Synergistic effects of 2,4-D and Cytokinins on callus culture establishment in rare medicinal plant- *Gymnema sylvestre*

Sunita Khatak*, Sonia Baches[1], Nitin Chauhan[1] and Amandeep Kaur[1]

Abstract– *Gymnema sylvestre* R.Br. belongs to *Ascelpediaceae* family and is a potent antidiabetic and diuretic medicinal plant. The vulnerability of species emphasizes the need to micropropagate pathogen free plants in a shorter time in small space. Field grown *Gymnema* axilary nodes, leaf and hypocotyls explants were aseptically cultured on MS basal medium fortified with different concentrations of 2,4-D alone or in combination with cytokinins (BAP, Kinetin) and auxins (NAA) along with sucrose and maltose as altered sugar source. 2,4-D proved outstanding in providing a stimulus to generate callus with different color and texture from leaf and hypocotyls on different media tested. However, 2,4-D alone generated green friable callus in almost 40 days which get shortened to 28 days when used in combination with kinetin. Further inclusion of BAP as cytokinin, the days to callus induction decreased to 20 days. The induced callus when subcultured on MS basal medium fortified with 2,4-D (1.0 mg/L, 3.0 mg/L and 5.0 mg/L) alone with maltose as altered sugar source resulted in friable fluorescent green color callus only in 5-10 days. BAP at variable concentration (0.25 mg/L, 0.5 mg/L and 2.5 mg/L) along with 2.5 mg/L NAA and 2,4-D at 0.5 mg/L at a constant concentration with dextrose as a sugar source resulted in lustrous green callus with embryogenic characteristics which turned brownish and harder with the passage of time. Being a plant of rare origin callus induction in shorter period of time is a prerequisite for mass propagation and our study establish this first step for *Gymnema* which is declared as vulnerable plant in red data book.

Key words– 2, 4-D, Micropropagation, Embryogenic, *Gymnema sylvestre*, Kinetin (KN).

1 INTRODUCTION

Gymnema sylvestre R. Br. is a woody, vine-like plant which climbs on bushes and trees in the Western Ghats in South India and to the west of those mountains in the territory around the coastal city of Goa. *G. sylvestre* is a plant used in India and parts of Asia as a natural treatment for diabetes. The herb's active ingredient, gymnemic acid, is extracted from leaves and roots and helps to lower and balance blood sugar levels. Indian physicians first used *Gymnema* to treat diabetes almost 2,000 years ago [1]. The leaves were also used for stomach ailments, constipation, water retention and liver disease. In the 1920s, preliminary scientific studies found some evidence that *Gymnema* leaves can reduce blood sugar levels [2]. The primary chemical constituents of *Gymnema* include Gymnemic acid, tartaric acid, gurmarin, calcium oxalate, glucose, stigmasterol, betaine and choline, while studies have shown that a water-soluble acidic fraction provides the hypoglycemic actions [3]. Gurmarin (another constituent of the leaves) and gymnemic acid have been shown to block sweet taste in humans [4]. This action is gradual in nature, differing from the rapid effect of many prescription hypoglycemic drugs. *Gymnema* leaves raise insulin levels according to research based on animal studies, which may be due to regeneration of the cells in the pancreas that secrete insulin. *Gymnema* can also improve uptake of glucose into cells and prevent adrenaline from stimulating the liver to produce glucose, thereby reducing blood sugar levels as reported. *G. sylvestre* has been used for thousands of years and has proven over time to be a non-toxic remedy. It is used for many conditions including diabetes, digestion, urinary tract problems, obesity, hypoglycemia, allergies, anemia, cholesterol and hyperactivity. The leaves were also reported for lowering serum cholesterol and triglycerides [5]. It has been believed that *G. sylvestre* has benefits of anti-allergic, antiviral and lipid lowering activities. The chemicals of *G. sylvestre* are believed to be able to lower the sugar available in the stomach for absorption, raise the insulin levels and block the dietary fat absorption. Although the plant has already been exploited for its medicinal aspects [6], [7] but regarding its extinction and existence in threatened species, its mass multiplication is a prerequisite for incoming years [8]. The normal agricultural practice needs 6 to 8 months to develop into a complete plantlet from seed which in turn requires constant manuring and observation.

