In vitro Seeds Germination and Seedling Growth of *Gymnema sylvestre* R.Br. an important antidiabetic medicinal plant.

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Abstract: Efficient culture medium and culture condition (dark /light period) for each step of *In vitro* seeds germination and growth of seedlings were determined in *Gymnema silvestra* R.Br. Different ages (immature, mature and dry seeds) of seeds germination were found within 6 to 10 days on MS half /MS full medium and growth of seedlings were obtained on MS medium in six weeks. The maximum percentage of seeds germination (98±0.30%) were found on MS half strength medium incubated 120 hours in dark period from immature seeds. Numbers of seeds germination were observed on MS half and full basal medium with 3% sucrose and 0.8% agar with longer dark period. Germinated seeds were subculture on MS salt supplemented with BAP, NAA and KI (alone and in combination) with different concentration. The best seedlings growths (6.0±0.31cm.) were obtained on MS medium containing (BAP-1.0mgL^{-1}+ NAA-0.1mgL^{-1} + KI-0.5mgL^{-1}) within six weeks culture.

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Key words- BAP – (6- Benzyl amino purine), NAA – (1- Naphthalene acidic acid), Kinetin-(6- furfuryl aminopurine), MS- Murashige & skoog, Immature seeds (green seeds), Mature seeds (yellow seeds), Dry seeds (brown seeds), *Gymnema sylvestr R.Br.*

1. Introduction-

Gymnema sylvestre R. Br. Ex. is an expensive medicinal perennial woody climber belong to the family Asclepiadaceae and distributed from tropical and subtropical regions of India (Anonymous, 1997). The use of plants as medicine is as old as human civilization. It is a very effective medicinal plant use in the treatment of asthma, eye complaints, inflammations, family planning and snake bite (Anonymous, 1956; Uniyal, 1993; Selvanayagam et al., 1995). It is a potent antidiabetic plant and used in folk, Ayurvedic and Homeopathic system of medicine, studied by Kapoor, (1977), Ravi and Wahi, (1995), Mitra et al, (1995). Property of leaves of this plant, to appreciate the taste of sugar has been tested by Mr. D. Hooper in (1887). *Gurmar* is very slow growing and high demanding medicinal plant, at present 85% collection of plant material is from Natural resource. This was not full filling the demand of this impotent medicinal plant. Propagation of *Gymnema Sylvestre* is rare, due to the very short span of seeds viability and poor rooting ability of vegetative cuttings; no alternative mode of multiplication is available to propagate and to conserve genetic stock of this useful plant. However, the seeds viability period are also poor and less in nature, limiting its natural propagation (Reddy et *al*., 1998). Due to short span viability *In vitro* seeds (green seeds) germination is one of the most beneficial process to save this elite vine. Tissue culture offers an alternative propagation method would be beneficial in accelerating large scale multiplication, improvement and conservation of *Gymnema sylvestre.* In view of its inherent qualities and restricted distribution, rapid *In vitro* multiplication of this endangered species is needed. *Gymnema sylvestre*, natural strands are fast disappearing and threatened due to indiscriminate collection and over exploitation from natural resources for commercial purposes and to meet the requirements of the pharmaceutical industry (Choudhury, 1988). Limited tissue culture work has been done on *Gymnema* species (Komalavalli and Rao, (1997); Reddy et. al, (1998); N. Komalavalli & M. V. Rao, (2000); C. Subhathra Devi and V. Mohana Srinivasan, (2008). To date only little study has been reported on micropropagation of *Gymnema sylvestre* through seeds explants like N. Komalavalli & M. V. Rao in (2000); C. Subhathra Devi and V. Mohana Srinivasan in (2008); A.V. Jaybhaya and S. S. Deokule in (2010). Therefore, the investigation has to carry out to ascertain the most appropriate basal culture media, dark and light condition for each step of *In vitro* seeds germination and growth of seedlings for micro propagation of *Gymnema sylvestre R.Br.*

2. Materials and Methods-

2.1. Source of plant material and sterilization-

Seeds of different ages (immature, mature, and dry) were collected from pods of an elite vine (5-7year old plant) from Kasturi Herbal farm Misrod, Bhopal, in the month of Dec to Feb. Plant identified by Laghu Vanupaj Prasannskarn & Anusandhan Kendra Barkheda Pathani, Bhopal (M.P.). The pods were

washed under running water for 15 min and five to six times rinse with tap water and then with liquid soap solution followed by washing with tap water. Further surface sterilization treatment was conducted in a laminar air flow chamber. The pods were surface sterilized with 70% aqueous ethanol for 30 seconds, and dipped into 0.1% (w/v) fresh prepared mercuric chloride solution for 6-10 min, and then washed with 4-6 times in double sterilized distilled water. The sterilized pods were split to open and isolated seeds were inoculated on culture medium.

