In-vitro Callogenesis and Multiple Shoot formation of *Rauvolfia serpentina* (L.) Benth.

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Abstract- Medicinally important and endangered plant species *R. serpentina* belongs to family Apocynaceae, is a woody perennial shrub which has been used for treatment of different mental disorders. In this study, *In-vitro* propagation (Callus and shoot formation) of *R. serpentina* was studied by using different explants (Shoot tips, Nodal parts, internode parts and leaves) under the influence of different concentrations of BAP in combination with different concentrations 2-4 D and NAA. In both combinations of PGRs (BAP+2,4-D and BAP+NAA), highest percent shoot induction and highest percent callus formation was achieved from nodal part and leave explant respectively. PGRs concentration (1.0mg/l BAP + 0.5 mg/L 2-4 D) result higher number of shoot and highest callus formation (70%) from nodal and leaf explant respectively while no callus was observed all explant part used except leave explant. Direct shoot formation was not observed in internode explant in all concentrations and combinations of PGRs used. PGRs concentrations (1.0mg/l BAP + 0.5 mg/L 2-4 D) and (0.8mg/l BAP + 0.2 mg/L NAA) gave explant gave highest percent callus formation from leave explant. The combination of BAP with 2-4 D gave less number of multiple shoots. The combined effect of BAP, 2-4 D and NAA at different concentrations was thus found significant and gave moderate formation of callus and multiple shoots from the explants. The combined effect of BAP, 2-4 D and NAA at different concentrations was thus found significant and gave moderate formation of callus and multiple shoots from the explants. The optimized protocol developed in our work could be useful in micro-propagation, large-scale multiplication and conservation of this endangered plant *R.serpentina.*

Index Terms- Tissue culture techniques, Medicinal plant, BAP, multiple shoot formation, callogenesis

I. INTRODUCTION

Rauwolfia serpentina L. Benth. ex. Kurz belongs to family Apocynaceae, is a woody perennial shrub, commonly known with different names; sarpagandha, snake root plant, chotachand, chandrika etc. The roots of this plant have been used for centuries in ayurvedic medicines under the name sarpagandha and nakuli for the treatment of mental disorders. This plant contains alkaloids such as reserpine, ressinamine and yohmbine are used to cure various neurological ailments (1). The root contains ophioxylin (an alkaloid having orange colored crystalline principle), resin, starch and wax. The total alkaloid yield is 0.8%. Five crystalline alkaloids isolated are ajmaline, ajmalicine, serpentine, serpentinine and yohimbine. It has been stated that the drug is useful in mental disease, epilepsy, sleeplessness and several other ailments. According to Ayurveda root is bitter, acrid, heating, sharp, and pungent and anthelmintic. Drug *Rauwolfia* consists of air-dried roots. *Rauwolfia* preparations are used as antihypertensive and as sedative. It is also used for the treatment of various central nervous system disorders associated with psychosis, schizophrenia, insanity, insomnia, and epilepsy. It is an endangered medicinal plant found in Bangladesh, Bhutan, China, Indonesia, India, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka and Vietnam (2). Plant tissue culture, or the aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions *in-vitro*, is an important tool in both basic and applied studies as well as in commercial application. It is an applied science and forms the backbone of plant biotechnology. The best quality of planting material is the basic need of growers for increasing productivity (3). Plant tissue culture techniques have become powerful tools for studying and solving the basic and applied problems in plant propagation and genetic manipulation. In recent years these techniques have gained greater momentum in commercial applications in the field of plant propagation, mainly in horticulture as well as for medicinal plant species (4). A number of medicinally important plant species have been successfully propagated on a mass scale with the use of *in-vitro* techniques (5). *In-vitro* propagation offers not only a means for mass multiplication of existing germplasm stocks, but also for the conservation of important elite and RET (Rare, endangered & threatened species) which are facing the danger of extinction (6). All the cells in an organism carry the same genetic information, yet show variation in expression. Our knowledge of cell and tissue culture has been developing with full swing specially in biotransformation, forestry, genetic engineering, Morphogenesis, somatic hybridization, secondary metabolite production, hybridization variety development and their conservation, maintaining pathogen free plants and rapid clonal propagation,

