BAP. The same pattern was also observed in case of total soluble protein of the callus tissue.

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