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Mamatha DS
M.Sc. (Hort.), Research Scholar, Department of Biotechnology and Crop Improvement, KRC College of Horticulture, Arabhavi, Karnataka, India

Dr. Sandhyarani Nishani
Assistant Professor and Head, Department of Biotechnology and Crop Improvement, KRC College of Horticulture, Arabhavi, Karnataka, India

Dr. Mahantesha BN Naika
Assistant Professor, Department of Biotechnology and Crop Improvement, KRC College of Horticulture, Arabhavi, Karnataka, India

Dr. Laxminarayan Hegde
Professor and Dean CHEFT, Department of Plantation Spices Medicinal and Aromatic, College of Horticulture, Devihosur, Karnataka, India.

Dr. Ratnakar Shet
Assistant Professor, Department of Genetics and Plant Breeding, College of Horticulture, Sirsi, Karnataka, India

Srikanta Prasad D
Assistant Professor, Department of Plantation Spices Medicinal and Aromatic, KRC College of Horticulture, Arabhavi, Karnataka, India

Corresponding Author:
Mamatha DS
M.Sc. (Hort.), Research Scholar, Department of Biotechnology and Crop Improvement, KRC College of Horticulture, Arabhavi, Karnataka, India

Tissue culture studies on garsden Rauvolfia (*Rauvolfia tetraphylla* L.)

Mamatha DS, Dr. Sandhyarani Nishani, Dr. Mahantesha BN Naika, Dr. Laxminarayan Hegde, Dr. Ratnakar Shet and Srikanta Prasad D

Abstract
Rauvolfia tetraphylla (L.) is one of the important medicinal shrubs exhibiting a range of therapeutic importance. A study was conducted to standardize the micro propagation protocol from shoot tips and nodal segment of Garden Rauvolfia. Two different explants with four different media composition were used in this experiment. The experimental results revealed that the highest percentage of aseptic culture and lowest percentage of contamination was observed in treatment with Bavistin(1%), mercury chloride (0.1%) and ethanol (70%). Maximum multiplication rate was observed in cultures of MS media with nodal segments. Highest percentage of shoot proliferation and highest number of multiple shoots were seen in MS media with 2.5 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} IAA. Shoot elongation of shoot was found best (9.30 cm) on MS media supplemented with 2.5 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IAA. The *in-vitro* raised shoot successfully produced the roots in half strength MS media supplemented with 2.5 mg/L IBA and 0.5 NAA mg/L. The protocol was optimized by manipulations of different PGRs for enhanced multiplication. Protocol explained nodal segments on MS media produced the more number of multiple shoots which provides large number of true to the type, uniform, disease free, elite, plantlets right through the year, which helps large scale cultivation of this endangered important medicinal plant.

Keywords: *Rauvolfia tetraphylla* (L.), shoot tip, nodal segments, *in-vitro* regeneration

Introduction
Rauvolfia tetraphylla (L.) is one of the important medicinal shrubs commonly known as Four-leaved Garden Rauvolfia. It belongs to the milkweed family Apocynaceae with chromosome number 2n=22 (Kline, 1954) [5]. *Rauvolfia tetraphylla* L. is a pubescent, perennial shrub with woody stem grows up to the height of four to six feet. There are approximately 85 species of the genus *Rauvolfia* mainly found in tropical regions, among them *R. serpentina* and *R. tetraphylla* contains more than 50 alkaloids including the therapeutically important indole alkaloids namely, reserpine, rescinnamine, deserpidine, ajamalacine, ajmaline, neoajmalin, serpentine and yohimbine (Hines and Eckman, 1993) [3]. Various parts of *R. tetraphylla* are shown to exhibit a range of therapeutic importance such as anti-microbial, anti-inflammatory, antioxidant, cyto-toxic, anti-hemolytic, anxiolytic, anti-hypertensive, insecticidal, allelopathic, cardioprotective, platelet anti-aggregant, antipsychotic and antiparasitic activity. Poor seed viability, low vegetative propagation rate through root cuttings and low seed germination rate has hampered large scale commercial cultivation of *R.serpentina* through conventional mode of propagation. Further *R.serpentina* is threatened with extinction due to its limited cultivation and over exploitation by local people, government agencies and various pharmaceutical houses (Mamgain *et al*., 1998) [6]. Due to the prevailing problems in *R. serpentina* there is a huge need for *in vitro* propagation of *R. tetraphylla* to satisfy the growing commercial demand of the plant for the production of life supporting alkaloids. *R. tetraphylla* has wider adaptability and can be easily propagated compared to *R. serpentina*, but the only limitation is lower reserpine content. Hence improvement in plant tissue culture techniques for the mass propagation of *R. tetraphylla* are highly desirable which can further lead to increase the reserpine content by addition of elicitors.