2.2. Culture condition and statistical analysis-

The explants (immature, mature and dry seeds) were inoculated on MS (half &full) medium and incubated different light or dark period. Medium were supplemented with 3% w/v sucrose, adjust 5.5 to 5.8 pH (1N HCl or 1N NaOH) and solidified with 0.8 % agar than Autoclaved 121^0C for 15-20 min, poured into culture bottles or test-tube (66mm in diameter and 15mm in height). All cultures were incubated in culture room at 26±2 ^{0}C in darkness or cool white light of 3,500 Lx. at plant level provided by fluorescent tubes with 55-60% relative humidity. After 8-10 days seeds were germinated and *In vitro* germinated seeds were used as a source of explants for latter experiment. Experiments were investigated to optimize salt and hormonal requirements for maximum seedling growth. Germinated seeds were cultured on MS basal medium (Murashige & Skoog 1962) supplemented with growth regulators like Benzyl amino purine (BAP) alone and in combinations with different concentrations of Auxins (NAA-1,Naphthalene acetic acid) and cytokines (Kinetin- 6, furfuryl amino purine) were incorporated in to the basal media, containing 3 %(w/v) sucrose and gilled with 0.8% agar having pH 5.4 to 5.8 with 1N NaOH/1N HCl before autoclaving, and autoclaved at 1.06 kg cm^{-2} pressure ,at 120^0C tem for 15-20 min. Culture were maintained at 25±2^{0}C tem in a culture room with 70 µmol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes and were exposed to a photoperiod of 16/8 light/dark and 55±5 of relative humidity. Series of experiments were conducted and analyze statistically. Data were showed some prosperous effect, included in the table and presented in mean±SE of 10 explants per treatment and repeated three times. Mean values were not significantly different more than 1.

3. Results and Discussion

3.1. Effect of light or dark period and suitable medium on seeds germination (immature, mature and dry seeds)

The two different medium (MS1/2 and MS full) were tested in experiment, showed similar trend in responses to the medium and light or dark condition although seeds (immature, mature and dry seeds) germination percentages differ among them. Namely, the highest percentages of seeds germination (98±0.30%) on Ms Half and (88±0.20%) on MS full medium under 120 hours dark period were obtained from immature seeds (green). When seeds were incubated in 120 hours light condition green seeds germination percentage (70±0.06%) on MS ½ and (61±0.31%) on MS basal medium comparatively reduced. On the other hand, mature seeds (66+0.26%), dry seeds (21±0.31%) on MS1/2 and mature seeds (57±0.31%),dry seeds(09±0.23%) on MS full medium shows less germination under 120 hours dark in comparison to immature seeds were markedly reduced. No significant differences in 120 hours light period for seeds germination percentages were observed on MS Half and MS full basal medium. Seeds were incubated in darkness for 10 days and then transferred to light condition, on the other hand seeds were incubated in continuous light condition 10 days, less seeds germination have been observed. Experiment showed that MS half medium were most beneficial for seeds germination of *Gymnema sylvestre*, and increasing the length of dark period and decreasing seeds age, the seeds germination percentages were increased. Explants age plays a significant role to induce multiple shoots in a number of plants were studied by Distabanjong and Geneve, (1997) and Morus Alba Thomas, (2003). The culture's incubated in darkness for one week and longer, observed no significant difference. Similar response was also observed in the effect of light and dark condition of in vitro seed germination of *Paphiopedilum* species observed by Lee-Jiuan Tay, Kiyoltoshi Takeno and Yutaka Hori in (1988). *In vitro* propagation of plants belonging to family Asclepidiaceae have also been shown to have optimum overall growth in MS medium reported by Chi, Won and Jhon, (1985); Patnaik and Debata, (1996); Komalavalli and Rao, (1997). The degree of growth and differentiation consider with the medium constitution studied by Shekhawat et al., (1993); Das et al., (1996). Result of comparative study of seeds germination (immature, mature, and dry) on different medium followed by MS half and MS basal under different light and dark period show in table- 1, fig-1(A, B, C, D, E, and F), 2, 3, 4 and 5.

