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REGULAR ARTICLE

A RAPID *IN VITRO* PROPAGATION AND ESTIMATION OF SECONDARY METABOLITES FOR *IN VIVO* AND *IN VITRO* PROPAGATED *CROTALARIA* SPECIES, A *FABACEAE* MEMBER

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ABSTRACT

The secondary metabolites extracted from both *in vivo* and *in vitro* propagated *Crotalaria* species were comparatively estimated. The *in vitro* propagated *Crotalaria* species were obtained from the explants of three medicinally important *Crotalaria* species (*C.prostrata, C. retusa* and *C. medicagenea*) on Murashige and Skoog (MS) medium fortified with variant concentrations of growth regulators (6-Benzylaminopurine (BAP), α – Naphtahlene acetic acid (NAA), 2, 4-Dichlorophenoxy acetic acid (2,4-D) and Kinetin). An optimal response of 12.6 shoots per explant (6.2 cm length) was obtained from *Crotalaria retusa* on Murashige and Skoog medium fortified with 13.31 µM 6-Benzylaminopurine and 2.15 µM α – Naphthalene acetic acid. The shoots raised were rooted optimally on Murashige and Skoog medium containing Indolebutyric acid (7.38 µM*)* with 4.4 roots per shoot. Rooted plantlets thus developed were transferred to greenhouse after hardening with a mix of soil and compost (1:1). Nearly 90% of *in vitro* raised plants of *Crotalaria* species were acclimatized. The growth and morphology of *in vitro* regenerated plants resemble wild species. Therefore *in vitro* propagated plants of all three species were subjected to comparative estimation of secondary metabolites with both callus cultures and wild species of *Crotalaria*. Obtained optimal alkaloid content with 29.0% per gram of leaf dry weight was for *in vitro* propagated

Crotalaria retusa amongst the three. Hence from the present investigation it was proved that the quantity of secondary metabolites of an *in vitro* propagated *Crotalaria* species is higher than field grown for pharmaceutical preparations.

Keywords: *Crotalaria* species, secondary metabolites, growth regulators, phyto-chemical studies, thin layer chromatography

INTRODUCTION

Plants are obviously diverse source of flavors, fragnances, natural pigments and pesticides for pharmaceuticals and other bio-chemicals (**Saurabh *et al.,* 2002**). *Crotalaria*, one of the medicinally recognized genus belonging to the *Fabaceae* family, mostly found in the tropical regions (**Daimon *et al.,* 2002**). The different species of *Crotalaria* and their applications are described as follows. *Crotalaria retusa* is used in the treatment of leukemia, spasmodicity, neoplasticity, cardiac diseases (**Sridhar *et al.,* 2007**). *Crotalaria medicagineae* is used in the treatment of diabetes (**Pullaiah and Chandrasekhar Naidu, 2003**). *Crotalaria prostrata* is applied on against gout and its root paste is used to diagnose diarrhea, skin infections, snake bite and stomachache (**Vardhana, 2008; Roeder and Wiedenfeld, 2009**). These species are having the property of accumulating pyrrolizidine alkaloids, saponins and flavonoids as a notable chemical markers with basic *N*-oxides having medicinal properties (**Nwude, 1986; Steglich *et al.,* 1997**). It is in the light of these facts *Crotalaria* species occupies their role in veterinary pharmacy (**Nuhu *et al.,* 2009**). Furthermore, these would provide important information for future exploitation of the plants and offer a fast way to the discovery of new medicinally or industrially useful compounds from plants (**Ramar *et al.,* 2008**).

Due to the medicinal importance and large scale unrestricted exploitation to meet increasing demands by the pharmaceutical industries, coupled with limited cultivation and insufficient attempts for its reforestation, the wild stock of this important plant species has been markedly depleted (**Saurabh *et al.,* 2002**). Hence these plants have to be conserved through micropropagation. Micropropagation is advantageous over traditional plant breeding methods as it helps in the mass production of plants (free of somaclonal variations). The present investigation was focused on comparative studies on regenerability and estimation of

secondary metabolites for both *in vitro* propagated and field grown (wild) plants of three *Crotalaria* species.

MATERIAL AND METHODS

Plant Material

The central section of *Crotalaria retusa, Crotalaria prostrate* and *Crotalaria medicaginea* leaves were used as explants for *in vitro* propagation and extraction for secondary metabolites (wild plant). The plants herbarium were deposited in Andhra University, Andhra Pradesh, India. vide voucher numbers A.U. (B.D.H.) 5527, A.U. (B.D.H.) 2359 and A.U. (B.D.H.) 1850 respectively.