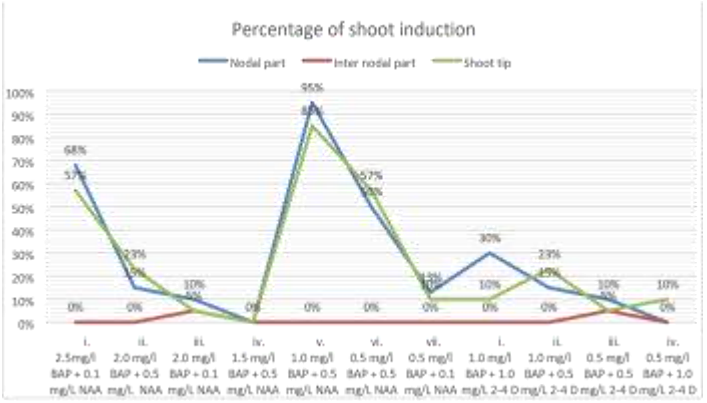
totipotency, differentiation, cell division, cell nutrition, metabolism, radio biology, cell preservation, etc. It is now possible to cultivate cell in quantity, or as clones from single cells, to grow whole plant from isolated meristems and to induce callus or even single cell to develop into complete plant either by organogenesis or directly by embryogenesis *in-vitro* (7) for conserving this medicinal plants.

II. MATERIALS AND METHODS

Shoot tips, Nodal parts, internode parts and leaves of *Rauvolfia serpentina* grown in Angiosperm Garden of Department of Botany, University of Peshawar were collected. The experiments were performed at Plant tissue culture laboratory Agriculture research institute ARI Quetta and Tissue culture laboratory department of Botany, University of Peshawar. Collected plant parts were washed under running tap water for 30 min to remove soil and mud from the surface. After washing, explants sterilization was done by treating them with 1.0% mild detergent (Tween-20) for 5 min, rinsed off with autoclaved distilled water followed by treatment with mercuric chloride treatment (0.1% w/v) for 2 min and then washed 2 - 3 times with autoclaved distilled water. After that, the explants were treated with sodium hypochlorite (50%, v/v) for 2 min and gave final wash with distilled water 2 - 3 times. Finally they were washed 4 - 5 times properly with sterile double distilled water and then placed on sterile filter paper sheets to remove moisture about 0.5-1 cm explants (Shoot tips, Nodal parts, internode parts and leaves) were excised and inoculated in glass test tubes (150 mm X 25 mm) containing Murashige and Skoog (MS) basal media supplemented with 3.0% sucrose (w/v), 100 mg.L -1 myoinositol and 0.7% agar in laminar cabinet. To check the effect of different plant growth regulators (BAP, 2-4 D and NAA) which are used in different concentrations and combinations (BAP + 2-4 D and BAP + NAA) on direct multiple shoot formation and Callus Induction of *Rauvolfia serpentina* Table 1. The pH of medium was adjusted to 5.8 before autoclaving at 121˚C for 20 min (8). The explants cultures were incubated under a 16/8-h (light/ dark) photoperiod with light supplied by cool-white fluorescent lighting at an intensity of 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity at a constant temperature of 25˚C ± 2˚C. The callus cultures were sub cultured at regular intervals of 4 weeks.

III. RESULTS & DISCUSSION

Multiple *in-vitro* experiments which involved two different combinations of hormones, (BAP + 2, 4-D and BAP + NAA) with different concentrations were performed to study the effect of growth regulators on the callogenesis and direct shoot formation of *Rauwolfia serpentina*. The mother plant was grown with the help of tissue culture technique (9). The experiments were performed on the medicinal plant "*Rauwolfia serpentina*". The callus and direct shoot were formed through tissue culture technique by using leaf, nodal part, internodal part and shoot tip as an explant as shown in (Fig 1 and 2).