Apart from this setback getting disease free plant from the seeds of previous mother plant is quite difficult. Plant cell tissue culture has offered a very novel technique to mass multiply, true to type and providing disease resistant plants in controlled conditions [9], [10], [11], [12], [13]. To extract active constituents from the plant requires no need to regenerate complete plant [9]. Pathogen free callus generated in shorter duration in vitro can prove to be a better alternative. So, present investigation serves this first step for mass proliferation of callus in *G. sylvestre*.

2 MATERIAL AND METHODS

2.1 Plant Material

The seedling of *G. sylvestre* used in the present study for callus induction and mass proliferation was procured from Tau Devi Lal Herbal Park, Near Khizrabaad Highway, District Yamunanagar, Haryana, India. The seedling was cultured in pot house with all recommended agricultural practices.

2.2 Explants Sterilization

The disease free, young leaf, hypocotyls and auxiliary nodes were collected from 4 weeks old healthy plant. The explants were excised and the contaminants were washed under running tap water for 45 minutes, followed by washing in the liquid detergent Tween- 20 (few drops/ 100ml) and then rinsed with running tap water. The cleaned explants were surface sterilized with variable concentration of mercuric chloride (0.1-0.5%) under laminar air flow. The explants were subjected to different time intervals to optimize the sterilization procedure and then rinsed 4-5 times with sterilized distilled water. After trimming the cut ends, surface sterilized explants were cultured on the culture medium.

2.3 Callus Initiation and Callus Induction

The sterilized explants were cultured on Murashige and Skoog [14] medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with naphthalene acetic acid (NAA) and cytokinins viz. 6-Benzyladenine (BAP) and kinetin (KN) at different concentration for callus initiation and proliferation. Nine different callus induction media (Table 2) were used. Out of nine different media tested, 2,4-D was initially used alone at three different concentrations of 1.0 mg/L, 3.0 mg/L and 5.0 mg/L. These concentrations were used in combination with kinetin at a constant concentration of 1.0 mg/L to study the effect of inclusion of cytokinins. Further NAA was included along with 2,4-D both at constant concentration of 2.5 mg/L and 0.5 mg/L, respectively, along with BAP at variable concentration of 0.25 mg/L, 0.50 mg/L and 2.50 mg/L in different combinations. The concentration of BAP was raised to 2.5 mg/L to see the effect of altering cytokinin from kinetin to BAP on callus induction. After inoculation the culture tubes and conical flask were properly covered with non absorbent cotton plugs. After labeling these were transferred to the culture room for incubation at 28±2°C in the rack. The cultures were incubated under cool fluorescent lights with 1500-2000 lux for 16 hours at a temperature of 28±2°C and 80% relative humidity. Each experiment had replicates repeated at least three times. Data was recorded up to seven weeks of culture.

2.4 Embryogenic Callus Induction

The callus induced was further subcultured on different MS basal media fortified with higher concentrations of BAP, Kinetin, NAA and 2, 4-D (Table 3). Sugars like maltose, dextrose and sucrose were altered for mass proliferation of callus induction and to explore embryogenic potential. 2, 4-D was used alone at three different concentrations of 1.0 mg/L, 3.0 mg/L and 5.0 mg/L with maltose (3.0%) as an altered sugar source. BAP was used at a higher concentration of 5mg/L as suggested by earlier reports in three combination by altering kinetin, 2, 4-D and NAA. Similarly, NAA was included along with 2,4-D both at constant concentration of 0.5 mg/L and 2.5 mg/L respectively along with BAP at variable concentration of 0.25 mg/L, 0.50 mg/L and 2.50 mg/L in different combination but dextrose was altered from sucrose at a concentration of 3.0%.