Surface sterilization

The leaf explants are washed thoroughly with 2% (volume/volume) Tween-20 followed by running tap water for 10 min and then washed thrice with distilled water. The explants were then dipped in 70% ethanol for 1 min, followed by surface sterilization with 0.1% mercuric chloride for 5 min and finally rinsed thrice with sterile distilled water. The explants were surface-dried on sterile filter paper and cultured on Murashige and Skoog (MS) basal medium supplemented with different concentrations of plant growth regulators (**Murashige and Skoog, 1962**).

Culture media and culture conditions

The surface sterilized explants were inoculated on 15 mL Murashige and Skoog (MS) medium containing 30 gL^{-1} sucrose and 8gL^{-1} agar. The pH of all media were adjusted to 5.8 prior to autoclaving at 121^{o}C for 20 min. Leaf cuttings (1 cm) of all three species of *Crotalaria* were cultured on Murashige and Skoog (MS) medium supplemented with BAP (2.21-17.75 µM), Kn (0.46-18.84 µM), NAA (0.53-16.12 µM) and 2, 4-D (2.26-15.83 µM) alone or in combination for callus induction and multiple shoots induction (Table 1 & 2). All the cultures were incubated at 25±2^{o}C for 16 hours photoperiod. Thereafter, the clumps of shoots produced were separated and divided into single shoots after 30 days. These shoots were sub-cultured once in 30 days on same media for multiplication. Due to the lack of

growth regulators on Murashige and Skoog medium, the explants inoculated on it are served as control.

Multiple shoot induction

The leaf explants inoculated on Murashige and Skoog media fortified with BAP (2.21–13.31 µM) alone or in combination with NAA (0.53-4.50 µM) induce multiple shoots. Further, these aroused shoots were subcultured on Murashige and Skoog (MS) medium containing various concentrations of IBA & NAA for rooting leading to plantlet development.

Rooting of shoots

The aroused shoots were cultured on half-strength Murashige and Skoog (MS) medium supplemented with different concentrations of IBA (2, 4, 7, 9 and 19 µM) and NAA (2, 5, 8, 10 and 21 µM) for rooting. Aroused Plantlets were gently extracted from the glass vessels and washed thoroughly with tap water to remove adhered agar and traces of the medium in order to avoid contamination. These plantlets were given a final wash with distilled water for 5 min and transferred to a plastic cups (8 cm in diameter) containing garden soil subsequently transferred to pot. The pots were maintained in a polyethylene chamber in the culture room and irrigated every alternative day with a solution of half-strength Murashige and Skoog (MS) medium. Polyethylene covers were removed gradually, and then the plants were transferred to garden soil for further growth in a greenhouse. All the above experiments were repeated thrice and recorded the observations and results. The results of phytochemical studies conducted with field grown (wild) plants and *in vitro* propagated plants of *Crotalaria* species were compared.

Extraction

The shade dried leaf powder of field grown (wild) plant species, callus induced from leaf explants and *in vitro* regenerated leaves of *Crotalaria* species were subjected to cold extraction with 98% petroleum ether, 98% chloroform, 95% ethanol, and distilled water for 24hours each in the order of increasing solvent polarity. The condensed extracts were used for conducting the preliminary screening of phytochemicals such as alkaloids, flavonoids,

phenols, saponins and tannins (**Mallikharjuna *et al.*, 2007; Prabhu *et al.*, 2011; Savithramma *et al.*, 2011**).

Quantitative estimation of Secondary Metabolites

The presence of secondary metabolites in wild, *in vitro* propagated and callus induced from *Crotalaria* species were quantitatively determined by adopting standard protocols. Alkaloids were estimated using Ikan's method (**Ikan, 1981**), flavonoids using Swain and Hillis method (**Swain and Hillis, 1959**), phenols using Bray and Thorpe method (**Akharaiyi and Bolatito, 2010**), tannins using Folin-Denis method (**Schanderi, 1970**) and saponins using Sanchez method (**Nishanthi *et al.*, 2012**).

Separation of Secondary Metabolites by thin layer chromatography (TLC)

The secondary metabolites extracted from *Crotalaria* were qualitatively separated by TLC using Silica gel – G (Fischer Scientific Pvt Ltd) coated on glass plate.

