Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; SP5: 250-254

ML Jakhar
Department of Plant Breeding & Genetics, SKN College of Agriculture SKN Agriculture University, Jobner, Rajasthan, India

Rajani Verma
Department of Plant Breeding & Genetics, SKN College of Agriculture SKN Agriculture University, Jobner, Rajasthan, India

Divya Dixit
Department of Plant Breeding & Genetics, SKN College of Agriculture SKN Agriculture University, Jobner, Rajasthan, India

Correspondence
ML Jakhar
Department of Plant Breeding & Genetics, SKN College of Agriculture SKN Agriculture University, Jobner, Rajasthan, India

(Special Issue- 5)
International Conference on
"Food Security through Agriculture & Allied Sciences"
(May 27-29, 2019)

Effect of antioxidants on *in vitro* degree of browning and culture establishment of Guggul [*Commiphora wightii* (Arnott)]: A valuable desert medicinal plant

ML Jakhar, Rajani Verma and Divya Dixit

Abstract
Commiphora wightii (Arnott) is an important medicinal plant, belongs to *Burseraceae* family. This plant is considered as an endangered plant in India and listed as "Data Deficient" in the IUCN Red Data List because of excessive exploitation for taping of gum causes mortality of plants, lack of knowledge for its conservation, poor germination capacity of seed, lack of proper vegetative propagation method and unscientific cultivation. Therefore, there is an urgent need to conserve this species *ex situ* through *in vitro* methods. However, under *in vitro* medium browning is a major problem in *Commiphora wightii* due to the exudation of high amount of phenols, especially in mature explants (nodal segment and shoot apex). In present study an attempt has been made to demonstrate the effect of antioxidants on *in vitro* degree of browning and culture establishment of guggul. Different antioxidants *viz*; activated charcoal, PVP, ascorbic acid and citric acid have been tried to eliminate medium browning problem through incorporation on the responsive culture medium for shoot proliferation (nodal segment, 1.5 mg/l BAP and shoot apex, 2.0 mg/l Kn) and callus induction (leaf explants, 2.0 mg/l 2,4-D). Among the different antioxidants, activated charcoal (150-200 mg/l) was found better in reducing browning of medium, establishment of culture and promoted shoot proliferation and callus differentiation because it controls the accumulation of inhibitory substances and it immediately absorbs plant growth regulators and vitamins from the medium and gradually releases them again in the medium. Maximum callus differentiation in leaf explants was observed at 250 mg/l activated charcoal followed by 200 mg/l. Among all the antioxidants polyvinyl pyrroli done was least effective to proliferate shoot bud and callus induction.

Keywords: Nodal segment, shoot apex, leaf, callus, antioxidants, *in vitro*, phenolic, browning, establishment, guggul

Introduction
Plants are an important source of medicines and play a key role in health in almost all regions of the world, from ancient times to till now (Kala *et al*., 2006) [6]. *Commiphora wightii* (Arnott) is a plant of immense medicinal important plant belongs to family Burseraceae and having the chromosome number 2n = 26 (Sobti and Singh, 1961) [17] and is listed in IUCN Red Data List. Due to poor germination, slow growth, uncontrolled exploration for gum, lack of proper vegetative propagation method and unscientific cultivation this plant considered as critically endangered plant. Therefore, there is an urgent need to conserve this species ex situ through *in vitro* methods. Under *in vitro* culture condition there is problem of browning of media due to leaching of some phenolic substances from cut surfaces of explants (Ozyigit, 2008) [12]. Phenolic compounds occur as secondary metabolites in all plant species (Kefeli *et al.*, 2003) [7]. These compounds oxidize later and turn the media brown. After oxidation these compound releases the free radicals which becomes toxic to the explants and results in retardation of growth and eventually leads to complete failure to survivability of the explants. Medium browning is a major problem in *Commiphora wightii* due to the exudation of high amount of phenols, especially in mature explants (nodal segment and shoot apex). In perennial bushes, establishment of explants requires special procedures to escape the problem that associated with exudation of phenolic compounds from cut surface. Different attempts has been made to eliminate browning problem in woody plant species like pre-soaking of explants

In antioxidant (Activated charcoal, PVP, ascorbic acid, citric acid etc.) Solution, incorporation of oxidants into medium, incubation of culture in to dark period and frequent subculturing of explants.