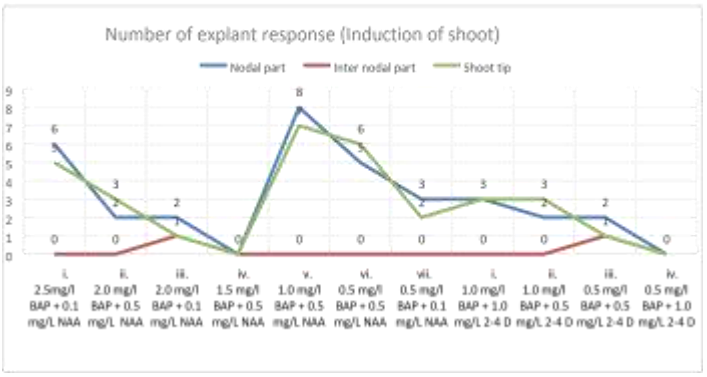
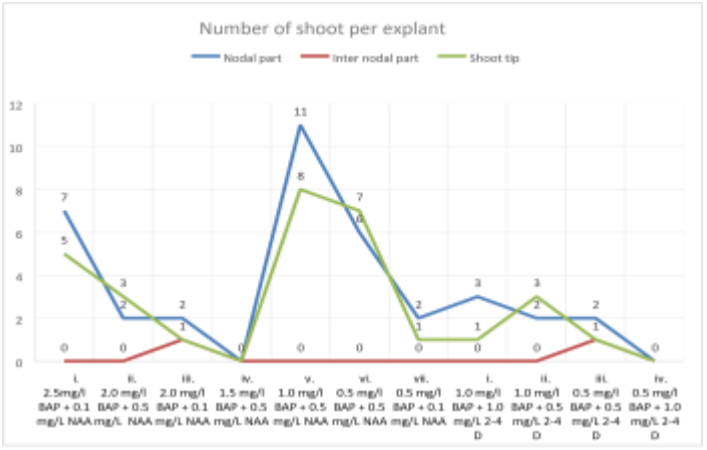


Fig. 1: Percentage of shoot induction (A) & induction of shoot (B) of node, intermodal and shoot tip.



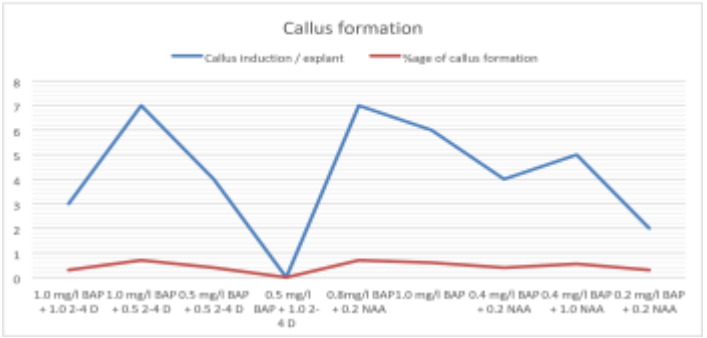


Fig. 2: Number of shoot per plant (A) & callus formation (B) of node, intermodal and shoot tip.

Effect of BAP and 2-4D on direct multiple shoot formation

Direct multiple shoot formation was observed during 2nd week of culture. The shoots were about 4 cm in height. Higher number of shoot formation was observed in nodal explant at a concentration of 1.0mg/l BAP + 0.5 mg/L 2-4 D. From the results it is evident that higher concentration of cytokinin in combination with auxin could induce multiple shoot elongation. Richa Bhatt also reported that higher conc of BAP to low conc of 2-4 D promotes multiple shoot induction as shown in (Table 1) (1). Multiple shoot formation from nodal segments was reported by Salma *et al*., (10). In their experiments, an addition of BAP to the medium also increased the number of explants forming shoots. Similarly, other sources show that 1mg/l BAP enrichment is the most effective concentration of the regulator for this species (11). It has been previously found that regeneration of flax plants is more likely to succeed from hypocotyl segments, and relatively harder from leaf explants, the callus or the protoplasts fusion (12). The available literature demonstrates that flax appears to be very prone to any modifications in the media including the addition of various growth regulators at different concentrations. Not only the growth regulators presented in this study are commonly used, there were also others e.g. Thidizuron (TDZ) or 2iP (13,14). According to several studies, BAP is one of the most effective hormones that may be used to improve the regeneration abilities of flax under *in-vitro* conditions (15,16).

Table 1: **Effect of BAP and 2-4 D on direct multiple shoots formation in 2 weeks of 10 explants each.**

GR	mg/l	TOE	NOER	SI %age	CF	No. of shoots/ explant
BAP + 2-4 D	1.0 + 1.0	Nodal part	3	30%	++	3
		Inter nodal part	-	0%	-	-
		Shoot tip	2	10%	++	1
	1.0+0.5	Nodal part	2	15%	+++	2
		Inter nodal part	-	0%	+	0
		Shoot tip	3	23%	+++	3
	0.5+ 0.5	Nodal part	2	10%	++	2
		Inter nodal part	1	5%	++	1
		Shoot tip	1	5%	++	1
	0.5+1.0	Nodal part	-	0%	++	-
		Inter nodal part	-	0%	++	-
		Shoot tip	-	0%	+	-

