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SM Mahadik
Plant Biotechnology Centre, College of Agriculture, Dapoli. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

SV Sawardekar
Plant Biotechnology Centre, College of Agriculture, Dapoli. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

VG Kelkar
Plant Biotechnology Centre, College of Agriculture, Dapoli. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

NB Gokhale
Plant Biotechnology Centre, College of Agriculture, Dapoli. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

Corresponding Author:
SM Mahadik
Plant Biotechnology Centre, College of Agriculture, Dapoli. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

In vitro regeneration technique in *Rauwolfia serpentina* and quantification of reserpine

SM Mahadik, SV Sawardekar, VG Kelkar and NB Gokhale

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Abstract
The reliable technique for the indirect regeneration of *R. serpentina* was standardised in the present investigation. The secondary metabolites extracted from callus using methanol and analysed using Thin Layer Chromatography (TLC) and UV-Vis Spectrophotometer. The *in vitro* induction of callus influenced by several factors such as media composition and explants quality. From the various media combinations of 2, 4-D and BAP the frequency of highest callus induction (94.67%) was observed on MS + 2.0 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP when leaf disc used as explant. Throughout the organogenic callus induction, different calli were observed having variation in colour and texture. The shoot regeneration frequency was observed highest (98.33%) in 4.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA. Maximum shoots (15.33 per callus) were observed in leaf callus. The rooting was induced (95.33%) in *in vitro* regenerated shoots in Murashige and Skoog (MS) medium which contain 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP + 1 gL^{-1} activated charcoal (AC). The *in vitro* regenerated plantlets through callus culture were hardened in the greenhouse with 75 per cent establishment. The quantity and quality of reserpine was measured in callus and root of *R. serpentina*. The TLC reveals the accumulation of reserpine at callus as well as roots of cultivated plant. Spectrophotometric estimation showed that concentration of reserpine observed in callus extract was higher (6.8 μgml^{-1}) than the root extract (6.4 μgml^{-1}).

Keywords: *Rauwolfia serpentina*, callus, reserpine, secondary metabolite

Introduction
Rauwolfia serpentina (L.) family Apocynaceae is a medicinal shrub which is commonly known as sarpagandha. In case of *R. serpentina* roots are rich source of Indole alkaloids such as, serpentine, recinnamine, reserpine ajamalicine, ajamaline, *etc*. According to ayurveda, whole plant of *R. serpentine*, specially roots are used for the treatment of various diseases, such as snake bite, rheumatism, cardiovascular disorder, hypertension, insanity, epilepsy, eczema (Joshi *et al*., 2010). *R. serpentina* endanger in India because of non-selective plantation and overexploitation of naturally available sarpagandha to fulfill the requirement of industry (Nayar and Sastry, 1987) [9]. IUCN has declare the sarpagandha plant is under endangered condition. The endangered status of sarpagandha is due to poor seed viability and low seed germination rate (Chaudhari *et al.*, 2015) [4]. To satisfy the increasing commercial demand of alkaloids and conservation of this important endangered plant there is a need to produce *R. serpentina* under *in vitro* condition (Bhatt *et al*., 2008) [2]. The quality of alkaloids extracted for in vitro callus culture is almost same as to that of natural sources of alkaloids. (Yoshimatsu and Shimomura, 1991) [18]. Sometimes, higher production capacity of producing secondary metabolites observed in callus culture (Benavides and Caso, 1993) [1] and the callus culture can also efficient and reliable to produce alkaloids at high mass (Yamamoto and Yamada, 1986). The methanolic extraction of alkaloids is also reliable in case of callus culture (Kirillova *et al*., 2001) [6]. After understanding this problems, the present study deals with standardization technique for *in vitro* regeneration through callus of leaf, leaf node and stem, and analysis of estimated of secondary metabolite.

Materials and Methods
1. Collection of explant
The experimental material of the present investigation was collected from the Charak nursery, College of Forestry, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist.-Ratnagiri. The seedlings used as source of explants were potted and maintained in the greenhouse.