Material and Methods
Standardization of protocol for regeneration of Garden Rauvolfia was carried out in the Tissue Culture Unit, Department of Biotechnology and Crop Improvement during 2019-20. The explants (shoot tips and nodal segments) for the experiment was collected from one year old

mother plant of *R. tetraphylla* grown at Department of Biotechnology and Crop Improvement, KRC college of Horticulture, Arabhavi. The explants were washed with running tap water thoroughly for 15 minutes followed by washing with sterile water for two to three times before culture under laminar air flow chamber served as control. Similarly explants were treated with different chemicals sodium hypochlorite, Tween-20, mercuric chloride and ethanol in different concentrations with specific time period. Finally four to five times rinsed with sterile water in the laminar air flow chamber. Basal portion was placed adaxially on four different media (Murashige and Skoog medium, B5, Woody plant medium and Modified MS medium) with 30g/l^{-1}sucrose, 8g/l^{-1} agar and Benzyl amino purine (BAP) in combination with Naphthalene acetic acid (NAA) and Indole acetic acid (IAA) for shoot regeneration. The pH of the medium was adjusted to 5.75 before autoclaving at 121^{0}C, 15 lbs for 15 min. Media was supplemented with 30g l^{-1} sucrose as a carbon source and 7g l^{-1} agar-agar as a gelling agent. The effect of different medias on shoot regeneration were studied. The regenerated shoots were placed on MS media for elongation. The effect of BAP at different concentrations and combinations of BAP with NAA and IAA were studied on the induction of multiple shoot formation in MS media.

The regenerated shoots were transferred for rooting after one month to Half strenghth MS medium with 3% sucrose and containing different concentration of IBA and NAA separately. In rooting media activated charcoal has been added at 2g l^{-1}. All the media were kept and observed for contamination for minimum three days before using for culture. Baby jar bottles (250 ml) with autoclavable polypropylene caps were used as culture containers. Culture room maintained at a temperature of 25 ± 2^{o}C with uniform light intensity (ca 1000 lux) was provided by fluorescent tubes (7200^{o}K) over a light and dark cycle of 16 and eight hours respectively. Each treatment had three replications arranged in a completely randomized design.

Result and Discussion

The data on percentage of aseptic culture was recorded one week after culture initiation. The highest percentage (77.78%) of aseptic culture was observed in treatment with 1% bavistin, $HgCl_2$ and ethanol. No aseptic culture was recorded in untreated control in all the cultures of Garden Rauvolfia (Table 1). Significantly lower level of contamination (5.57%) was observed in cultures treated with Bavistin, Ethanol and $HgCl_2$. Among the cultured media, highest percent response for shoot bud initiation (90.33%) was observed in MS medium and among the different explants used, shoot tips have shown highest per cent response for shoot bud initiation (85.00%). Mukhopadhyay *et al*. (1991) [7] in *R. serpentina* reported that the rate of multiplication and growth of individual shoot tip was better in the MS medium containing NAA (0.5 mg l-1) and BAP (2.0 mg l-1). Among cultured media, higher number of shoots (2.18) was produced in MS medium and among the different explants, nodal segments recorded the highest number of shoots per explant (2.10). Alatar *et al.* (2012) [2] also reported high frequency shoot regeneration and plant establishment from nodal segment explants of *R.serpentina*. MS medium resulted in significantly higher number of leaves per shoot (4.17) while among different explants highest number of leaves (4.03) were observed in nodal segments. The results for the effect of BAP at different concentrations and combinations of BAP with NAA and IAA on MS media for induction of multiple shoot formation in MS media showed that highest number of adventitious buds (5.33) was observed in MS media with 2.5 mg/L BAP, 0.5 mg/L NAA and 0.5 mg/L IAA. Significantly highest number of shoots (4.33) were recorded in 2.5 mg/L BAP, 0.5 mg/L NAA and 0.5 mg/L IAA. Significantly higher shoot length (9.30 cm) was observed in MS media 2.5 mg/L BAP, 0.5 mg/L NAA and 0.5 mg/L IAA. Hoque *et al*. (2020) [4] in *R. tetraphylla*. Best response towards *in vitro* shoot regeneration was obtained from nodal segment when they were cultured on MS supplemented with 2.2 mg/L BA and 0.1 mg/L NAA. About 9.9 ± 0.87 shoots were obtained per explants. The highest number of roots (5.40) were observed in half MS media supplemented with 2.5 mg/L IBA and 0.5 NAA mg/L and Significantly higher length of root (3.98cm) was recorded in half MS media supplemented with 2.5 mg/L IBA with 0.5 NAA mg/L. Number of primary roots were recorded highest (3.73) in half MS media supplemented with 2.5 mg/L IBA with 0.5 NAA mg/L and the number of secondary roots were also found highest (1.33) in 2.5 mg/L IBA with 0.5 mg/L NAA on half MS media. Ahamed *et al*. (2005) [1] where they, identified that MS medium along with different concentration of IAA and IBA in combination or separately can induce roots during micro propagation.