TLC study of alkaloids

The extracted fractions of wild, callus and *in vitro* propagated *Crotalaria* species were wetted with half diluted ammonium hydroxide (NH_4OH) and lixiviated with Ethyl acetate (EtOAc) for 24hours at room temperature. The organic phase is separated from the acidified filtrate and basified with NH_4OH (pH 11.0-12.0). These phytochemicals were extracted with chloroform (3X), condensed by evaporation and then used for chromatography. The alkaloid spots were separated using the solvent mixture chloroform and methanol (3:1). The colour and hRf values of the separated alkaloids were recorded under both ultraviolet (345 nm) and visible light after spraying Dragendorff's reagent (**Wagner and Bladt, 1996**).

TLC study of flavonoids

The methanolic extracts of all the samples of *Crotalaria* species were condensed by evaporation and used for chromatography. Later the flavonoid spots were separated using chloroform and methanol (9:1) solvent mixture. The colour and hRf values of these spots were recorded under ultraviolet (345 nm) light (**Wagner and Bladt, 1996**).

TLC study of phenols

The methanolic extracts of all the samples of *Crotalaria* species were used for chromatography. The phenols were separated using chloroform and methanol (27:0.3) solvent mixture. The colour and hRf values of these phenols were recorded under visible light after spraying Folin-Ciocalteu's reagent at 80^{o}C/10min (**Harborne, 1998**).

TLC study of saponins

The aqueous extracts of all the samples of *Crotalaria* species were enriched with saturated n-Butanol, and thoroughly mixed. The n-Butanol fraction of the mixture was condensed and used for chromatography. The saponins were separated using chloroform: acetone (1:1) solvent mixture. The colour and hRf values of these spots were recorded by exposing chromatogram to the iodine vapours (**Wagner and Bladt, 1996**).

TLC study of Tannins

All the Condensed extracts of *Crotalaria* species were determined by oxidation of Condensed-Tannin with Butanol-Hydrochloric acid reagent in the presence of iron and estimated for the tannins (**Porter *et al.*, 1986**).

The significant differences among the means were assessed by Duncan's Multiple Range Test (DMRT) and Tukey's test using Statistical Package for Social Sciences (SPSS version 17.0).

RESULTS AND DISCUSSION

The effect of growth regulators on medicinally important *Crotalaria* species for regeneration and the comparative secondary metabolites estimation for both *in vivo* and *in vitro* propagated plants were conducted in present investigation.

Callus and multiple shoots induction

Leaf explants of *Crotalaria* species inoculated on Murashige and Skoog (MS) medium with 30 gL^{-1} sucrose supplemented with different concentrations of growth regulators induce callus and multiple shoots. Multiple shoots optimally initiating from leaf cuttings of *Crotalaria retusa*, cultured on Murashige and Skoog (MS) medium fortified with 13.31 µM BAP in combination with 2.15 µM NAA (Fig. 1). *Crotalaria prostrata* had shown lesser response (79.5%) than *Crotalaria medicaginea* (87%) for regeneration (Table 1-2). Multiple shoots were induced on Murashige and Skoog (MS) medium containing various concentrations of Kn and BAP alone or in combination with NAA. The addition of NAA into the medium considerably enhanced the multiple shoots induction. Cytokinins are necessary in concert with auxin in many cases for cell division at G1-S and G2-M transitions in plant cells (**Mercier *et al.*, 2003**). BAP induce multiple shoots induction has been reported in *Ulmus* (**Thakur and Karnosky, 2007**). The proliferation ability of leaf depends on the plant growth regulators concentration and the age of explants. Multiple shoots formed for all the species with all the combinations investigated. However, there were no shoots on MS basal medium (control). The percentage response, number of shoots and average shoot length were controlled by the type and concentration of the growth regulator employed. A maximum of 93.0% response was obtained from the cultures of *C.retusa* with 11.4 shoots per explant on MS media supplemented with 13.31 µM BAP alone. The average length of induced shoots was limited to 3.2 cm after 30 days of culture. A visible improvement was observed for *Crotalaria retusa* and *C. medicaginea*, when NAA (0.53–4.3 µM) was supplemented along with 13.31 µM BAP but no improvement was observed for *C. prostrata*. The addition of NAA enhanced the response for *C.retusa* to 96.4% with 12.6 shoots per explant having a length of 6.2cm on MS media containing 13.31 µM BAP and 2.15 µM NAA (Figure 2). However, the average shoots length show much variation from the earlier treatment (Table 1). Similar results were observed for the leaf explants of *Strobilanthes flaccidifolious* cultured on Murashige and Skoog (MS) medium supplemented with BAP alone and in combination with NAA (**Pickens *et al.*, 2005**).