2. Surface sterilisation
Different explants such as leaf disc, leaf node and meristem were washed with running tap water for 5 minutes and subsequently with distilled water. Explants were treated with carbendazim ethanol, mercuric chloride and subsequently with distilled water in laminar airflow cabinet. Sterilised filter paper used to blot the explants before inoculating on Murashige and Skoog (MS) media. The MS media prepared as per the standard procedure with addition of heat resistant growth regulators and pH was adjusted to 5.8.

3. Callus induction
For callus induction juvenile leaf disc, leaf node and meristem of 5 mm length were cut with sterile scalpel and inoculated on MS medium having appropriate concentrations of growth hormones which enhance callus induction. The inoculated cultures were kept in incubation room up to 21 days by adjusting 16 hrs. light and 8 hrs. dark period.

4. Shoot regeneration from callus
The callus growing on proliferation nutrient media was cut with sterile scalpel into 0.5 mm size and inoculated on shooting media for shoot initiation and multiplication. To obtain the more number of shoots the MS media supplemented with various concentration of BAP and NAA. After that culture was incubated at 16 hrs. light and 8 hrs. dark period on a constant temperature (25 ^{o}C) in incubation room. The sub culturing was continued every after 21 days to get more number of shoots.

5. Rooting and hardening
After development of multiple shoots, the well grown elongated shoots were separated from each other and transferred on media which is favorable for rooting. The MS media was prepared with different concentration of auxin (NAA) and cytokinin (BAP) and activated charcoal for further root development. Hardening is a critical step, the tissue culture plants are taken out from MS media, carefully washed roots with water The potting mixture (coco peat + vermiculite) was used for primary hardening and incubated plants in growth chamber for 15 to 20 days for acclimatisation by maintaining humidity temperature and light. Then the plants are transferred for secondary hardening on another potting mixture (soil, sand and vermiculite; 1:1:1) and kept under natural condition.

6. Extract preparation
The methanolic extraction of *R. serpentine* was done using 100 g callus which is soaked in 10 mL methanol and kept for 30 min. at room temperature. The extract was filtered using Whatman filter paper No.1, and residue obtained after filtration again dissolved 5 mL of methanol and kept for 10 min. at room temperature. Repeated this process to get 50 mL volume of total filtrate. The filtrate was evaporated by keeping the extract in beaker (without lid) for 20 to 24 hrs. After that the crude extract was dissolved properly in 100 mL HCL (0.001M) and pH was adjusted to 6.00 using NaOH (0.001M). The obtained extract was used for the TLC as well as spectrophotometric analysis.

7. Qualitative analysis of secondary metabolites by Thin Layer Chromatography (TLC)
The TLC was used to check the quality of major groups of alkaloid derivatives present in extract obtained from callus of *R. serpentina*. The chloroform and methanol (97:3) were used to prepare solvent system for TLC. After completion of the TLC, the Dragendorff´s reagent used to visualise the spots which develop orange s.pots. The spots intensified by using HCl or 50 per cent water-phosphoric acid and then R_f value was calculated for the extract.

8. Quantitative estimation of secondary metabolites using UV-Vis Spectrophotometer:
One milligram of reserpine was taken and dissolved in 10 ml of methanol and various dilutions are made from it having concentration (2 μgmL^{-1}-10μgmL^{-1}). All the various dilutions of reserpine were observed under UV spectrophotometer using λmax 268 nm. Absorbance of all the samples and standard was calculated. The experiment was done in triplicate. Calibration curves series of standard curves were prepared with a concentration range 2-10 µg (n=3, five standards). The data of concentration versus absorbance was analyzed by linear test square regression analysis.

Result and Discussion
1. Surface sterilisation
Explants (leaf disc, leaf node and meristem) washed thoroughly with running tap water for 5 min. and then with distilled water. After that explants were treated with 0.1% carbendazim for 10 min. and then rinsed thoroughly with sterile distilled water. The leaves were treated with 70 per cent ethanol for 30 sec., washed with distilled water and then treated with 0.1 per cent mercuric chloride $HgCl_2$ for 5 min. and again washed with distilled water, showed the maximum frequency (95.33%) of aseptic callus (Data not presented).