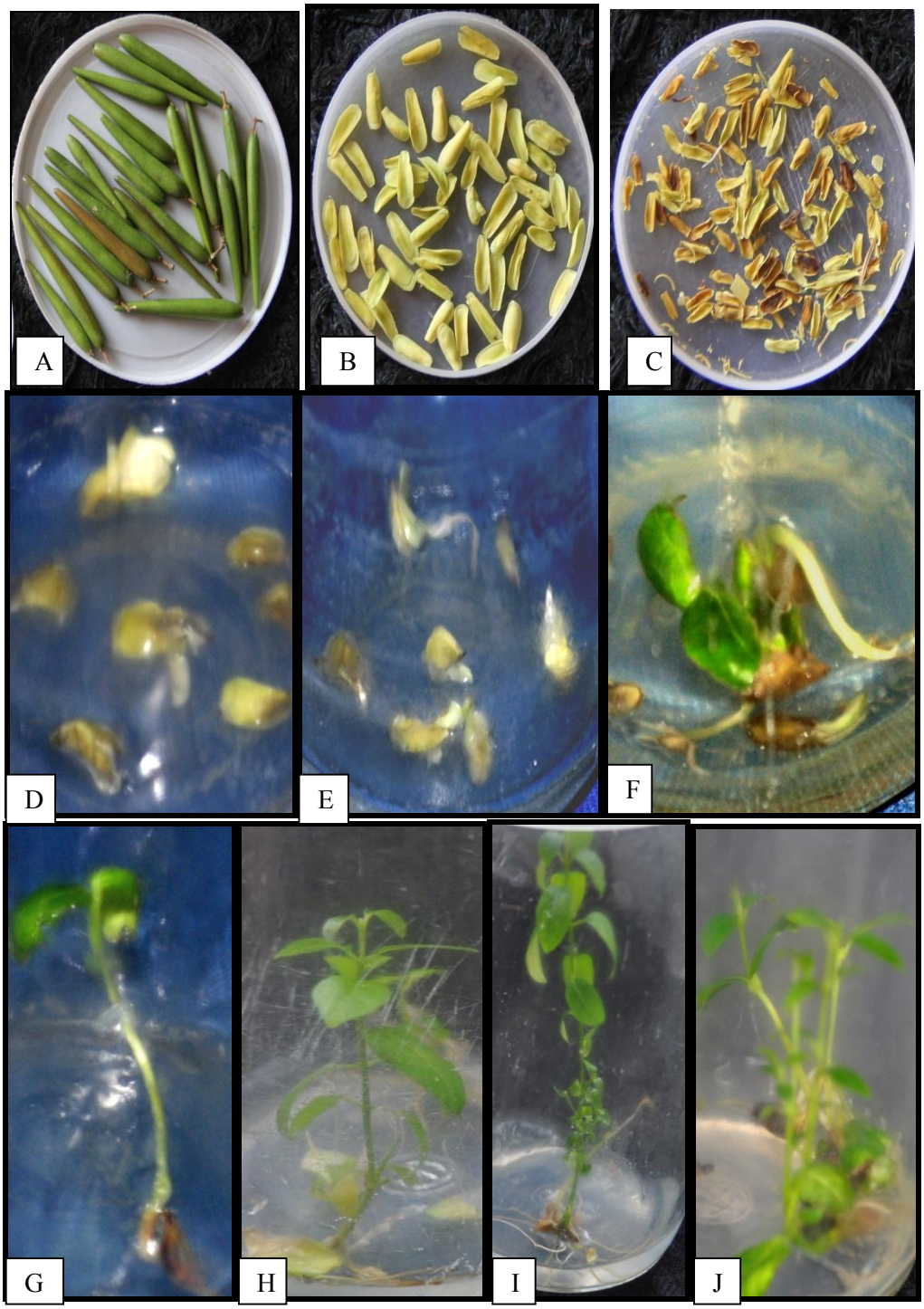

Figure 1. A. Green pods, B. immature seeds, C. mature dry seeds D. Green immature seeds in MS half medium after 6 to 10 days, E. Green immature seeds in MS full medium after 6 to 10 days, F. germinated seeds after 20 days, G. 15 days old seedling in MS 10, H. 30 days old seedling in MS-10, I. 45 days old seedling in MS 10, J. Seedling having multiple shoots in MS-10 medium after 45 days.