Different antioxidants are commonly used in tissue culture media to improve cell growth and development (Pan and Staden, 1998 and Thomas, 2008) [13, 18]. These plays important role in irreversible adsorption of inhibitory compounds in the culture medium and substantially reduces the toxic metabolites, phenolic exudation and exudate accumulation (Fridborg *et al*., 1978 and Thomas, 2008) [5, 8]. They are able to scavenge oxygen radicals produced when the plant tissue is wounded, therefore protecting the cells from oxidative injury. Therefore, antioxidant growth regulators should be considered as one of the most important factor for the development of a standard tissue culture protocol for mass multiplication of any plant species (Dayarani *et al*., 2013) [3]. Thus present investigation was carried out to assess the effect of antioxidants on *in vitro* culture

Materials and Methods

The present investigation was carried out in Tissue Culture Laboratory, Department of Plant Breeding and Genetics, S. K. N. College of Agriculture, Jobner. Three explants *viz*., nodal segments, shoot apex and leaves were used as explant in the present investigation. All the explants were washed with liquid detergent under running tap water for 20 minutes to remove dust particles. These were again washed with liquid detergent (Rankleen) for ten minutes with vigorous shaking. After washing with detergent, explants were again washed with running tap water to remove any trace of detergent for 5 minutes. After it were sterilized with bavistin for 5-10 minutes and then washed with double distilled water 4-5 times, then sterilized with 0.1 per cent HgCl2 for 2-5 min depending upon the nature of explants. Thereafter, the explants were again washed 4-5 times with autoclaved distilled water. After sterilization the explants were inoculated on culture media aseptically.

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 20C under fluorescent light in a 14:10 hour's photoperiod. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). Different antioxidants *viz*., activated charcoal, ascorbic acid, citric acid and polyvinylpyrrolidone were added to control the accumulation of phenolic compounds in the culture medium to enhance the rate of micro propagation were worked out at most responsive level of plant growth regulators differentiation (Nodal segment, 1.5 mg/l BAP and shoot apex, 2.0 mg/l Kn and callus induction in leaf explants, 2.0 mg/l 2, 4-D). The following levels of antioxidants were tested.

1. Activated charcoal (50, 100, 150, 200, 250 and 300 mg/l).
2. Ascorbic acid (50,100,150, 200, 250 and 300 mg/l).
3. Citric acid (10, 20, 30, 40, 50 and 60 mg/l).
4. Polyvinylpyrrolidone (5, 10, 15, 20, 25 and 30 mg/l).

Result

When different antioxidants incorporated singly in MS medium supplemented with responsive level of plant growth regulators for shoot bud differentiation (Nodal segment, 1.5 mg/l BAP and shoot apex, 2.0 mg/l Kn) and callus induction (leaf explants, 2.0 mg/l 2, 4-D) elicited different response for shoot bud induction and callus differentiation because it controls the accumulation of inhibitory substances (phenolic compounds) in the growth medium (Table 1- 2).

Effect of activated charcoal

When activated charcoal (50 - 300 mg/l) was added in the basal medium with micro propagation protocol for nodal segment (1.5 mg/l BAP), it induced shoots at all the level of activated charcoal ranging from 1.7 – 2.1 within 16 – 20 days of incubation. Maximum number of shoot bud (2.1) was observed at 200 mg/l activated charcoal with low browning intensity in the culture medium (Fig. 1). All other level showed medium browning except 200 mg/l BAP. Similarly, when shoot apex explant incubated on MS medium supplemented with 2.0 mg/l Kn along with different levels (50-300 mg/l) of activated charcoal. Maximum shoot bud indication (1.9) was observed at 150 mg/l activated charcoal followed by 200 mg/l activated charcoal with low intensity of browning in the medium (Fig. 2). All other levels of activated charcoal showed medium browning in the medium, however, (>150 mg/l) activated charcoal showed low to medium browning in the medium with comparatively less number of shoot buds due to inhibitory effect on growth through hampering the nutrient absorption.