2. Callus induction
Murashige and skoog media in the absence of growth hormone was not efficient to induce callus (Shah *et al*., 2003). All the three explants (Leaf, leaf node and meristem) produced callus in MS medium prepared by using 2,4-D (0.5-5.0 mgL^{-1}) and BAP (1.0 mgL^{-1}). From all growth hormones, 2,4-D was more efficient in callus induction. The best response was obtained in MS medium, prepared by using 2,4-D (2.0 mgL^{-1}) in all three explants. Among the three explants the maximum callus induction frequency was noticed in leaf disc (94.67%) followed by leaf node (91.33%) and meristem (80.67%) (Table-1) (Figure 1). Sarkar *et al.* (1996) reported that MS medium prepared by using auxins and cytokinins specifically 2,4-D and BAP having more efficiency of callus induction. Callus cultures initiated through explants taken from any plant parts and the percent callus induction varies according to explant. The type of explant used for the callus induction have efficient role in healthy growth of callus. Among various explants leaf disc explants gave good response for callus induction have been recorded in *R. serpentina* by Roja *et al*. (1987), Upadhyay *et al*. (1992) and Panwar *et al*. (2011).

3. Proliferation of callus
One-month-old callus was sub-cultured in the medium which is used for callus induction for proliferation of healthy callus. Green-friable callus was obtained in leaf disc and leaf node explants while yellowish-friable callus was obtained when meristem used as explants after 25 to 30 days of subculture (Figure 2). Leaf explant produced loose callus while meristem explant produces tough and compact callus. Growth regulators and its concentration in the medium also plays important role in dedifferentiation of tissues. Proper combination of auxins and cytokinins resulted in efficient

callus proliferation. Pant and Joshi (2008) [10] reported that after 10 to 14 days, all sources of the explants (leaf disc, leaf node and meristem) were swelled and callus initiation stared from only cut surface of explants because of injury.

4. Shoot induction

The callus obtained from leaf disc, leaf node and meristem explants was failed to generate shoots in MS medium (without growth regulators). The medium prepared by using BAP (1.5-5.5 mgL^{-1}) generate multiple shoots in all explants and the shoot induction percentage was calculated which lies between 24.33-98.33 per cent. Table-2 shows that the medium containing 4.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA was observed as the most effective combination for multiple shoot induction (98.33%) when the callus derived from leaf disc. Leaf node and meristem derived callus showed maximum per cent shooting 67.33 per cent and 97.17 per cent, respectively on MS medium supplemented with 4.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA. Medium containing 4.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA was observed as the most efficient combination for getting multiple shoot induction, about 25 shoots per culture were observed (Figure 3). These results are showing similarity with the result obtained by Sushila *et al*. (2013) [16] that the high concentration of cytokinin in combination with auxin have higher efficiency of producing multiple shoots. The multiple shoot induction can be also influenced by physiological status and level of endogenous hormones.

5. Rooting and hardening

The *in vitro* produced multiple shoots were successfully transferred on rooting medium supplemented with NAA and BAP. However, when the medium was prepared by using NAA 1.0 mgL^{-1} + BAP 0.1 mgL^{-1} 95.33 per cent rooting was achieved (Figure 4, Table 3). The plantlets having healthy well developed roots were hardened in green house condition and slowly acclimatized with environment with a success rate of 75 per cent (Figure 5). Roy *et al*. (1995) [14] reported that the overall success of organogenesis depends on the efficiency of transferred plants to survive in environmental condition. The plants produced through *in vitro* technique are acclimatized to unique set of growth conditions which may increase rapid growth and multiplication in the field.

6. Qualitative analysis of secondary metabolites by Thin Layer Chromatography

The methanolic extract obtained from leaf callus, *in vivo* root and standard were spotted on TLC Silica gel 60F plate (Figure 6). The spot of callus in the figure shown R_f value of 0.96. The standard R_f value of reserpine is 0.96 and spot of root extract shown a R_f value 0.957 (Table-4). These observations clearly revealed the presence of reserpine in callus and root extracts, and the intensity of the spot showed extract obtained from callus had higher concentration of reserpine. Callus culture contains more or less homogenous clumps of dedifferentiated cells are used for secondary metabolite production. Similarly, alkaloids from Bael (*Aegle marmelos*) were extracted by Borde *et al*. (2011) [3] in 95 per cent ethanol and separated with chloroform by using synthetic antioxidant as a standard.