Table 1: Effect of surface Sterilants on explant contamination and intensity of browning in *R. tetraphylla*

Treatment	No. of explants taken	No. of explants established	Per cent Aseptic culture (%)	Per cent contamination (%)	Days to initiate sprouting	Intensity of browning
T_0	15	0	0.00(0.28)	100.00(89.71)	0.00(0.28)	0
T_1	15	2	15.55(23.14)	70.62(57.20)	9.87(18.30)	0
T_2	15	6	39.99(39.20)	42.93(40.93)	8.16(16.58)	+
T_3	15	4	31.55(34.13)	35.47(36.54)	10.85(19.23)	++
T_4	15	12	77.78(61.93)	5.57(13.65)	9.25(17.71)	+
T_5	15	3	22.22(28.07)	2.54(9.17)	10.67(19.06)	++
T_6	15	0	0.00(0.28)	0.47(3.30)	0.00(0.28)	+++
CV (%)	S.E.m±		2.26	1.13	0.28	
	CD @ 1%		9.51	4.76	1.17	
			14.64	8.69	6.93	

'0' – no browing, '+'-low browning, '++'-moderate browning, '+++'-high browning

The values given in parenthesis are arc sine transformed values (Sin-1√X/100)

Table 2: Treatment details

Treatments (T)	Chemicals	Concentration	Duration
1	Control	0.0	15 min
2	Bavistin	0.1%	30 min
	$HgCl_2$	0.1%	4 min
3	Bavistin	0.5%	30 min
	$HgCl_2$	0.2%	4 min
4	Bavistin	0.1%	30 min
	$HgCl_2$	0.3	3min
5	Bavistin	0.1%	30 min
	$HgCl_2$	0.1%	4 min
	Ethanol	70%	30sec
6	Bavistin	0.1%	30 min
	$HgCl_2$	0.1%	4 min
	Ethanol	70%	30 sec
	NaO Cl_2	5%	5 min
7	Bavistin	0.1%	30 min
	Ethanol	70%	30 sec
	Tween 20	4 drops	20 min
	NaO Cl_2	5%	5 min
	$HgCl_2$	0.1%	4 min

Table 3: Effect of explants source and media on shoot proliferation in *R. tetraphylla*)