3 Results and Discussion

The callus induction, proliferation and exploring the embryogenic potential in *G. sylvestre* is an utmost requirement for propagation of this plant being of rare origin and to explore the antidiabetic aspects.

3.1 Sterilization of Explants

Sterilization of explants is a crucial step in plant tissue culture and to achieve 100 percent sterilization explants were subjected to various concentration of mercuric chloride (0.1-0.5%) along with antifungal supplement and a prior ethanol treatment (Table 1, Fig. 1).

TABLE 1

DIFFERENT CONCENTRATION OF MERCURIC CHLORIDE USED FOR STERILIZATION OF LEAVES AS EXPLANT

S. No.	Concentration of Mercuric Chloride (%)	Time (min.)	Number of Explants (5explant/ conical flask)	After 10 days no. of explants left without contamination	After 30 days no of explants left without contamination	Sterilization (%)
1)	0.1	3	30	20	13	43
2)	0.15	3	30	18	12	40
3)	0.2	3	30	24	16	53
4)	0.25	3	30	28	23	76
5)	0.3	3	30	27	24	80
6)	0.35	3	30	23	11	36
7)	0.4	3	30	21	9	30
8)	0.45	3	30	17	10	33
9)	0.5	3	30	20	7	23

Maximum sterilization (of 80%) was observed using a concentration of 0.3% of mercuric chloride for 3 min. A higher concentration of 0.5% of mercuric chloride burns the tissue and cause blackening at the edges resulting in 23% sterilization while a lower concentration of 0.1% of mercuric chloride resulted in only 40% sterilization. It was found that mercuric chloride alone at a concentration of 0.3% for 3

minutes is sufficient to sterilize the explants without a prior treatment of ethanol and antifungal supplements.

3.2 Callus Initiation

Callus was initiated using leaf and hypocotyl explants from seedling of *G. sylvestre*. Different MS basal media supplemented with different plant growth regulators were used to initiate callus consisting of 2,4-D, NAA as auxin and kinetin, BAP as cytokinin (Table 2).

TABLE 2

DAYS OF INITIATION, TEXTURE AND COLOUR OF CALLUS OBTAINED USING LEAF EXPLANTS OF *G.* SYLVESTRE

S. No.	Medium Code	Medium Details	Days	Texture	Color
1)	MS-1	MS + 1.0 mg/L 2,4-D + 3.0% Sucrose	40 days	Friable	Fluorescent Green
2)	MS-2	MS + 3.0 mg/L 2,4-D + 3.0% Sucrose	38 days	Friable	Fluorescent Green
6)	MS-3	MS + 5.0 mg/L 2,4-D + 3.0% Sucrose	38 days	Friable	Fluorescent Green
7)	MS-4	MS + 1.0 mg/L 2,4-D + 1.0 mg/L Kinetin + 3.0% Sucrose	30 days	Friable	Fluorescent Green
8)	MS-5	MS + 3.0 mg/L 2,4-D + 1.0 mg/L Kinetin + 3.0% Sucrose	28 days	Friable	Fluorescent Green
9)	MS-6	MS + 5.0 mg/L 2,4-D + 1.0 mg/L Kinetin + 3.0% Sucrose	28 days	Friable	Fluorescent Green
10)	MS-7	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.25 mg/L BAP + 3.0% Sucrose	20 days	Friable	Dark Green
11)	MS-8	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.5 mg/L BAP + 3.0% Sucrose	18 days	Friable	Pale Green
12)	MS-9	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 2.5 mg/L BAP + 3.0% Sucrose	19 days	Friable	Pale Yellow