Key: GR= Growth regulator, TOE= Type of Explant, NOER= No. of explant response, SI= Shoot induction, CF= Callus formation

Effect of BAP and NAA on direct multiple shoot formation

In our set of experiments, it was observed in the nodal explant at 1.0mg/l BAP + 0.5mg/l NAA after 2 weeks show shoot induction from explant. The concentration was reviewed from Bahuguna *et al.,* (17). These were again reviewed by (18,19). Elimination of NAA will affect the shoot induction adversely if NAA not added it will give u no shoot as shown in (Table 2).

Table 2: **Effect of BAP and NAA on direct multiple shoot formation in 2 weeks of 10 explants each.**

GR	mg/l	TOE	NOER	SI %age	CF	No. of shoots/ explant
BAP + NAA	2.5+0.1	Nodal part	6	68%	+	7
		Inter nodal part	-	0%	-	-
		Shoot tip	5	57%	+	5
	2.0+0.5	Nodal part	2	15%	+++	2
		Inter nodal part	-	0%	+	0
		Shoot tip	3	23%	+++	3
	2.0+ 0.1	Nodal part	2	10%	++	2
		Inter nodal part	1	5%	++	1
		Shoot tip	1	5%	++	1
	1.5+0.5	Nodal part	-	0%	++	-
		Inter nodal part	-	0%	++	-
		Shoot tip	-	0%	+	-
	1.0+0.5	Nodal part	8	95%	+++	11
		Inter nodal part	-	0%	+	0
		Shoot tip	7	85%	+++	8
	0.5+ 0.5	Nodal part	5	50%	++	6
		Inter nodal part	-	0%	++	-
		Shoot tip	6	57%	++	7
	0.5+0.1	Nodal part	3	13%	++	2
		Inter nodal part	-	0%	-	-
		Shoot tip	2	10%	++	1

Key: GR= Growth regulator, TOE= Type of Explant, NOER= No. of explant response, SI= Shoot induction, CF= Callus formation

Effect of BAP along with 2, 4-D on Callus Induction

In our set of experiments, callus induction was observed in leaf explant when 1.0 mg/l BAP + 0.5 mg/L 2-4 D, after 2 weeks of culture. It has been reported that using comparatively low concentrations (0.125 and 0.250 mg/l) of 2-4 D have also initiate callus induction. They have also reported that BAP (1.0 and 1.5 mg/l) alone did not induce direct regeneration from apical and nodal explants of *R. serpentina* (1). But according to Bahuguna *et al.,* (17) 2, 4-D (2.0) + BAP (1.0) show good response in callus formation as shown in (Table 3). These concentrations were also suggested by Panwar *et al.,* (20). A callus culture medium containing low concentration of cytokinin often enhances callus induction (21,22). Prabhat Singh in his work used different concentrations (1 to 2.5 mg/L)

of BAP and maximum callusing frequency (77 %) was observed at lowest concentration 1 mg/L used.

Table 3: **Effect of BAP along with 2, 4-D on Callus Induction 10 leaf explants in 2 weeks.**

BAP + 2-4 D	Callus induction / explant	%age of callus form	Callus Color
1.0 + 1.0	3	30%	Yellowish green
1.0 + 0.5	7	70%	White
0.5 + 0.5	4	40%	Yellowish Brown
0.5 + 1.0	0	0%	Brown & dead