7. Quantitative estimation of secondary metabolites by using spectrophotometer

One milligram of reserpine dissolved in 10 ml of methanol and various dilutions are made from it having concentration (2 μgml^{-1}-10 μgml^{-1}). Absorbance of standard was recorded (Table-5), with respect to absorbance of standard the unknown concentration of reserpine present in extract was determined by extraploitation (Figure 7). The concentration of reserpine observed in callus extract was 6.8 μgml^{-1} whereas the concentration of reserpine observed in root extract was 6.4 μgml^{-1} (Table 6). In the present study, it was observed that the concentration of reserpine observed in callus extract was 6.8 μgml^{-1} whereas the concentration of reserpine observed in root extract was 6.4 μgml^{-1}. Callus contain higher amount of reserpine than the roots of cultivated plant, some compounds which are infecting the reserpine in the field should be avoided during callus culture (Panwar and Guru, 2011; Mallick *et al*., 2012) [11, 12, 7].

Table 1: Frequency of callus induction

Sr. No.	Combination details	Explants			Mean (%)
		Leaf disc (%)	Leaf node (%)	Meristem (%)	
CIM_1	MS medium (control)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
CIM_2	MS + 0.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
CIM_3	MS + 1.0 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP	55.33 (48.06)	51.33 (45.76)	12.67 (20.84)	39.78 (39.10)
CIM_4	MS + 1.5 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	73.33 (58.90)	68.67 (55.96)	65.33 (53.92)	69.11 (56.23)
CIM_5	MS + 2.0 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	94.67 (76.64)	91.33 (72.87)	72.67 (58.47)	86.22 (68.21)
CIM_6	MS + 2.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP	91.33 (72.87)	84.67 (66.94)	80.67 (63.91)	85.56 (67.66)
CIM_7	MS + 3.0 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	80.67 (63.91)	76.67 (61.11)	73.33 (58.90)	76.89 (61.26)
CIM_8	MS + 3.5 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	71.33 (57.62)	64.67 (53.52)	51.33 (45.76)	62.44 (52.20)
CIM_9	MS + 4.0 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	64.00 (53.13)	59.33 (50.37)	41.33 (40.00)	62.44 (47.80)
CIM_{10}	MS + 4.5 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	37.33 (37.66)	31.33 (34.03)	27.33 (31.52)	32.00 (34.44)
CIM_{11}	MS + 5.0 mgL^{-1} 2.4-D + 1.0 mgL^{-1} BAP	19.33 (26.08)	16.67 (24.09)	15.33 (23.05)	17.11 (24.43)
	Mean (%)	53.39 (46.94)	49.52 (44.72)	40.00 (39.23)	47.64 (43.64)
		SE (m)±	CD at 1%		
	Media combination	3.66	13.74		
	Explants	1.91	7.17		
	Interaction	6.34	23.80		

Table 2: Per cent shooting

Sr. No.	Combination details	Callus			Mean (%)
		Leaf disc (%)	Leaf node (%)	Meristem (%)	
SIM_1	MS medium (control)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
SIM_2	MS + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

SIM_3	MS + 1.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	52.33 (46.33)	24.33 (29.56)	40.50 (39.52)	39.06 (38.67)
SIM_4	MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	48.33 (44.04)	36.33 (37.07)	45.50 (42.41)	43.39 (41.20)
SIM_5	MS + 2.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	61.67 (51.74)	40.67 (39.62)	55.33 (48.06)	52.56 (46.46)
SIM_6	MS + 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	91.33 (72.87)	45.00 (42.13)	86.67 (68.58)	74.33 (59.56)
SIM_7	MS + 3.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	95.33 (77.52)	67.33 (55.14)	92.33 (73.92)	85.00 (67.21)
SIM_8	MS + 4.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	98.33 (82.58)	64.50 (53.42)	90.67 (77.52)	82.67 (68.07)
SIM_9	MS + 4.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	97.33 (79.48)	60.67 (51.16)	97.17 (72.21)	85.22 (65.39)
SIM_{10}	MS + 5.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	85.67 (67.75)	42.83 (40.58)	65.33 (53.93)	64.61 (53.39)
SIM_{11}	MS + 5.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA	75.67 (60.44)	40.33 (39.42)	58.00 (49.60)	58.00 (49.60)
	Mean (%)	64.12 (53.20)	38.32 (38.24)	57.24 (49.16)	53.23 (46.85)
		SE (m)±	**CD at 1%**		
	Media	1.73	6.51		
	Explants	0.90	3.40		
	Interaction	3.00	11.28		