Treatments	Percent response for shoot bud initiation	Number of shoots per explants	No. of leaves per shoot
MS (M_1)	90.33(9.50)	2.18(1.48)	4.17 (2.03)
WPM(M_2)	78.33(8.85)	1.69(1.30)	3.56 (1.87)
B5(M3)	80.33(8.96)	1.99(1.41)	3.94 (1.98)
MMS(M4)	86.72(9.31)	2.10(1.45)	4.11(2.01)
S.E.m±	0.07	0.02	0.06
CD @ 1%	0.28	0.10	0.25
Shoot tip(E1)	85.00(9.21)	1.87(1.37)	3.86(1.95)
Nodal segment(E2)	82.86(9.10)	2.10(1.45)	4.03(1.99)
S.E.m±	0.05	0.02	0.04
CD @ 1%	0.20	0.07	0.17
M_1 x E_1	96.11(9.80)	2.08(1.44)	4.11(2.01)
M_1 x E_2	84.55(9.19)	2.28(1.51)	4.22 (2.05)
M_2 x E_1	77.00(8.77)	1.51(1.23)	3.56 (1.87)
M_2 x E_2	79.67(8.92)	1.86(1.36)	3.56(1.86)
M_3 x E_1	79.44(8.91)	1.90(1.38)	3.78 (1.94)
M_3X E_2	81.22(9.01)	2.08(1.44)	4.11 (2.02)
M_4 x E_1	87.45(9.35)	2.01(1.42)	4.00(1.98)
M_4 x E_2	86.00(9.27)	2.18(1.48)	4.22(2.05)
S.E.m±	0.09	0.03	0.08
CD @ 1%	0.39	0.14	0.35
CV (%)	1.79	4.16	7.39

Values in the parenthesis represent square root values of mean.

Table 4: Effect of different growth regulators used in MS media on *in vitro* shoot regeneration of *R. tetraphylla*

Treatment	No of adventitious buds per explant	Number of shoot	Length of the shoot	No of leaves	% Response
Control	1.110(1.04)	0.890(0.93)	3.800(1.95)	3.223(1.76)	78.890(8.88)
BAP 1.5 mg/L	1.130(1.05)	0.780(0.87)	4.433(2.10)	3.996(1.99)	84.333(9.17)
BAP 2.0 mg/L	2.110(1.44)	1.000(1.00)	4.210(2.05)	5.663(2.38)	89.220(9.44)
BAP 2.5 mg/L	2.223(1.48)	1.446(1.20)	4.976(2.23)	6.003(2.44)	87.776(9.36)
BAP 1.5 mg/L+NAA 0.5 mg/L	2.780(1.67)	1.780(1.34)	4.066(2.02)	6.446(2.53)	84.110(9.18)
BAP 2.0 mg/L+NAA 0.5 mg/L	3.443(1.84)	1.886(1.37)	5.580(2.35)	6.223(2.49)	91.333(9.55)
BAP 2.5 mg/L+NAA 0.5 mg/L	3.890(1.96)	2.446(1.56)	6.033(2.45)	10.890(3.30)	90.220(9.50)
BAP 1.5 mg/L+IAA 0.5 mg/L	2.443(1.55)	1.666(1.28)	5.176(2.27)	7.113(2.67)	85.333(9.23)
BAP 2.0 mg/L+IAA 0.5 mg/L	1.996(1.40)	1.333(1.14)	6.490(2.54)	9.333(3.05)	91.113(9.54)
BAP 2.5 mg/L+IAA 0.5 mg/L	5.333(2.31)	3.670(1.92)	8.490(2.91)	10.333(3.21)	92.220(9.60)
BAP 1.5 mg/L+NAA 0.5 mg/L+IAA 0.5 mg/L	1.443(1.19)	1.110(1.05)	7.510(2.73)	9.776(3.12)	83.890(9.15)
BAP 2.0 mg/L+NAA 0.5 mg/L+IAA 0.5 mg/L	3.00(1.73)	2.000(1.42)	9.036(3.01)	9.333(3.06)	90.556(9.52)
BAP 2.5 mg/L+NAA 0.5 mg/L+IAA 0.5 mg/L	5.333(2.30)	4.333(2.08)	9.300(3.04)	11.553(3.40)	94.886(9.75)
S.E.m±	0.39	0.26	0.45	0.51	2.73
CD @ 1%	1.407	0.925	1.610	1.846	9.856
CV (%)	22.259	21.664	11.719	10.582	4.933

Values in the parenthesis represent square root values of mean.