*******Effect of BAP and NAA on Callus Induction***

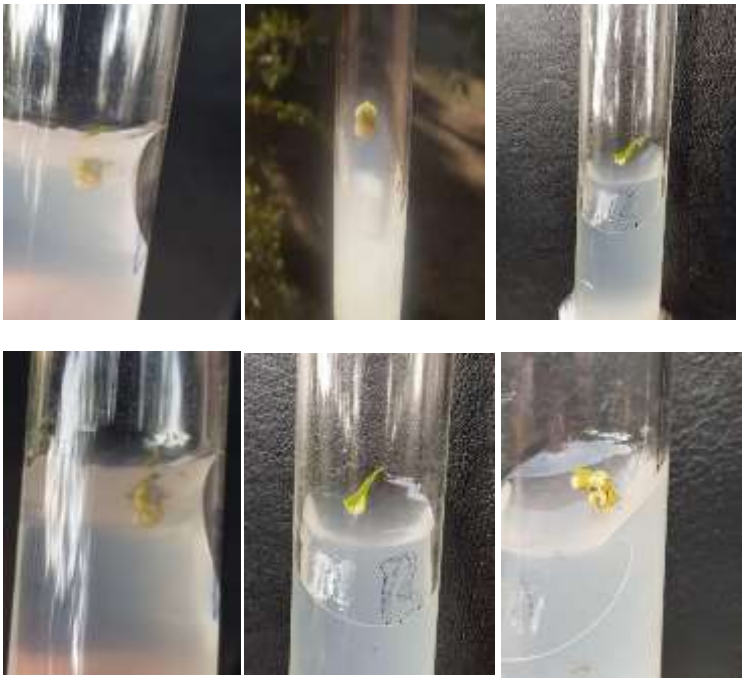
The combination of BAP and NAA also show good callus induction. In our set of experiments the callus induction was observed in leaf explant at 0.2mg/l BAP + 0.8mg/l NAA in full strength MS medium in 2 weeks. Pant *et al.,* (23) has also reported that lower concentration of BAP (cytokinin) to high concentration of NAA (auxin) promotes callus induction of *R. serpentina*. In their work, they achieved high callus induction by using low concentration of cytokinin (0.1 mg/l BAP) in combination with high concentration (1.0 mg/l NAA) of auxin. These concentrations were also reported by Choudhary *et al.,* (24) but Panwar *et al*., (20) got no result on the combination of NAA and BAP as shown in (Table 4).

Table 4**: Effect of BAP along with NAA on Callus Induction 10 leaf explants in 2 weeks.**

BAP + NAA	Callus induction / explant	% age of callus formation	Callus Color
08 + 0.2	7	70%	Yellowish green
1.0 + 0	6	60%	White
0.4 + 0.2	4	40%	Yellowish Brown
0.4 + 1.0	5	55%	Green
0.2 + 0.2	2	20%	Brown

Multiple shoots induction

Callus induction



IV. CONCLUSION

It could be concluded that direct multiple shoot formation was the best from the nodal part of *Rauwolfia serpentina* L. using 1.0 mg/L BAP + 0.5 mg/L NAA. This combination induced higher number shoots. On the other hand, callus was also observed at a concentration of 1.0 mg/L BAP + 0.5 mg/L 2, 4-D. The use of 0.5mg/l NAA also induced callus formation. Similarly, moderate amount of BAP + NAA also induced shoots formation and callus induction.

REFERENCES

[1]. Bhatt, R., Arif, M., Gaur, A. K., & Rao, P. B. (2008). Rauwolfia serpentina: Protocol optimization for *in-vitro* propagation. *African Journal of Biotechnology*, *7*(23).

[2]. De, S., Dey, Y. N., & Ghosh, A. K. (2010). Phytochemical investigation and chromatographic evaluation of the different extracts of tuber of Amorphaphallus paeoniifolius (Araceae). *Int J Pharm Biol Res*, *1*(5), 150-7.

[3]. Rout, G. R., Mohapatra, A., & Jain, S. M. (2006). Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. *Biotechnology advances*, *24*(6), 531-560.

[4]. Teng, W. L., Sin, T., & Teng, M. C. (2002). Explant preparation affects culture initiation and regeneration of Panax ginseng and Panax quinquefolius. *Plant cell, tissue and organ culture*, *68*, 233-239.

[5]. Sahoo, Y., Pattnaik, S. K., & Chand, P. K. (1997). In vitro clonal propagation of an aromatic medicinal herb

Ocimum basilicum L.(sweet basil) by axillary shoot proliferation. *In Vitro Cellular & Developmental Biology-Plant*, *33*, 293-296.

[6]. Srivastava, A. K., Diengdoh, L. C., Rai, R., Bag, T. K., & Singh, B. P. (2012). In vitro micropropagation and micro-tuberization potential of selected potato varieties. *Indian Journal of Hill Farming*, *25*(2), 14-17.

[7]. Vyas, P., Debnath, S. C., & Igamberdiev, A. U. (2013). Metabolism of glutathione and ascorbate in lingonberry cultivars during in vitro and ex vitro propagation. *Biologia plantarum*, *57*(4), 603-612.