When activated charcoal (50 - 300 mg/l) was added in the basal medium for callus induction in leaf explants (2.0 mg/l 2, 4-D), it induced callus at all the level of activated charcoal. Maximum callus proliferation (1.15 g) was observed at 250 mg/l activated charcoal followed by (0.98 g) at 200 mg/l activated charcoal with low intensity of browning in the medium (Table 2 and Fig. 3).

Effect of ascorbic acid

When ascorbic acid was added in the MS medium with 1.5 mg/l BAP for nodal segment explants along with different level of ascorbic acid. Maximum shoot bud induction (1.9) was observed at 150-200 mg/l ascorbic acid in nodal segment explants with low intensity of browning in the medium. Both lower and higher levels of ascorbic acid showed medium browning in the medium. When shoot apex explants incubated on MS medium supplemented with 2.0 mg/l Kn along with different level of ascorbic acid. Maximum shoot bud induction (1.8) was observed at 100 mg/l ascorbic acid in shoot apex explants with low intensity of browning in the medium followed at 50,150, 200 and 250 mg/l ascorbic acid levels with low browning in the medium (Table 1).

Similarly, when leaf explant incubated on MS medium supplemented with 2.0 mg/l 2, 4-D along with different level (50-300 mg/l) of ascorbic acid. Maximum callus proliferation (0.95 g) was observed at 200 mg/l ascorbic acid followed by at 150 mg/l ascorbic acid with low intensity of browning in the medium. Lower (50-100 mg/l) and higher (300 mg/l) levels of ascorbic acid showed intense browning in the medium (Table 3).

Effect of citric acid

Supplementation of citric acid in the basal medium containing 1.5 mg/l BAP induced shoots at all levels (10-60 mg/l) in nodal segment explants. Maximum shoot bud induction was observed at 40 mg/l level of citric acid followed by 20, 30 and 40 mg/l level. In case of shoot apex, maximum shoot bud induction (1.8) was observed at 30 mg/l level of citric acid followed by 20 and 40 mg/l level. The effectiveness of citric acid was less in comparison to activated charcoal (Table 1).

In case of leaf explants, Maximum callus proliferation (0.95 g) was observed at 30 mg/l citric acid followed by at 40 mg/l

citric acid with low intensity of browning in the medium. However, 50-60 mg/l ascorbic acid induced comparatively less callus at the cut ends of leaf explants due to inhibitory effect on growth through hampering the nutrient absorption (Table 2).