Figure 1 Initiation of multiple shoots induction from leaf explant on Murashige and Skoog (MS) medium.

Figure 2 Increase in length and frequency of multiple shoots after supplementation of NAA along with MS medium.

a) **b)** **c)**

Figure 3 Induction of diverse colored organogenic callus on Murashige and Skoog (MS) medium was obtained: **a)** brown **b)** green **c)** white.

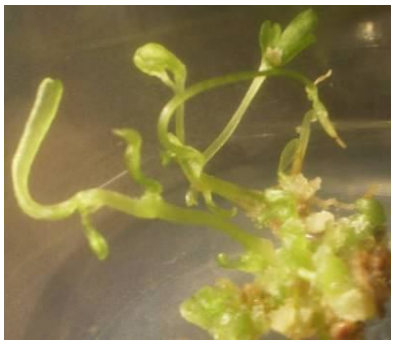

Figure 4 Rooting of aroused shoots on Murashige and Skoog (MS) medium fortified with IBA.

Table 1 Effect of BAP alone or in combination with NAA for adventitious shoot multiplication of leaf explants of *Crotalaria retusa* after 30 days

Plant growth regulators (µM)		% response explants producing shoots			Number of shoots per explant			Average shoot length (cm)		
BAP	**NAA**	***Crotalaria retusa***	***Crotalaria medicaginea***	***Crotalaria prostrata***	***Crotalaria retusa***	***Crotalaria medicaginea***	***Crotalaria prostrata***	***Crotalaria retusa***	***Crotalaria medicaginea***	***Crotalaria prostrata***
0.00	0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
2.21		48.0±0.76g	22.6±0.02i	37.3±0.01g	1.6±0.24i	0.9±0.11l	0.4±0.21g	2.2±0.20c	1.9±0.71c	1.3±0.02c
4.43		69.0±0.07e	36.1±0.01g	56.2±0.03d	2.6±0.37h	1.4±0.04k	1.1±0.04f	2.2±0.20c	1.9±0.62c	1.3±0.10c
6.65		74.0±1.41d	49.2±0.11e	67.4±0.01c	4.0±0.44g	2.9±0.12h	2.5±0.09de	2.2±0.20c	1.9±1.32c	1.3±0.09c
8.87		88.4±0.92b	54.0±0.06d	49.3±0.03f	6.6±0.50f	4.1±0.15ef	4.9±0.13b	3.2±0.20b	1.9±1.08c	3.0±0.61b
11.09		89.8±0.66b	67.1±0.03c	34.7±0.06h	7.0±0.44e	6.8±0.03c	5.1±0.06a	3.2±0.20b	2.8±0.96b	3.0±0.12b
13.31		93.0±0.10a	79.8±0.06b	24.5±0.08i	11.4±0.5 b	**8.4±0.04a**	3.6±0.05c	3.2±0.20b	2.8±0.63b	3.0±0.18b
15.53		70.2±0.86e	**87.3±0.07a**	15.1±0.09j	7.8±0.37e	7.6±0.01b	2.4±0.010de	3.2±0.20b	2.8±0.16b	3.0±0.31b
17.75		61.6±0.92f	69.2±0.01c	10.3±0.00k	6.0±0.31f	5.3±0.02d	1.0±0.74f	3.2±0.20b	3.4±0.41a	4.6±0.20a
13.31	0.53	78.8±0.86c	32.2±0.02h	53.4±0.01e	8.2±0.37d	3.8±0.15g	3.3±0.18c	6.2±0.31a	3.4±0.91a	4.6±0.15a
13.31	1.07	85.4±0.67b	56.5±0.01d	70.0±0.04b	10.4±0.50c	4.6±0.19e	3.8±1.02c	6.2±0.31a	3.4±0.62a	4.6±0.17a
13.31	**2.15**	**96.4±0.87a**	40.1±0.13f	**79.7±0.01a**	**12.6±0.4 a**	2.4±0.20i	2.9±0.24d	**6.2±0.31a**	**3.4±0.62a**	**4.6±0.91a**
13.31	4.30	89.6±0.50b	20.4±0.06	65.1±0.04c	9.2±0.37c	2.0±0.09j	1.3±0.91f	6.2±0.31a	3.4±0.62a	4.6±0.50a

Data were recorded after 4 wk of culture.

n.d not determined. Values represent means±SE from 5 replicates.

Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test (P<0.05).

Table 2 Effect of different concentrations of BAP, Kn, NAA and 2,4-D on organogenesis from leaf cuttings derived callus

Plant growth regulators (µM)				% of response		
NAA	**2,4-D**	**BAP**	**Kn**	***Crotalaria retusa***	***Crotalaria medicaginea***	***Crotalaria prostrata***
	2.26	2.21		20.2±1.58j	30.2±0.41n	21.6±0.21k
	4.52	2.21		40.6±0.81hi	39.4±1.12l	46.8±1.18f
	6.78	2.21		59.6±0.82fg	55.1±0.71hi	63.3±0.50cd
	9.04	**2.21**		81.8±1.86c	70.5±0.65ef	**79.4±1.62a**
	11.31	2.21		90.0±0.31b	83.9±1.15c	58.2±0.73d
	13.57	**2.21**		**98.4±1.21a**	90.6±0.85b	41.0±1.81g
	15.83	2.21		77.8±0.80d	70.2±0.33ef	25.1±0.61jk
2.68			2.32	13.4±0.50k	27.5±1.12n	8.3±1.39n
5.37			2.32	24.2±1.66ij	36.8±0.32m	19.4±0.42m
8.06			2.32	47.4±0.53h	50.1±1.33j	34.2±0.90hi
10.75			2.32	84.2±0.86c	66.2±0.53g	53.4±0.01e
13.44			2.32	70.6±0.92de	43.0±0.92k	47.2±0.11f
16.12			2.32	45.4±0.40h	31.1±1.52m	35.3±1.16hi
10.75		0.44		54.2±1.06g	44.7±0.11k	20.4±0.26l
10.75		0.85		59.6±0.97fg	59.3±0.08h	27.2±1.76j
10.75		1.77		72.4±1.36de	72.0±1.19e	41.6±0.45g
10.75		**3.55**		69.0±0.71de	**92.4±0.93a**	66.3±0.64c
10.75		7.10		63.0±1.30f	78.6±0.49d	52.1±1.11e
10.75		14.20		50.4±0.92g	50.0±0.66j	45.0±0.13f
10.75			0.46	43.8±1.24h	38.6±1.54l	32.4±0.34hi
10.75			0.92	50.2±1.15g	40.3±1.02k	38.1±1.09h
10.75			1.85	60.8±1.15fg	57.1±1.08hi	52.3±1.64e
10.75			3.71	64.4±1.63f	71.3±1.00ef	60.5±0.29d
10.75			7.42	42.2±1.28h	42.7±0.93k	76.2±0.52b
10.75			14.84	28.0±0.70i	30.0±0.36n	59.4±1.62d

Data were recorded after 4 wk of culture.

n.d not determined. Values represent means±SE from 5 replicates.

Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test ($P<0.05$).

Another cytokinin, Kn was added to the Murashige and Skoog (MS) medium at concentrations of 2-6 µM in order to investigate whether it plays any critical role in multiple shoot induction for all the species (Table 2). A comparative study for the effect of BAP and Kn on multiple shoots induction in mulberry (*Morus*) resulted that BAP enhances the multiple shoots induction (**Thomas and Surabhi, 2009**). However the number of shoots showed a slight decreased when 13.31 µM BAP alone was used for multiple shoots induction. Hence

the addition of NAA enhanced the average shoot length which plays a crucial role in the rooting of shoots for plantlet development. However, the individual use of cytokinins was not initiative in giving an optimal response in most of the systems; the best result was obtained when Kn or BAP was employed in combination with NAA. The promotory effect of cytokinin on culture proliferation is reported, while BA, Kn and NAA in combination was found to be superior over other treatment in *Acacia confusa, Morus indica* (**Vijaya Chitra and Padmaja, 1999; Selvaraj *et al.,* 2006; Arumugam *et al.,* 2009; Baskaran *et al.*, 2009**). Similar results were compared where combinations of BAP and NAA have induced maximum shoot multiplication from nodal explants of *Clitoria ternatea* (**Rout, 2005**). In *Acharus sapota*, the induction of multiple shoots was found to be low when BAP alone was employed for shoot proliferation. Hence this single step procedure can save time, since subsequent subcultures are not necessary to achieve optimal response.