3.2. Effects of P.G.R on seedling growth

Plant growth regulators pay an important role in plant growth. Germinated seeds (immature, mature, and dry seeds) were isolated and transferred into different growth hormones with MS salt under photoperiod 16/8 hour's light/dark. Best results were obtained on MS medium containing BAP ($1.0mgl^{-1}$) with NAA ($0.1mgl^{-1}$) and KI ($0.5mgl^{-1}$) for sub sequent growth of seedlings within six weeks (6.0±0.031cm). Seedling growth and developed two to three shoots at all concentration of BAP, KI and NAA (alone and in combination)] in table-2 and fig-1 (G, H, I, and J), 6. Similar response was also observed in the propagation of *Gymnema sylvestre* by C. Subathra Devi and V. Mohan Srinivasan, (2008). MS medium containing BAP was more effective than kinetin and NAA for seedling growth as experiment. Reddy et al. in (1998), reported that kinetin did not improve significantly the shoot length and the number of proliferating shoots, superiority of BA and kinetin in combination has been found for micro propagation of other woody perennials reported by Das et al. (1996); Komalavalli and Rao, in (1997).

Table 1. Seeds (green, yellow, brown) germination in *Gymnema sylvestre* on different culture medium in darkness and light, value are mean±SE of ten replicates.

Time duration in hours	Seeds germination percentage in mean ±SE											
	MS half strength medium						MS full basal medium					
	Dark			Light			Dark			Light		
	Green seeds (Immature)	Yellow seeds (Mature)	Brown seeds (Dry)	Green seeds (Immature)	Yellow seeds (Mature)	Brown seeds (Dry)	Green seeds (Immature)	Yellow seeds(Mature)	Brown seeds (Dry)	Green seeds (immature)	Yellow seeds (Mature)	Brown seeds (Dry)
24	46±0.26	21±0.30	04±0.08	15±0.17	08±0.20	02±0.30	35±0.12	25±0.18	01±0.06	10±0.31	09±0.23	--
48	54±0.23	24±0.18	06±0.17	24±0.27	06±0.16	03±0.23	42±0.29	22±0.14	--	40±0.31	18±0.12	--
72	62±0.28	30±0.16	09±0.10	30±0.26	22±0.07	--	25±0.19	18±0.21	04±0.02	23±0.25	20±0.31	01±0.06
96	74±0.31	42±0.31	15±0.21	44±0.17	18±0.26	05±0.31	46±0.18	32±0.26	05±0.16	38±0.27	22±0.28	03±0.01
120	98±0.30	66±0.26	21±0.31	70±0.06	48 ±0.16	14±0.16	88±0.20	57±0.31	09±0.23	61±0.31	45±0.26	11±0.30
144	80±0.23	58±0.19	17±0.26	33±0.01	30 ±0.31	09±0.26	62±0.28	44±0.26	10±0.31	58±0.22	34±0.30	08±0.23
168	94±0.31	60±0.21	19±0.16	56±0.25	34 ±0.23	11±0.19	83±0.21	40±0.09	13±0.30	44±0.30	38±0.19	10±0.18
192	86±0.17	62±0.30	14±0.27	48±0.28	29 ±0.27	15±0.31	74±0.31	50±0.23	08±0.27	46±0.26	30±0.23	06±0.26
216	42±0.26	46±0.30	10±0.20	25±0.08	26 ±0.30	10±0.27	38±0.26	38±0.29	06±0.23	27±0.21	24±0.16	--
240	35±0.25	37±0.23	03±0.08	10±0.29	17 0.08	--	30±0.29	30±0.08	02±0.12	17±0.23	17±0.30	02±0.21