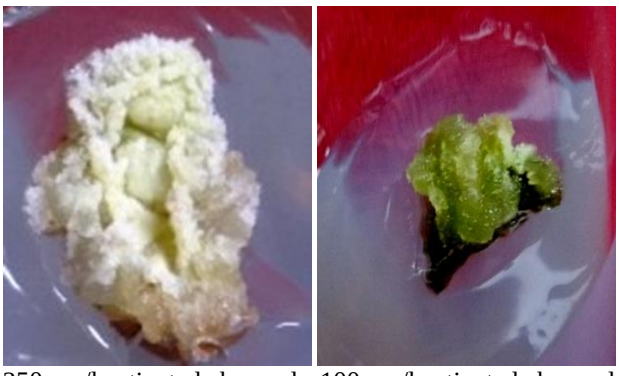
Effect of polyvinylpyrrolidone

Addition of polyvinylpyrrolidone in culture vessels with responsive level of plant growth regulator induced shoot bud from nodal segment at all levels. Supplementation of polyvinylpyrrolidone in the basal medium containing 1.5 mg/l BAP induced shoots at all levels (5-30 mg/l) in nodal segment explants. Maximum bud shoot induction (1.8) was observed at 15 mg/l level of polyvinylpyrrolidone. Shoot apex exhibited maximum shoot bud induction (1.8) at 20 mg/l level of polyvinylpyrrolidone followed by 15 and 20 mg/l level. Both higher and lower level of polyvinylpyrrolidone showed medium browning in culture vessels. In case of leaf explants, Maximum callus proliferation (0.96 g) was observed at 20 mg/l polyvinylpyrrolidone followed by at 25 mg/l polyvinylpyrrolidone with low intensity of browning in the medium (Table 2).

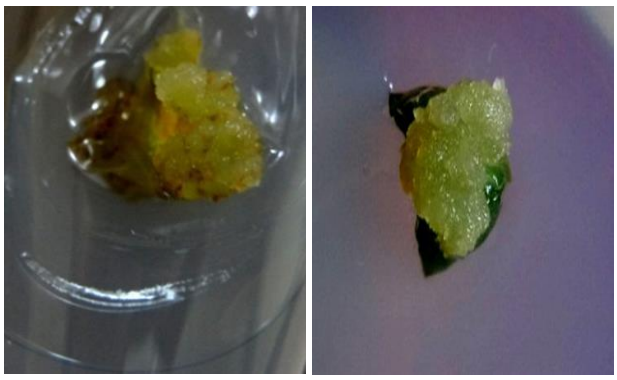
Perusal of Table 1 and 2 further, revealed that addition of 200, 150 and 250 mg/l activated charcoal in Ms medium along with responsive level of plant growth regulators for bud proliferation (nodal segment, explants 1.5 mg/l and shoot apex, 2.0 mg/l Kn) and callus induction (leaf, 2.0 mg/l 2, 4-D) appeared most optimum level of antioxidants for shoot bud proliferation in nodal segment, shoot apex and for callus induction in leaf explants, respectively. Among all the antioxidants polyvinylpyrrolidone was less effective to proliferate shoot buds and callus induction.

Fig 1: Effect of activated charcoal (200 mg/l) on shoot bud induction in nodal segment explants of guggul, supplemented with 1.5 mg/l BAP.

Fig 2: Effect of activated charcoal (150 mg/l) on shoot bud induction in shoot apex explant of guggul, supplemented with 2.0 mg/l Kn



250 mg/l activated charcoal 100 mg/l activated charcoal



200 mg/l activated charcoal 50 mg/l activated charcoal

Fig 3: Effect of activated charcoal on callus induction in leaf explant on MS medium supplemented with 2.0 mg/l 2, 4-D.

Table 1: Effect of antioxidants on *in vitro* shoot bud break in nodal segment and shoot apex explants on MS medium supplemented with responsive levels of plant growth regulators

Antioxidant	Concentration (mg)	Days taken in shoot induction		Number of shoot bud induction		Shoot length (cm)		Browning intensity	
		Nodal segment	Shoot apex	Nodal segment	Shoot apex	Nodal segment	Shoot apex	Nodal segment	Shoot apex
Activated Charcoal	50	18.6	18.8	1.7±0.26	1.6±0.22	1.75±0.03	1.68±0.04	++	++
	100	17.5	18.4	1.8±0.24	1.7±0.26	1.79±0.04	1.71±0.06	++	++
	150	17.3	16.8	2.0±0.14	1.9±0.23	1.85±0.04	1.97±0.02	++	+
	200	17.1	17.1	2.1±0.23	1.8±0.20	1.93±0.05	1.89±0.02	+	+
	250	18.1	18.7	1.9±0.18	1.7±0.15	1.83±0.03	1.79±0.04	++	++
	300	18.8	19.1	1.8±0.20	1.6±0.16	1.81±0.04	1.74±0.04	++	++
Ascorbic acid	50	18.4	17.3	1.7±0.26	1.7±0.21	1.76±0.04	1.79±0.06	++	+
	100	18.2	16.9	1.8±0.24	1.8±0.13	1.79±0.03	1.93±0.04	++	+
	150	17.8	17.6	1.9±0.18	1.7±0.21	1.81±0.04	1.89±0.03	+	+
	200	17.4	17.9	1.9±0.23	1.7±0.15	1.84±0.04	1.81±0.04	+	+