Before inoculation, the explants were surface sterilized using standard mercuric chloride concentration of 0.3% for three minutes which served best for giving 80% sterilization for the leaf and hypocotyls explants. Explants cultured on MS- medium supplemented with 1.0 mg/L of 2,4-D turned pale but generated fluorescent green, well developed, albino, spongy and loosely arranged callus in 40 days although the callus obtained was friable in texture and after sometime, the fluorescent green color turned into white (Fig. 1). On raising the concentration of 2,4-D to 3.0 mg/L and 5.0 mg/L, friable and fluorescent green color callus was obtained in 38 days. A higher concentration of 2,4-D does not produce detrimental effects on callus initiation instead the days to callus initiation were observed approximately the same (38-40 days). Moreover the color and texture of callus was almost same being friable, fluorescent and green even on minimum concentration of 1.0 mg/L of 2,4-D. The present results contradict the results of [15] who reported callus induction from hypocotyls and cotyledons in *Medicago littoralis* in the presence of 2,4-D alone and when cultured on NAA the explants either died out or show little differentiation. Amoo and Ayisire [16] reported a specific callus initiation and order of callus size on different concentration of 2,4-D (0.4-1.0 mg/L) using cotyledon explants placed with abaxial surface on the media in *Parkia biglobosa*. The fact that callus was not induced by 2,4-D alone suggests that gurmar plant is not auxin specific. In comparison, MS medium supplemented with 2,4-D at three different concentration of 1.0 mg/L, 3.0 mg/L and 5.0 mg/L along with Kinetin (1.0 mg/L) at a constant concentration, produced fluorescent green color, less hard, compact textured callus (Fig. 2) in 28-30 days. This is similar to the observations of [17] in *Acacia mangium* where calli were reportedly induced from cotyledon explants on MS basal medium supplemented with both 2,4-D and kinetin. Roy [12] reported friable green callus in 2,4-D and in combination with kinetin in 25 days only using leaf explants in *Gymnema* itself. The time required for callus induction was lessening by 10 days as compared to media supplemented with 2,4-D alone.

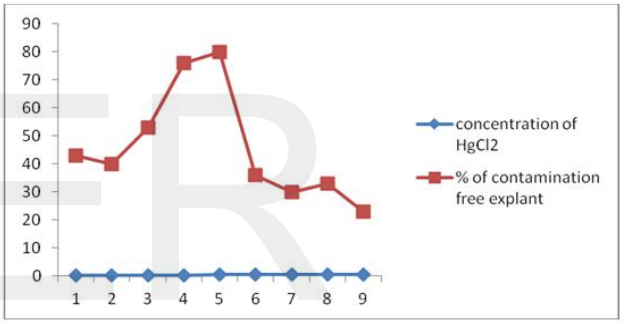

Fig. 1 Graphical representation of percentage of sterilization achieved using different concentrations of mercuric chloride.

The callus obtained when subcultured on same media proliferated into nodular, lustrous callus showing embryogenic characteristics, but embryos were not observed. The time required to initiate callus was further lessen significantly by 10 more days on MS media supplemented with 2.4-D, at a lower concentration of 0.5 mg/L along with NAA at a concentration of 2.5 mg/L and BAP at variable concentrations of 0.25 mg/L, 0.5 mg/L and 2.5 mg/L as compared to media supplemented with 2,4-D, Kinetin and 2,4-D alone. The cytokinins along with auxins were useful in reducing the time required for callus induction.

The callus obtained was dark green to pale green in color and friable in texture on lower concentrations of BAP (0.25 mg/L and 0.5 mg/L) but on raising the concentration to 2.5 mg/L, the callus color turned pale yellow only in 18-20 days. Abdelmageed [18] reported highest rate of callus proliferation on all combination of BAP and 2,4-D in *Michelia champaca* using axillary bud explants when cultured on MS basal medium consisted of 0-0.2 mg/L IAA, 0-2.0mg/L 2,4-D along with 0-2.0mg/L BAP. Similarly, Sundersekar [19] reported the combination of 2,4-D along with BAP resulted

in 93.75% callus induction within 15 days, while same combination at higher concentrations took 40 days from inoculation to produce yellow friable callus in *Hymenocallis littoralis* using bulbs dissected from 4 weeks old plants. Kumar [20] observed in vitro callus induction using leaf explants on 2.0 mg/L of 2,4-D along with 0.05mg/L of BAP, while a higher concentration of 3.0 mg/L of 2,4-D resulted in compact brown callus in 20 days. This suggests that auxins along with cytokinins have critical role in callus proliferation in medicinal plants.