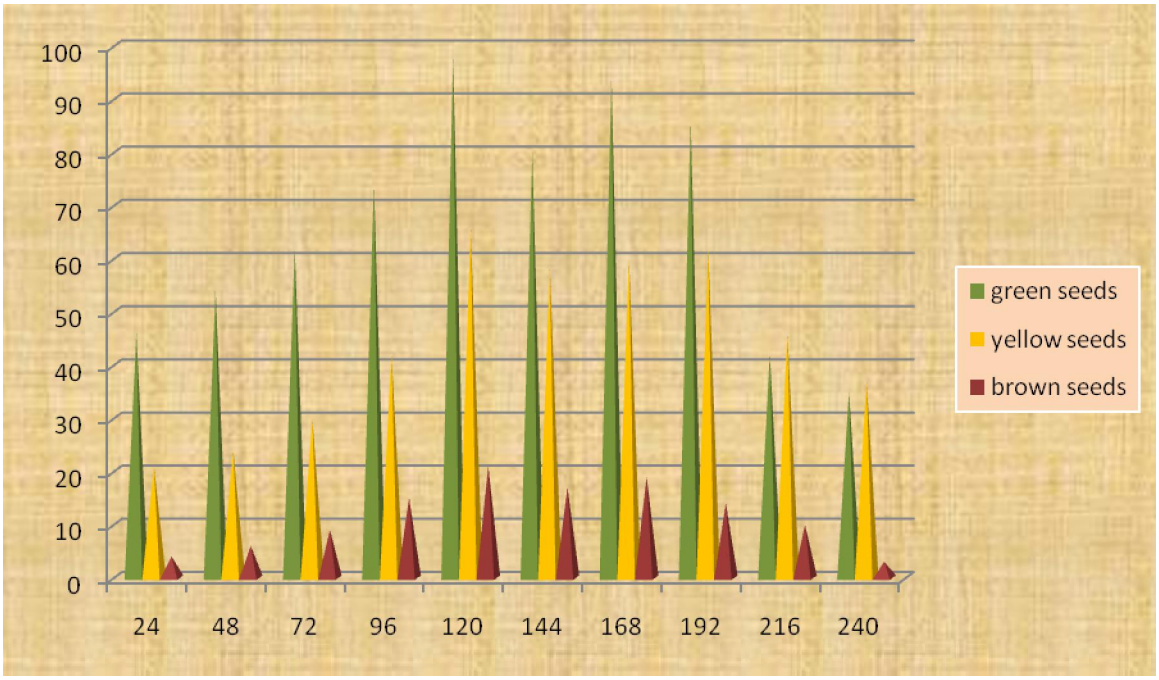

Fig.2. Seeds (green, yellow, brown) germination in *Gymnema sylvestre* incubated on MS-half strength medium in dark period and then transferred to light condition, percents germination were obtained for 15 days after the seeds inoculation.