	250	18.3	18.1	1.8±0.20	1.7±0.15	1.80±0.02	1.79±0.04	++	+
	300	18.5	18.6	1.7±0.26	1.6±0.16	1.75±0.04	1.73±0.05	++	++
Citric acid	10	18.6	18.4	1.7±0.21	1.6±0.16	1.75±0.02	1.69±0.06	+++	++
	20	18.2	17.9	1.8±0.20	1.7±0.15	1.76±0.02	1.75±0.07	+++	++
	30	17.3	17.1	1.8±0.24	1.8±0.20	1.81±0.03	1.82±0.04	+	+
	40	17.1	17.3	1.9±0.23	1.7±0.15	1.83±0.04	1.81±0.04	+	+
	50	17.5	18.1	1.8±0.20	1.6±0.16	1.79±0.02	1.74±0.05	++	++
	60	18.2	18.6	1.7±0.21	1.5±0.16	1.76±0.01	1.68±0.05	++	+++
Polyvinylpyrrolidone	5	18.1	19.2	1.6±0.16	1.5±0.16	1.78±0.02	1.72±0.05	+++	++
	10	17.6	18.2	1.7±0.26	1.6±0.16	1.79±0.02	1.76±0.06	++	++
	15	16.9	18.1	1.8±0.20	1.7±0.13	1.85±0.02	1.85±0.03	+	+
	20	17.1	17.5	1.7±0.21	1.8±0.13	1.81±0.02	1.92±0.02	+	+
	25	17.8	17.6	1.7±0.26	1.7±0.15	1.77±0.02	1.86±0.02	++	++
	30	18.0	18.4	1.6±0.22	1.6±0.16	1.74±0.01	1.78±0.04	++	++

Table 2: Effect of antioxidants on callus proliferation from leaf explant in MS medium supplemented with 2.0 mg/l 2, 4-D in guggul

Antioxidant	Concentration (mg)	Days taken in callus induction	Fresh callus weight (g)	Browning intensity
Activated Charcoal	50	22.9	0.89	+++
	100	21.1	0.92	++
	150	20.5	0.91	++
	200	19.9	0.98	+
	250	19.1	1.15	+
	300	21.7	0.88	+++
Ascorbic acid	50	23.3	0.88	+++
	100	21.6	0.89	+++
	150	19.1	0.94	+
	200	19.2	0.95	+
	250	22.4	0.91	++
	300	24.9	0.88	+++
Citric acid	10	21.9	0.91	++
	20	20.8	0.93	++
	30	19.5	0.95	+
	40	19.8	0.94	+
	50	22.1	0.82	+++
	60	23.1	0.88	+++
Polyvinylpyrrolidone	5	22.1	0.86	+++
	10	21.2	0.88	+++
	15	20.9	0.92	++
	20	19.8	0.96	+
	25	21.9	0.94	+
	30	22.7	0.87	+++