TABLE 3

DAYS OF PROLIFERATION, TEXTURE AND COLOUR OF CALLUS OBTAINED AFTER SUB CULTURING

S. No.	Media Code	Media Details	Days of proliferation	Texture	Color
1)	MSC-1	MS + 1.0 mg/L 2,4-D + 3.0% Maltose	5-10 days	Friable	Fluorescent green
2)	MSC-2	MS + 3.0 mg/L 2,4-D + 3.0% Maltose	5-10 days	Friable	Fluorescent green
3)	MSC-3	MS + 5.0 mg/L 2,4-D + 3.0% Maltose	10-15 days	Friable	Dark green
4)	MSC-4	MS + 5.0 mg/L BAP + 1.0 mg/L Kinetin + 3.0% Sucrose	10-15 days	Friable	Dark Green
5)	MSC-5	MS + 5.0 mg/L BAP + 1.0 mg/L 2,4-D + 3.0% Sucrose	10-15 days	Friable	Dark green
6)	MSC-6	MS + 5.0 mg/L BAP + 1.0 mg/L NAA + 3.0% Sucrose	10-15 days	Friable	Light green,Nodular
7)	MSC-7	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.25 mg/L BAP + 3.0% Dextrose	15-20 days	Friable	Fluorescent green
8)	MSC-8	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.5 mg/L BAP + 3.0% Dextrose	15-20 days	Friable	Fluorescent green
9)	MSC-9	MS + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 2.5 mg/L BAP + 3.0% Dextrose	15-20 days	Friable	Fluorescent green

In overall, the combination of kinetin along with 2,4-D proves to be crucial for induction of green colored and brittle callus from leaf explants as compared to BAP and NAA as growth regulators. The presence of Kinetin along with 2,4-D reduces the time for callus induction .But the reduction in callus induction time period was achieved by the presence of BAP in the media (Fig. 2). This view is supported by findings of [21], [22] and [23].

3.4 Callus Induction and Mass Proliferation

The callus induced or initiated were further subcultured on nine different media having different combinations of 2,4-D with kinetin and BAP as cytokinins with altered sugar source like maltose and dextrose for mass proliferation of callus and to explore embryogenic potential (Fig. 3). Callus subcultured on MS medium supplemented with 2,4-D (1.0 mg/L) and maltose (3.0%) as altered sugar source produced nodular lustrous callus showing embryogenic characteristics. This suggests that 2,4-D as an auxin provide a necessary stimulus for callus initiation at the cut edges of the explants. Similar result were observed on medium containing a higher concentration of 2,4-D (3.0 mg/L) and maltose (3.0%), while a higher concentration of 2,4-D (5.0 mg/L) along with 3.0% maltose take 5-7 days more to induce callus which lack luster.

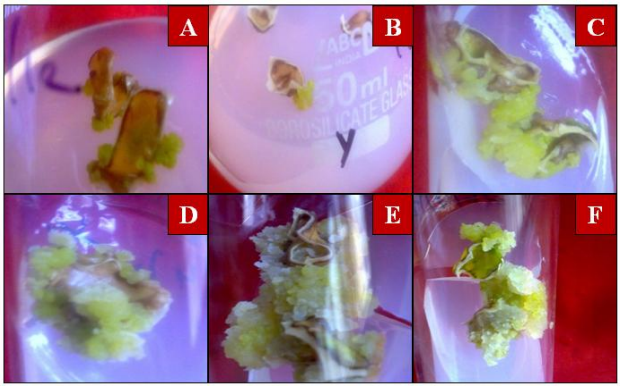

Fig. 2 Callus texture on MS basal medium fortified with (A) 3.0% sucrose + 1.0 mg/L 2,4-D (B) 3.0% sucrose + 5.0 mg/L 2,4-D (C) 3.0% sucrose + 1.0 mg/L 2,4-D + 1.0 mg/L Kinetin (D) 3.0% sucrose + 5.0 mg/L 2,4-D + 1.0 mg/L Kinetin (E) 3.0% sucrose + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.25 mg/L BAP (F) 3.0% sucrose + 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.5 mg/L BAP.