The leaf cuttings of three species were cultured on Murashige and Skoog (MS) medium supplemented with BAP (2.21 µM) and 2, 4-D (13.57 µM) for callus induction. The callus emerged from leaf cut ends of *Crotalaria retusa* on Murashige and Skoog (MS) medium shows 98.4% response. The fastest response for callus induction was observed for *Crotalaria retusa* over other two species after seven days of inoculation. The friable, green, brown and whitish organogenic callus was formed from leaf segments of *Crotalaria* on Murashige and Skoog (MS) medium supplemented with BAP (2.21 µM) and 2,4-D (13.57 µM) after 20 days of culture (Figure 3a-c).

The friable callus obtained from all the species were sub cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of BAP (0.44-14.2 µM) or Kn (0.46-14.8 µM) alone or in combination with NAA (2.68-16.12 µM) for shoot regeneration (Table 2). The percentage response and frequency of shoots per explant were greatest for *Crotalaria retusa* on Murashige and Skoog (MS) medium supplemented with 13.31 µM BAP. Here, a maximum of 93.0 % cultures responded and produced 3.2 shoots per explant (Figure 1). The addition of NAA along with BAP significantly increased the response and frequency of shoots. An optimum 96.4% cultures responded with 12.6 shoots was observed on Murashige and Skoog (MS) medium supplemented with 13.31 µM BAP and 2.15 µM NAA(Figure 2). Callus induction through an auxin-cytokinin combination has been reported for several systems including *Clematis gouriana*; *Valeriana edulis*; *Dioscorea zingiberensis* and *Clerodendrum serratum* (**Castillo *et al.,* 2000; Shu *et al.,* 2005; Vidya *et al.,* 2005; Raja Naika and Krishna, 2008a**). The emergence of several healthy green shoots was observed after four weeks of inoculation. The callus derived shoots had longer leaves

than the multiple shoots induced directly from leaf explants, but the stem sizes were same in both cases.

Individual shoots aroused with at least 6.0-6.2 cm long of all the species were separated and sub-cultured on Murashige and Skoog (MS) medium supplemented with half-strength Murashige and Skoog (MS) medium supplemented with NAA (2.68-21.50 µM) or IBA (2.46-19.68 µM) for root induction. The shoots with below 3.0 cm in length did not produce any roots when cultured on rooting medium (data not shown). The shoots produced roots at all concentrations of IBA and NAA, but the highest efficiency of 100% rooting was observed for *C. retusa* on MS media containing 7.38 µM IBA with an average of 4.4 roots per shoot, was recorded at the end of the 30th day of culture (Table 3; Figure 4). IBA was comparatively better than NAA in terms of percentage in rooting as well as the frequency of roots per shoot. Similar results were reported for *Clematis gouriana* and *Naravelia zeylanica* (**Raja Naika and Krishna, 2008a,b**). The rooted shoots were successfully transplanted to plastic cups containing garden soil, and the humidity was maintained at approximately 90% by covering it with polythene bag and watered twice every four days. The plantlets were transferred to larger pots for acclimatization and 100-day-old plants were transferred to the field. An optimum survival frequency of 86% was observed for *C.retusa*, whereas *C.medicaginesa* and *C.prostrata* had shown only 73% and 59% respectively. Plants transferred to the field established themselves in soil and are growing well.

Table 3 Effect of half-strength Murashige and Skoog (MS) medium with various concentrations of NAA and IBA on the induction of roots from multiple shoots derived *in vitro*