(+) = Low browning, (++) = Medium browning and (+++) = Intense browning

Discussion

One of the major problems associated with plant tissue culture is browning of the culture medium and the explants, which invariably leads to death of plants. In order to control browning of medium and explants *in vitro*, many workers have tried to incorporating nonspecific absorbents like activated charcoal, ascorbic acid and polyvinylpyrrolidone in to the culture medium but only met to limited success (Weatherhead *et al*., 1979 and Bharadwaj and Ramawat, 1993) [21, 1]. The majority of woody plants and some herbaceous species under *in vitro* culture shows browning of medium. If this browning was so extreme, the explants turn its colour brown to black and become necrotic and finally lead to die (Ko *et al*., 2009) [8]. The browning of the medium is due to releasing phenol by the explants which get oxidized, and this oxidation product could be phytotoxic. Thus, it needs a scrutinized investigation before incubating explants in the culture medium. The degree of browning is different from species to species and depend on the age of the tissue (old tissues show more browning than the younger one), season of culture initiation (more in winters and autumn) and composition of the medium (Saenz *et al*., 2010) [16]. Thomas (2008) [8] reported that activated charcoal can alleviate toxic metabolites, phenolic exudation, their accumulation and promotes regeneration by absorbing inhibitory compounds. Activated charcoal could promote growth by releasing substances that are naturally present in the charcoal. It also immediately adsorbed PGRs and vitamins from medium and gradually releases them again in the medium.

In the present investigation different antioxidants (activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone) were incorporated singly in MS medium supplemented with responsive level of plant growth regulators, elicited different response for shoot bud induction and callus differentiation because it controls the accumulation of inhibitory substances (phenolic compounds) in the growth medium. Among the different antioxidants, activated charcoal was found better in reducing browning of medium and explant. The results of present investigation for use of activated charcoal were in close agreement with observation of antioxidant by Madhusudhanan and Rahiman (2000) [9] in piper species (P. longum, P. attenuatum, P. betle, P. nigrum). They observed effective role of activated charcoal in minimization and elimination of browning of culture medium. North *et al*. (2012) [11] also reported significant reduction of the total phenol content of media by 53 per cent, compared with ascorbic acid in Strelitzia reginae. In current investigation addition of activated charcoal 250 - 300 mg/l reduced the growth of explants. It might be due to immediate absorption of nutrients from medium. Activated charcoal is a strong phenol adsorbent (Zhou *et al*., 2010) [22] that reduces phenolic browning in explants by way of absorption of toxic substances and phenols (Fernando *et al*., 2010) [4] in culture media. Parmar and Kant (2012) [14] and Sharma *et al*. (2012) [14] reported positive response of activated charcoal (0.3 per cent) for shoot bud break in nodal segment of Commiphora wightii and root induction *in vitro* derived shoot of Acacia leucophloea, respectively.

Ascorbic acid is an antioxidant used to control oxidation of phenols. In the present investigation ascorbic acid also played important role to reduce browning through control of oxidation of phenol in shoot apex explants incubated on MS medium supplemented with 2.0 mg/l Kn. Maximum shoot bud induction was observed at 100 mg/l ascorbic acid in shoot apex explants with low intensity of browning in the medium followed by 50,150, 200 and 250 mg/l ascorbic acid (Table 1). Many researchers, Ko *et al*. (2009) [8] in banana, Cosmos *et al*. (2014) [2] in Brahylaena huillensis and Munguatosha *et al*. (2014) [10] in banana also reported reduction of oxidation

of phenols in the presence of ascorbic acid in cultural medium.

PVP was also found almost effective in browning control. Tyagi *et al*. (1981) [19] and Prajapati *et al*. (2003) [15] effectively controlled explant browning with PVP when supplemented in medium. The effectiveness of different antioxidants in control of browning is varying among plants and species. This could be due to the specificity of these chemicals to certain plant and species. The specificity of PVP in browning control was also reported by Vaugh and Duke (1984) [20].