Proliferated Callus was transferred on medium supplemented with BAP (5.0 mg/L) at a higher and constant concentration and altering Kinetin (1.0 mg/L), NAA (1.0 mg/L) and 2,4-D (1.0 mg/L) one by one resulted in dark green color callus in 10-15 days, which turned brown with the passage of time. Callus subcultured on medium supplemented with 2,4-D (0.5 mg/L), NAA (2.5 mg/L), BAP (0.25 mg/L) along with dextrose at 3.0% concentration resulted in globular and fluorescent green color callus, while on increasing the concentration of BAP to 0.5 mg/L from 0.25mg/L and 3.0% dextrose resulted in globular and dark green color callus showing embryogenic characteristics (Fig. 3).

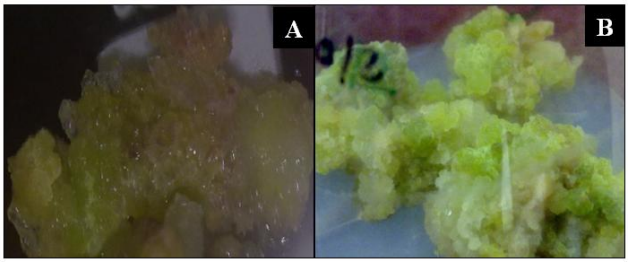

Fig. 3 Mass proliferation of callus on MS basal medium fortified with A) 0.5 mg/L 2,4-D + 2.5 mg/L NAA + 0.5 mg/L BAP + 3.0% dextrose B) 5.0 mg/L BAP + 1.0 mg/L NAA + 3.0% sucrose.

A further increase in the concentration of BAP to 5.0

mg/L resulted in a dark green color callus in 10-15 days, which resulted in increase in biomass only and it turned brown with the passage of time. Similar to our study, Gopi and Vatsala [24] reported higher callus yield on 0.5 mg/L of 2,4-D using leaf and nodal segments in 2-3 weeks, while a higher concentration of NAA (2.5 mg/L) resulted in maximum callus induction merely in 15 to 18 days .Our results does agree with the color and texture of callus obtained using different hormones as reported by [24]. Their results well suited to our results in using combination of 2,4-D , NAA along with BAP resulting in higher callus induction. On raising the concentration of BAP to 2.5 mg/L along with dextrose the callus turned fluorescent green in color in 15-20 days. Zhang [25] reported that MS medium supplemented with 2, 4-D and Kinetin and half concentration of NH4NO3 led to induction of embryogenic callus while media supplemented with KNO3 results in embryoid induction. The proembryoid development can be enhanced by transferring callus to media lacking NH4NO3 and containing double the amount of KNO3 as reported by [26] and was further supplemented with a different source of sugar i.e. maltose at a concentration of 3.0%. Addition of maltose separately produced yellow color callus in merely 10 days, while hypocotyls raised calli were transferred to medium consisted of a combination of BAP (1.5-3 mg/L) and NAA (1.0-1.5 mg/L) the callus proliferated into lustrous green, compact and globular having shiny appearance. A raised concentration of BAP and NAA induce stimulus for mass proliferation of callus which in turn is a prerequisite for any tissue culture technique. Similar findings were observed by [27] in *Leptadenia reticulate* where epicotyls cultured on 1.0 mg/L of 2,4-D produced regenerative cell culture when transferred to 1.0 mg/L BAP and 0.1mg/L NAA. The culture differentiated with high frequency through organogenesis into multiple shoots.