Table 3: Per cent rooting

Sr. No.	Combination details	Explants			Mean (%)
		Leaf disc (%)	Leaf node (%)	Meristem (%)	
RIM_1	MS medium (control)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
RIM_2	MS + 0.1 mgL^{-1} NAA + 1.0 mgL^{-1} BAP	24.67 (29.77)	20.67 (27.03)	16.67 (24.09)	20.67 (27.03)
RIM_3	MS + 0.5 mgL^{-1} NAA + 1.5 mgL^{-1} BAP	56.00 (48.44)	49.33 (44.61)	46.67 (43.08)	50.67 (45.38)
RIM_4	MS + 0.1 mgL^{-1} NAA + 2.0 mgL^{-1} BAP + 1 gL^{-1} Activated charcoal	77.33 (61.56)	72.67 (58.47)	68.67 (55.96)	72.89 (58.62)
RIM_5	MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP + 1 gL^{-1} Activated Charcol	95.33 (77.52)	91.33 (72.87)	87.33 (69.15)	91.33 (72.87)
RIM_6	MS + 1.0 mgL^{-1} NAA + 0.5 mgL^{-1} BAP + 1 gL^{-1} Activated Charcol	87.33 (69.15)	83.33 (65.90)	79.33 (62.96)	83.33 (65.90)
RIM_7	MS + 1.0 mgL^{-1} NAA + 1 mgL^{-1} BAP	46.67 (43.08)	40.67 (39.62)	37.33 (37.66)	41.56 (40.13)
RIM_8	MS + 1.0 mgL^{-1} NAA + 1.5 mgL^{-1} BAP	21.33 (27.50)	16.67 (24.09)	13.33 (21.41)	17.11 (24.43)
RIM_9	MS + 1.0 mgL^{-1} NAA + 2.0 mgL^{-1} BAP + 1 gL^{-1} Activated Charcol	85.33 (67.48)	76.67 (61.11)	73.33 (58.90)	78.44 (62.33)
RIM_{10}	MS + 2.0 mgL^{-1} NAA + 1.0 mgL^{-1} BAP + 1 gL^{-1} Activated Charcol	92.67 (74.28)	86.67 (68.58)	82.00 (64.89)	87.11 (68.96)
RIM_{11}	MS + 2.0 mgL^{-1} NAA + 3 mgL^{-1} BAP	12.67 (20.85)	10.00 (18.43)	8.67 (17.12)	10.44 (18.85)
	Mean (%)	54.48 (47.57)	49.88 (44.93)	46.61 (43.05)	50.32 (45.18)
		SE (m)±	CD at 1%		
	Media	3.20	12.02		
	Explants	1.67	6.27		
	Interaction	5.55	20.82		

Table 4: Thin layer chromatography

Sr. No.	Sample	Distance travelled by solute	Distance travelled by solvent	R_f Value
1	Standard	12.4	12.8	0.96
2	Callus	12.3	12.8	0.96
3	Root	12.2	12.8	0.957

Table 5: Absorbance of reserpine at various concentration

Sr. No.	Concentration (μgmL^{-1})	Absorbance ± SD
1	2	0.039 ± 0.00707
2	4	0.081 ± 0.002121
3	6	0.120 ± 0.000707
4	8	0.171 ± 0.00707
5	10	0.208 ± 0.001414

Table 6: Absorbance and content of reserpine in *R. serpentina*

Methanolic extract	Absorbance	Conc. of reserpine (μgmL^{-1})
Callus	0.139	6.8
Root	0.133	6.4