References

1. Bharadwaj L. Ramawat KG. Effect of antioxidants and adsorbents on tissue browning associated metabolism in Cocculus penclulus callus cultures. Indian Journal of Experimental Biology. 1993; 31:715-718.
2. Cosmos FN, Emerald M, Patrick AN. Effects of ascorbic acid in controlling lethal browning in *in vitro* culture of Brahylaena huillensis using nodal segments. American Journal of Plant Sciences. 2014; 5:187-191.
3. Dayarani M, Dhanarajan M, Uma S. *In vitro* response of ornamental banana (Musa spp.). International Journal of Chemical, Environmental & Biological Sciences (IJCEBS), 2013.
4. Fernando SC, Santha ES. Hewarathna DJA. Activated coconut shell charcoal as a component of tissue culture media of *Cocos nucifera* L. Journal of the National Science Foundation of Sri Lanka. 2010; 38(3):181-185.
5. Fridborg G, Pedersen M, Landstorm LE, Eriksson T. The effect of activated charcoal on tissue cultures adsorption of metabolites inhibiting morphogenesis. Physiologia Plantarum. 1978; 43:104-106.
6. Kala CP, Dhyani PP, Sajwan BS. Developing the medicinal plants sector in Northern India: challenges and opportunities. Journal of Ethnobiology and Ethno medicine, 2006, 2:32.
7. Kefeli VI, Kalevitch MV, Borsari B. Phenolic cycle in plants and environment. Journal of Molecular Cell Biology. 2003; 2:13-18.
8. Ko W, Su C, Chen C, Chao C. Plant Cell Tissue Organ Culture. 2009; 96:137-141.
9. Madhusudhanan K. Rahiman BA. The effect of activated charcoal supplemented media to browning of *in vitro* culture of Piper Species. Biological plantarum. 2000; 43(2):297-299.
10. Munguatosha N, Emerald M, Patric N. Control of lethal browning by using ascorbic acid on shoot tip cultures of a local Musa spp. (Banana) cv. Mzuzu in Tanzania. African Journal of Biotechnology. 2014; 13(16):1721-1725.
11. North JJ, Ndakidemi PA, Laubscher CP. Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micro propagation of Strelitzia reginae. International Journal of the Physical Sciences. 2012; 7(4):638-646.
12. Ozyigit II. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. African Journal of Biotechnology. 2008; 7(8):1145-1150.
13. Pan MJ, Van Staden J. The use of charcoal in *in vitro* culture: A review. Plant Growth Regulation. 1998; 26:155-163.
14. Parmar AK, Kant T. Efficient micro propagation and evaluation of genetic fidelity of *in vitro* raised plants of Commiphora wightii (Arn.): A medicinally important red listed species of arid regions. Journal of Plant Development. 2012; 19(1):29-40.
15. Prajapati HA, Mehta SR, Patel DH, Subramanian RB. Direct *in vitro* regeneration of Curculigo orchioides Gaertn: An endangered anti-carcinogenic herb. Current Science. 2003; 84(6):747-749.
16. Saenz L, Herrera-Herrera G, Uicab-Ballote F, Chan JL, Oropeza C. Plant Cell Tissue Organ Culture. 2010; 100:301-308.
17. Sobti SN, Singh SD. A chromosomal survey of medicinal plants. Indian Academy of Sciences. 1961; 54b:138-144.
18. Thomas TD. The role of activated charcoal in plant tissue culture. Biotechnology Advances Journal. 2008; 26:618-631.
19. Tyagi AK, Rashid A, Maheshiwari SC. Promotive effect of polyvinyl poly pyrroli done on pollen embryogenesis in Datura innoxia. Physiologia Plantarum. 1981; 53:405-406.
20. Vaugh KC. Duke SO. Function of polyphenol oxidase in higher plants. Physiologia Plantarum. 1984; 60:106-112.
21. Weatherhead MA, Burdon L, Henshaw GG. Effect of activated charcoal as an additive to plant tissue culture media. Part 2. Pflanzenphysiologie. 1979; 94:399-406.
22. Zhou B, Wei X, Wang R, Jia J. Quantification of the enzymatic browning and secondary metabolites in the callus culture system of Nigella glandulifera Freynet Sint. Asian Journal Traditional Medicines. 2010; 5(3):109-116.