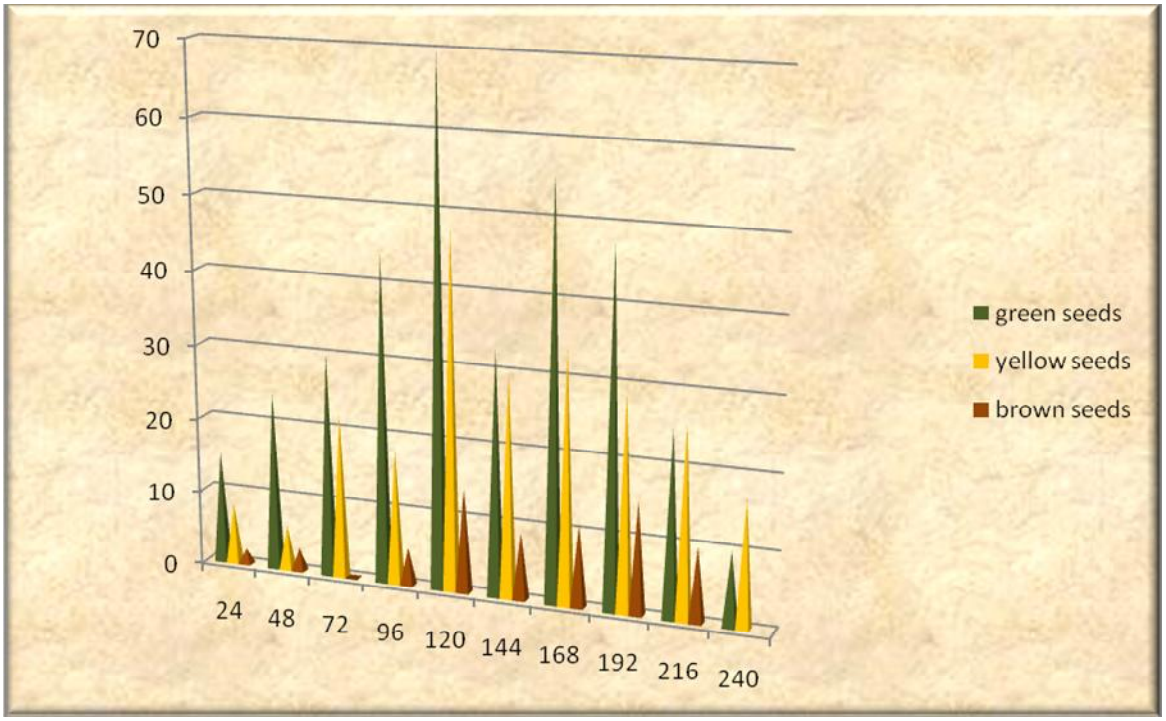

Fig.3. Seeds (green, yellow, brown) germination in *Gymnema sylvestre* incubated on MS-half strength medium in light condition and then transferred to dark condition, germination percentage were obtained for 15 days after the seeds inoculation.

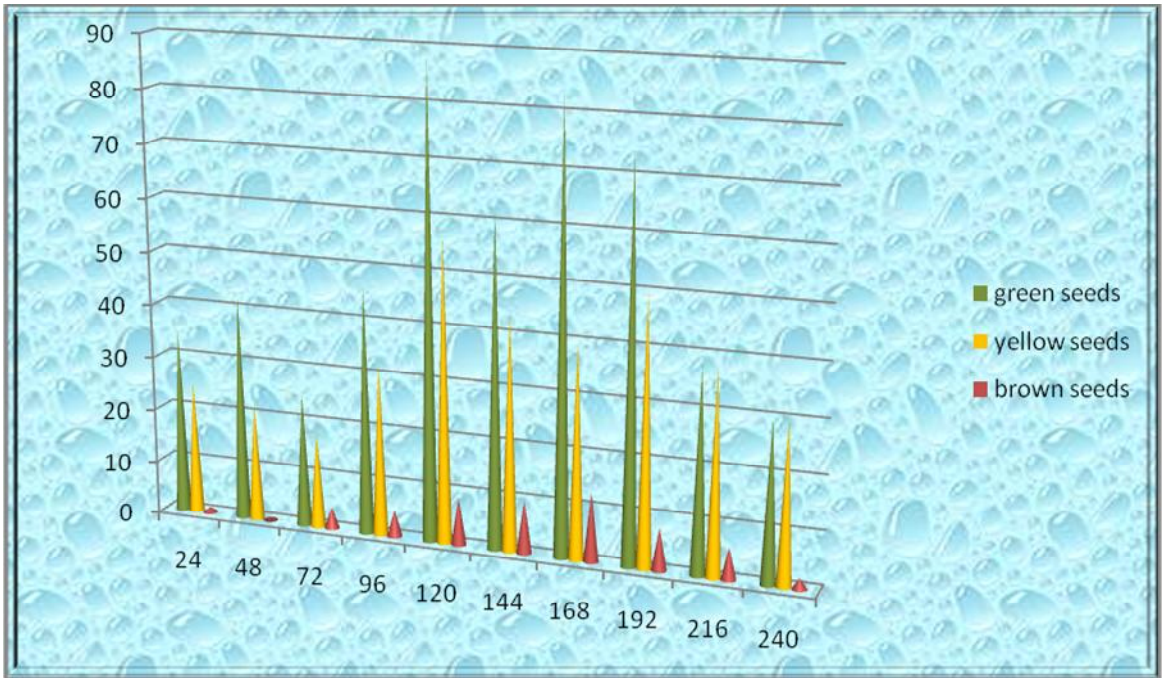

Fig.4. Seeds (green, yellow, brown) germination in *Gymnema sylvestre* incubated on MS basal medium in dark period and then transferred to light condition, percents germination were obtained for 15 days after the seeds inoculation.