Table 5: Effect of media and growth regulators concentration on *in vitro* rooting in *R. tetraphylla*

Treatment	Number of roots per explant	Root length	No. of primary roots	No. of secondary roots
IBA 2.0 mg/L	1.07 (1.18)	1.10 (1.19)	0.93 (1.15)	0.13 (0.79)
IBA 2.5 mg/L	2.20 (1.53)	1.39 (1.30)	1.60 (1.73)	0.47 (0.96)
IBA 2.0mg/L+ NAA 0.5 mg/L	4.13 (2.15)	3.40 (1.98)	3.07 (1.88)	0.47 (0.97)
IBA 2.5mg/L+ NAA 0.5 mg/L	5.40 (2.43)	3.98 (2.11)	3.73 (2.05)	1.33 (1.35)
S.E.m±	0.70	0.54	0.56	0.17
CD @ 1%	3.31	2.57	2.64	0.80
CV (%)	37.71	38.09	41.33	48.11

Values in the parenthesis represent square root values of mean.

Fig 1: Field grown mother block of Sarpagandha (*Rauvolfia tetraphylla*)

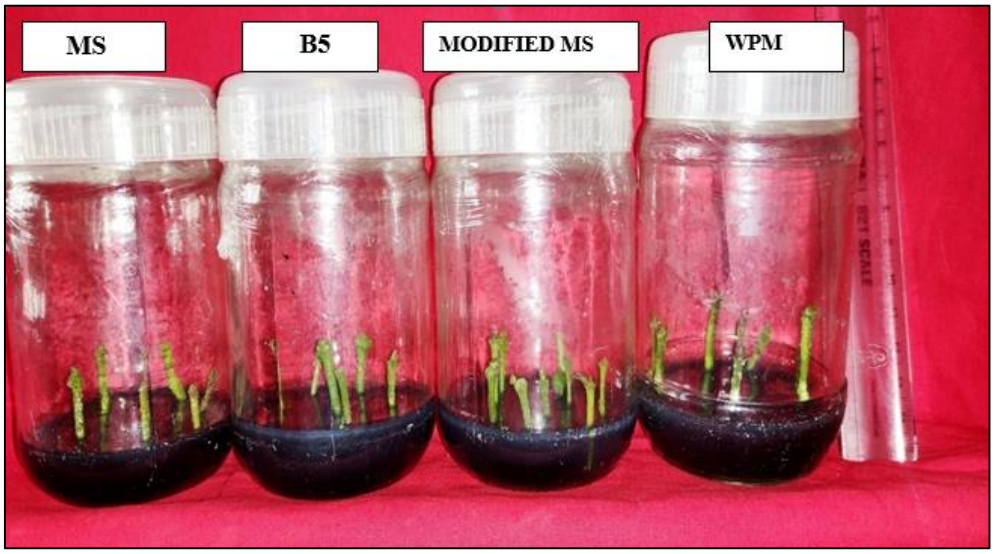

Fig 2: Effect of nodal segments with different media on shoot proliferation

Fig 3: Effect of different levels of growth regulator concentration on shoot proliferation

Fig 4: Rooted plants of Garden Rauvolfia (*Rauvolfia tetraphylla*)

Conclusion

The present study was carried out to develop efficient protocol for micro propagation of Garden Rauvolfia (*Rauvolfia tetraphylla*). Highest per cent of aseptic culture in the present study observed in the treatment of explants using sterilents bavistin, Ethanol and $HgCl_2$. Different explants and media were used for direct regeneration. Maximum multiplication rate was observed in cultures of MS media with nodal segments. In the shoot elongation study the maximum length of shoot was noticed in MS media containing 2.5mg/L BAP, 0.5mg/L NAA and 0.5mg/L IAA. In case of rooting, the maximum number roots were observed in half MS media supplemented with 2.5 mg/L IBA with 0.5 NAA mg/L. Considering the number of shoots and roots to be more important factors, MS media with 2.5 mg/L BAP in combination with 0.5 mg/L IAA and NAA for multiplication of shoot and half MS media augmented with 2.5 mg/L IBA with 0.5 NAA mg/L will be more economic and reproducible as well. The output of present study has brought novelty in micropropagation of *R. tetraphylla* on MS media which has proven to produce higher number of shoots. The optimized micropropagation also proved to give production of efficient and homogenous plant population. The regeneration method employed in the present study could provide a better supplement for high-rate production of quality planting materials to meet the commercial demands of industries and also a basic media for further scientific researches.

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