4 CONCLUSION

This study has established the callogenic potential of leaf explants of *Gymnema*. It has also established the embryogenic characteristics to produce somatic embryos from these induced calli. Further research to explore the possibility to generate complete plantlet from these embryos will be the next step. Meanwhile, this protocol serves itself as an efficient method to mass proliferate the callus and for clonal propagation of this plant for its conservation and vulnerability.

ACKNOWLEDGMENT

This study was financially supported by Department of Biotechnology Engineering, University Institute of Engineering & Technology, Kurukshetra University, Kurukshetra, Haryana, India. We also thank Tau Devi Lal Herbal Park, Yamunanagar, Haryana, India for proving us seedlings of the plant.

REFERENCE:

[1] P.V. Kanetkar, R.S. Singhal, K.S. Laddha, and M.Y. Kamat, "Extraction and Quantification of Gymnemic Acids Through Gymnemagenin from Callus Cultures of *Gymnema sylvestre*," Phytochemical Anaysis, vol. 17, pp. 409-413, 2007.

[2] T.B.B. Squier, and L.D. Peden, Herbal Folk Medicine. Henry Holt and Company, New York, pp. 34-37, 1997.

[3] L.H. Zhang, and P.G. Xiao, "Oleannae Saponins from *Gymnema sylvestre*," Phytochemistry, vol. 53, no. 8, pp. 893-899, 1993.

[4] T. Kuzuyama, "Pathways for the Biosynthesis of Gymnemic Acid Units," Bioscience Biotechnology and Biochemistry, vol. 66, no. 8, pp. 1619-1627, 2002.

[5] T. Kuzuyama, and H. Seto, "Diversity of the Biosynthesis of the Gymnemic Acids Units," Natural Product Reports, vol. 20, no. 2, pp. 171-183, 2003.

[6] P.S. Reddy, G.R. Gopal, and G.L. Sita, "*In-vitro* Multiplication of *Gymnema sylvestre* R.Br.- An Important Medicinal Plant," Current Science, vol. 75, pp. 843-847, 1998.

[7] N. Komalavalli, and M.V. Rao, "*In-vitro* Micropropagation of *Gymnema sylvestre*- A Multipurpose Medicinal Plant," Plant Cell and Tissue Organ Culture, vol. 61, pp. 97-105, 2000.

[8] P.S. Udayan, K.V. Tushar, George S., and Balachandran I., "Notes on a Few Rare, Endemic and Red Listed Plants from Trumula Forest of Tirupati Chittor District, Andhra Pradesh," Journal of Economic & Taxonomy Botany, vol. 33, pp. 277-284, 2009.

[9] T.B. Jha, and B. Ghosh, Plant Tissue Culture Basic and Applied. Universities Press, New Delhi, India, pp. 1-4, 2005.

[10] R. Baksha, M.A. Jahan, R. Khatun, and J.L. Munshi, "Micropropagation of Aloe barbadensis Mill. Through *in-vitro* Culture of Shoot Tip Explants," Plant Tissue Culture & Biotechnology, vol. 15, pp. 121-126, 2005.

[11] S. Biswas, N. Dasgupta, and B. De, "Antioxidant Activity of Some Indian Medicinal Plants used for the Treatment of Diabetes Mellitus," Pharmacologyonline, vol. 3, pp. 7-12, 2010.

[12] A. Roy, S. Ghosh, M. Chaudhuri, and P. Saha, "Effect of Different Plant Hormones on Callus Induction in *Gymnema* sylvestris R.Br. (Asclepiadaceae)," African Journal of Biotechnology, vol. 7, no. 13, pp. 2209-2211, 2008.

[13] R. Bhadra, S. Vani, and J.V. Shanks, "Production of Indole Alkaloids by Selected Hairy Root Lines of Catharanthus roseus," Biotechnology Bioengineering, vol. 41, pp. 581-592, 1993.

[14] T. Murashige, and F. Skoog, "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures" Physiologia Plantorum, vol. 15, pp. 473-497, 1962.