Fig 1: Callus Induction. MS + 2 mgL^{-1} 2, 4-D + 1.0 mgL^{-1} BAP for Leaf and leaf node MS + 2.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP for Meristem

Fig 2: Callus proliferation after 21 days. MS + 2 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP for Leaf and leaf node MS + 2.5 mgL^{-1} 2,4-D + 1.0 mgL^{-1} BAP for Meristem

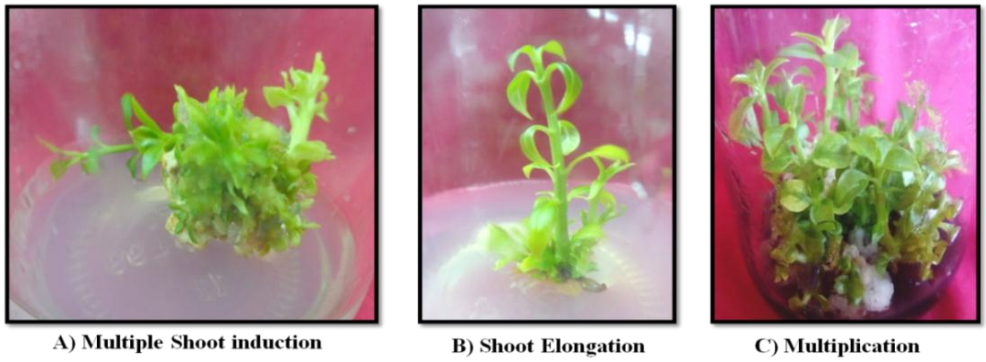

Fig 3: Shoot multiplication MS + 4.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

Fig 4: Rooting MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP + 1.0 gL^{-1} activated Charcol

Fig 5: Hardening Soil + Soilrite + Sand = 1:1:1

Fig 6: Qualitative analysis by Thin Layer Chromatography (A) Standard (Reserpine) (B) Extract From Callus (C) Extract From Root

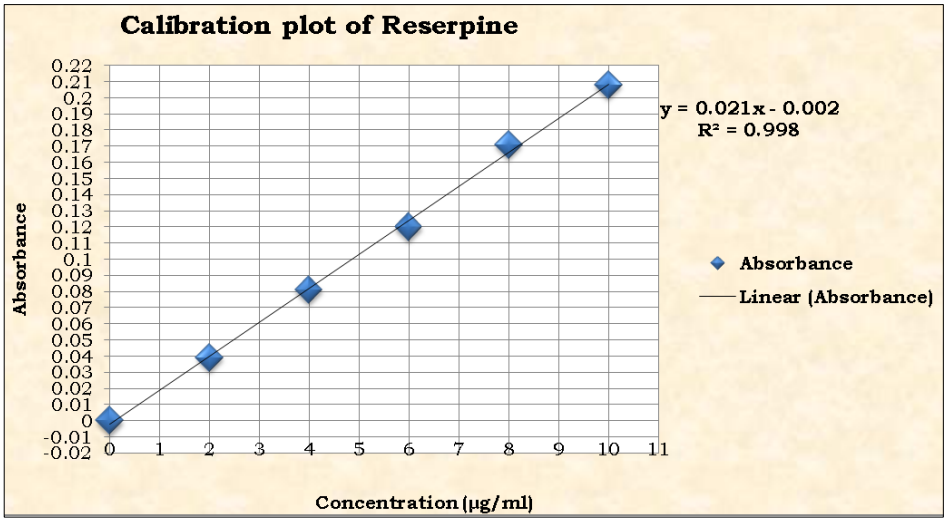

Fig 7: Calibration plot of Reserpine

Conclusion

The callus culture is a good alternative for production of secondary metabolites. The quality and quantity of reserpine is better in callus as compare to the root extract. The better quantification can be done using HPLC and LCMS technique and obtained compounds can be docked using molecular docking, which can be found as target against diseases. For increasing quantity of the reserpine the elicitors or precursor feeding is better option. Standardised regeneration protocol can be used in gene editing approaches.

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