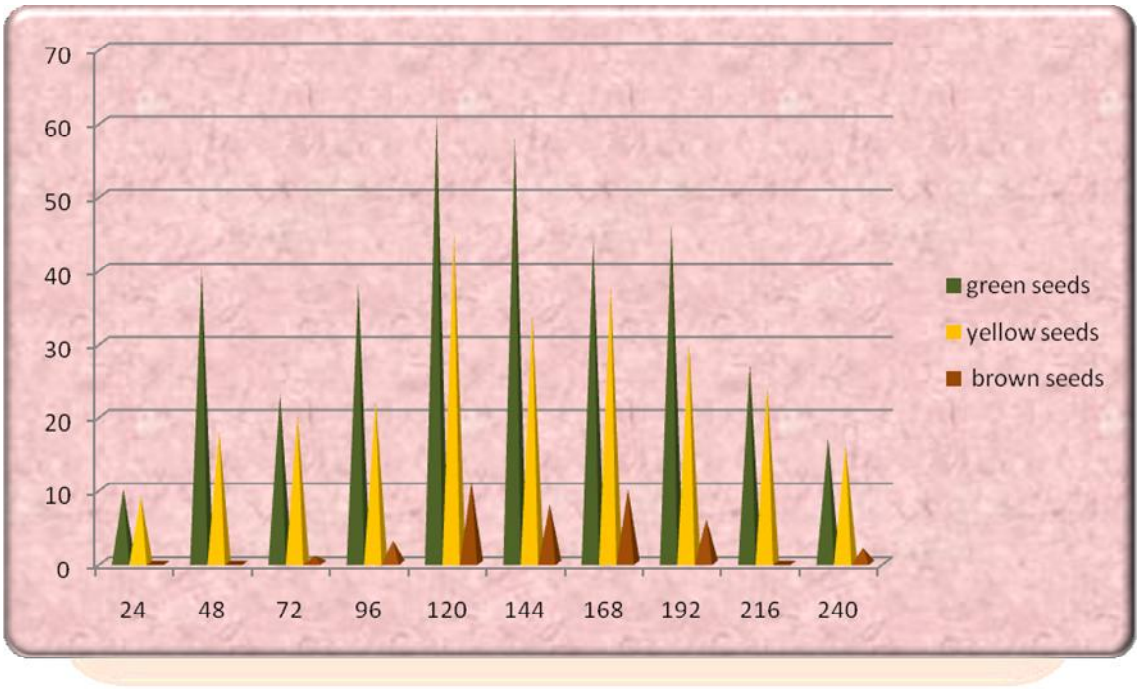

Fig.5. Seeds (green, yellow, brown) germination in *Gymnema sylvestre* incubated on MS basal medium in light condition and then transferred to darkness, germination percentage were obtained for 15 days after the seeds inoculation.