[8]. Asad, M., Ahmed, N., Sohail, A., Burni, T., Hadi, F., Ali, R., & Muhammad, A. (2020). 35. In vitro shoots multiplication from nodal explants of Sesame (Sesamum indicum L.). *Pure and Applied Biology (PAB)*, *9*(1), 303-308.

[9]. Jabeen, S., Khaliq, I., Togo, J., Sajjad, M., & ul Malook, S. (2016). Tissue culture technology is a breeding approach in wheat: An overview. *Molecular Plant Breeding*, *7*(13).

[10]. Salma, U., Rahman, M. S., Islam, S., Haque, N., Jubair, T. A., Haque, A. K., & Mukti, I. J. (2008). The influence of different hormone concentration and combination on callus induction and regeneration of Rauwolfia serpentina L. Benth. *Pakistan Journal of Biological Sciences: PJBS*, *11*(12), 1638-1641.

[11]. Burbulis, N., Blinstrubienė, A., Sliesaravičius, A., & Kuprienė, R. (2007). Some factors affecting callus induction in ovary culture of flax (Linum usitatissimum L.). *Biologia*, *53*(2).

[12]. Yildiz, M., & Özgen, M. (2004). The effect of a submersion pretreatment on in vitro explant growth and shoot regeneration from hypocotyls of flax (Linum usitatissimum). *Plant Cell, Tissue and Organ Culture*, *77*, 111-115.

[13]. Yildiz, M., & Özgen, M. (2006). A comparison of growth regulators for adventitious shoot regeneration from hypocotyls of flax (Linum usitatissimum L.).

[14]. Burbulis, N., Blinstrubiene, A., Kupriene, R., Jonytiene, V., Rugienius, R., & Staniene, G. (2009). In vitro regeneration of Brassica napus L. shoots from hypocotyls and stem segments. *Zemdirbyste-Agriculture*, *96*(3), 176-185.

[15]. Zhan, H. J., Levery, S. B., Lee, C. C., & Leigh, J. A. (1989). A second exopolysaccharide of Rhizobium meliloti strain SU47 that can function in root nodule invasion. *Proceedings of the National Academy of Sciences*, *86*(9), 3055-3059.

[16]. Janowicz, J., & Wojciechowski, A. (2009). The evaluation of regeneration ability of two flax (Linum usitatissimum L.) cultivars in in vitro culture. *Rośliny Oleiste*, *30*(1), 35-49.

[17]. Bahuguna, R. N., Joshi, R., Singh, G., Shukla, A., Gupta, R., & Bains, G. (2011). Micropropagation and total alkaloid extraction of Indian snake root (Rauwolfia serpentina). *Indian Journal of Agricultural Sciences*, *81*(12), 1124-9.

[18]. Sudha, G. G., & Seeni, S. (1996). *In-vitro* propagation of *Rauwolfia micrantha*, a rare medicinal plant. *Plant cell, tissue and organ culture*, *44*(3), 243-248.

[19]. Faisal, M., Ahmad, N., & Anis, M. (2005). Shoot multiplication in Rauvolfia tetraphylla L. using thidiazuron. *Plant Cell, Tissue and Organ Culture*, *80*(2), 187-190.

[20]. Panwar, G. S., Attitalla, I. H., & Guru, S. K. (2011). An efficient *in-vitro* clonal propagation and estimation of reserpine content in different plant parts of *Rauwolfia serpentina* L.

[21]. Altpeter, F. and U. K. Posselt (2000). "Improved plant regeneration from cell suspensions of commercial cultivars, breeding-and inbred lines of perennial ryegrass (Lolium perenne L.)." Journal of plant physiology 156(5-6): 790-796.

[22]. Singh, P., Singh, A., Shukla, A. K., Singh, L., Pande, V., & Nailwal, T. K. (2009). Somatic embryogenesis and in vitro regeneration of an endangered medicinal plant sarpgandha (Rauvolfia serpentina L.). *Life Science Journal*, *6*(2), 57-62.

[23]. Pant, K. K., & Joshi, S. D. (2008). Rapid Multiplication of Rauvolfia serpentina Benth. ex. kurz through tissue culture. *Scientific World*, *6*(6), 58-62.

[24]. Choudhary, A. K., Ranjan, A., & Kumari, P. (2016). In vitro shoot proliferation for rapid and mass production of quality planting materials of Bambusa nutans in the climatic conditions of Bihar, India. *Indian Journal of Energy*, *5*(2), 1-11.