[15] Y. Zafar, E. Nenz, F. Damiani, F. Pupilli, and S. Arcioni, "Plant Regeneration from Explants and Protoplast Derived Calluses of *Medicago littoralis*," Plant Cell Tissue and Organ Culture, vol. 41, pp. 41-48, 1995.

[16] S.O. Amoo, and B.E. Ayisire, "Induction of Callus and Somatic Embryogenesis from Cotyledon Explants of *Parkia biglobosa* (jacq) Benth," African Journal of Biotechnology, vol. 4, no. 1, pp. 68-71, 2005.

[17] D. Xie, and Y. Hong, "*In-vitro* Regeneration of *Acacia mangium* via Organogenesis," Plant Cell, Tissue and Organ Culture, vol. 66, pp. 167-173, 2001.

[18] A.H.A. Abdelmageed, Q.Z. Faridah, K.N. Suhada, and A.A. Julia, "Callus Induction and Plant Regeneration of *Michelia champaca* (Magnoliaceae): A Multipurpose Tree," Journal of Medicinal Plant Research, vol. 6, no. 17, pp. 3338-3344, 2012.

[19] J. Sundarasekar, J.J.J. Anthony, V. Murugaiyah, and S. Subramaniam, "Preliminary Responses of 2,4-D and BAP on Callus Initiation of an Important Medicinal Ornamental *Hymenocallis littoralis* plants," Journal of Medicinal Plant Research, vol. 6, no. 11, pp. 2088-2093, 2012.

[20] H.G.A. Kumar, H.N. Murthy, and K.Y. Paek, "Somatic

Embryogenesis and Plant Regeneration in *Gymnema sylvestre*," Plant Cell Tissue and Organ Culture, vol. 71, pp. 85-88, 2010.

[21] A.B. Dongre, S.B. Nandeshwar, K.R. Kranthi, S. Kranthi, and A.K. Basu, "Induction of Somatic Embryogenesis in a Range of Cotton Cultivars," Proc. World Cotton Research Conference Challenging the Future, pp. 356-358, 1994.

[22] I.S. Katageri, and B.M. Khadi, "Somatic Embryogenesis in Cultivated Cotton (Gossypium spp.)," Journal of Indian Society Cotton Improvement, 23, pp. 184-191, 1998.

[23] B.H. Zhang, R. Feng, F. Liu, and Q. Wang, "High Frequency Somatic Embryogenesis and Plant Regeneration of an Elite Chinese Cotton Variety," Bot. Bull. Acad. Sin, vol. 42, pp. 9-16, 2001.

[24] C. Gopi, and T.M. Vatsala, "*In-vitro* Studies on Effects of Plant Growth Regulators on Callus and Suspension Culture Biomass Yield from *Gymnema sylvestre* R.Br.," African Journal of Biotechnology, vol. 5, no. 12, pp. 1215-1219, 2006.

[25] H.S. Zhang, J.L. Li, P. Gao, and H.B. Wang, "Embryogenesis and Plant Regeneration from Somatic Cell Culture of Glandless Upland Cotton," Hebei agricultural University, vol. 22, pp. 9-12, 1999.

[26] G.H. Davidonis, and R.H. Hamilton, "Plant Regeneration from Callus Tissue of *Gossypium hirsutum* L," Plant Science letters, vol. 32, pp. 89-93, 1983.

[27] V. Arya, N.S. Shekhawat, and R.P. Singh, "Micropropagation of *Leptadenia reticulate* - A Medicinal Plant," In Vitro Cell Developmental Biology, vol. 39, pp. 180-185, 2003.

** Sunita Khatak is currently working as Assistant Professor in University Institute of Engineering & Technology, Kurukshetra University, Kurukshetra, Haryana (India). E-mail: sunkhatak@rediffmail.com*

[1]Sonia Baches, Nitin Chauhan and Amandeep Kaur are currently pursuing masters degree program in Biotechnology engineering in University Institute of Engineering & Technology, Kurukshetra University, Kurukshetra, Haryana (India).

IJSER