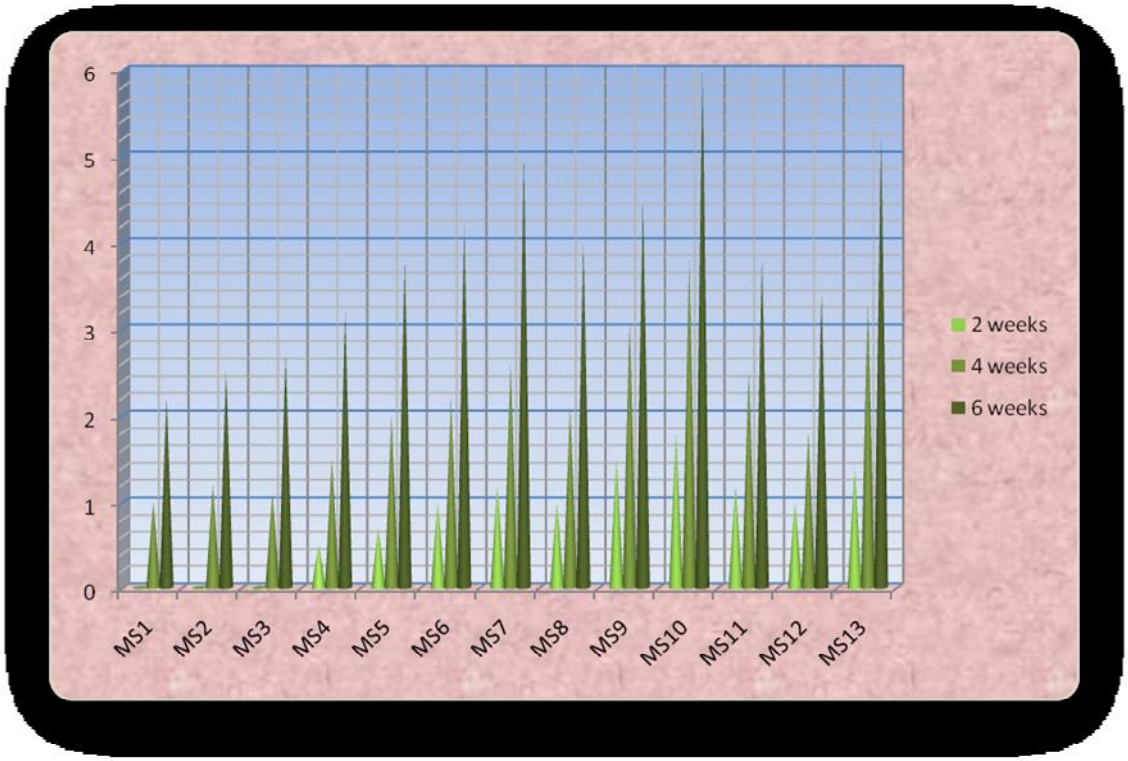

Fig.6. Effect of plant growth regulators on seedling growth from germinated seed of *Gymnema sylvestre* on MS medium after 6 weeks.

Table 2. Effect of plant growth regulators on seedling growth from germinated seed of *Gymnema sylvestre* on MS medium after 6 weeks, Followed by In Each column, mean±SE.

Plant growth regulators (mgL^{-1})				Length of seedling mean±SE in cm.		
Medium	**BAP**	**NAA**	**KI**	**2 weeks**	**4 weeks**	**6 weeks**
MS_1	0.0	0.5	0.0	0.0±0.000	1.0±0.027	2.2±0.020
MS_2	0.0	0.2	0.0	0.0±0.000	1.2±0.028	2.5±0.019
MS_3	0.0	1.0	0.0	0.0±0.000	1.1±0.022	2.7±0.022
MS_4	0.2	0.0	0.0	0.5±0.027	1.5±0.028	3.2±0.030
MS_5	0.5	0.0	0.0	0.7±0.026	2.0±0.020	3.8±0.029
MS_6	0.5	0.1	0.0	1.0±0.030	2.2±0.027	4.2±0.023
MS_7	0.5	0.5	0.5	1.2±0.018	2.6±0.021	5.0±0.028
MS_8	1.0	0.0	0.0	1.0±0.018	2.1±0.029	4.0±0.031
MS_9	1.0	0.5	0.1	1.5±0.031	3.1±0.031	4.5±0.031
MS_{10}	1.0	0.1	0.5	1.8±0.030	3.8±0.031	6.0±0.027
MS_{11}	2.0	0.5	0.5	1.2±0.026	2.5±0.027	3.8±0.023
MS_{12}	2.0	0.0	0.0	1.0±0.022	1.8±0.022	3.4±0.018
MS_{13}	3.0	0.5	1.0	1.4±0.029	3.3±0.016	5.2±0.030

4. Conclusion

The standardized protocol contributes improvement for the propagation of multipurpose medicinal plant as the rates of conventional seeds germination method. In nature *Gymnema sylvestre* R.Br. propagated by seeds, however their germination rate are very poor in natural conditions due to short viability of seeds and less endosperm. Furthermore, it propagation through stem cutting poses is very slow. Owing to these factors, the species is at the verge of extinction and will extinct soon if proper steps are not taken for its conservation. Thus tissue culture method represents an important potential for its propagation over conventional methods for improvement, conservation and large-scale planting of this medicinally important plant. The investigation shows, maximum seeds germination from immature (green) seeds incubated longer dark period in competition mature or dry seeds. The process of *In vitro* seed germination and seedling growth could be exploited to preserve intrinsic genetic variability and also prove useful in obtaining contamination free source plants and juvenile explants that have better regenerability in tissue culture.

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