Auxin	Concentration of plant growth regulator (µM)	% of shoots responded for rooting			Average number of roots per shoot		
		Crotalaria retusa	*Crotalaria medicaginea*	*Crotalaria prostrata*	*Crotalaria retusa*	*Crotalaria medicaginea*	*Crotalaria prostrata*
Control	0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
NAA	2.68	0.0±0.78i	0.0±0.10j	18.7±1.00i	0.0±1.32e	0.0±0.80e	1.2±0.61d
	5.37	30.0±0.92h	38.1±0.87i	26.2±0.36h	2.0±0.65d	1.4±0.79d	1.2±0.71d
	8.06	35.6±0.74h	42.0±0.67h	33.8±0.49f	2.0±0.76d	1.4±0.61d	1.2±1.09d
	10.75	52.6±0.71f	67.8±0.71f	59.3±0.57c	2.0±0.94d	2.1±0.90c	2.0±0.05b
	21.50	45.0±0.92g	49.2±0.93g	29.0±0.63g	3.0±0.70c	2.1±0.12c	2.0±0.62b
IBA	2.46	85.4±1.02d	72.0±0.21e	53.8±0.23d	3.0±1.06c	2.8±0.11b	1.8±0.19c
	4.92	93.6±0.82b	86.1±0.19c	69.1±0.11b	4.0±0.95b	2.8±1.08b	1.8±0.39c
	7.38	**100.0±0.00a**	95.6±1.06b	**79.5±0.14a**	**4.4±0.16a**	2.8±1.14b	**2.5±0.21a**
	9.84	91.2±0.58c	**98.2±1.02a**	42.3±0.02e	4.2±0.41b	**3.2±1.64a**	2.5±0.61a
	19.68	82.8±0.83d	76.8±0.09d	31.9±0.16f	4.2±0.74b	3.2±0.99a	2.5±1.09a

Data were recorded after 4 wk of culture.

n.d not determined. Values represent means±SE from 5 replicates.

Means followed by different *letters* within *columns* are significantly different by Duncan's multiple range test ($P<0.05$).

Estimation of metabolites

The therapeutic significance of medicinal plants is due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins *etc*. The preliminary screening helps in the detection of bioactive principles and subsequently provides the information useful in drug discovery and development.

The wild, *in vitro* propagated and callus cultures of *Crotalaria* species were subjected to cold extraction for the estimation of secondary metabolites. The alkaloid content of an *in vitro* propagated *Crotalaria* species was higher (22.9 % of metabolite/g dry weight) than in the field grown plants. The *in vitro* regenerated *Crotalaria retusa* species resulted with the highest content of medicinally important secondary metabolites (Table 4). The hRf (Retardation factor) value of alkaloid content of all the extracts were compared and analyzed that *Crotalaria retusa* accumulates higher content of medicinally important monocrotaline alkaloid, confirmed through further analysis with standard monocrotaline whose hRf value is 56.0. The higher content of secondary metabolites in *Crotalaria* species was observed in *in vitro* regenerated *C. retusa* than in the naturally growing plants. Hence, the present investigation on *Crotalaria* species evidenced that the accumulation of metabolite content is high in *in vitro* propagated plants than in wild plants. Similar comparative studies were reported for *Solidago chilensis, Solanum villosum* and *Exacum bicolor* (**Schmeda-Hirschmann *et al.*, 2005; Esam, 2011; Meethaley and Subramaniam, 2011**).

Table 4 A comparative quantitative estimation of secondary metabolites from *Crotalaria* species

Sl. No.	Type of Metabolite	Name of species	Percent (%) of secondary metabolites / gram dry weight ± SE		
			In vitro propagated	Callus	Wild
1	Alkaloids	*Crotalaria retusa*	**29.0 ± 0.20a**	19.0 ± 1.1a	14.5 ± 0.10b
		Crotalaria prostrate	17.0 ± 0.11c	12.0 ± 0.63b	4.03 ± 0.05e
		Crotalaria medicaginea	14.2 ± 0.23d	8.2 ± 0.41d	9.32 ± 0.30c
2	Flavonoids	*Crotalaria retusa*	**22.9 ±0.12b**	**19.6 ± 0.12a**	**18.42 ± 0.08a**
		Crotalaria prostrate	0.95 ± 0.16h	0.34 ± 0.16 ij	0.29 ± 0.05i
		Crotalaria medicaginea	0.21 ± 0.24j	0.18 ± 0.19j	0.02 ± 0.04j
3	Phenols	*Crotalaria retusa*	8.31 ± 0.30f	6.52 ± 0.21f	3.26 ± 0.07f
		Crotalaria prostrate	7.12 ± 0.27f	4.53 ± 0.10g	2.16 ± 0.02g
		Crotalaria medicaginea	1.03 ± 0.32g	0.91 ± 0.19i	0.25 ± 0.02j
4	Tannins	*Crotalaria retusa*	0.39 ± 0.12i	0.18 ± 0.02j	0.02 ± 0.01 k
		Crotalaria prostrate	0.02 ± 0.65j	0.04 ± 0.01k	0.04 ± 0.01k
		Crotalaria medicaginea	0.10 ± 0.11j	0.07 ± 0.01k	0.04 ± 0.02k
5	Saponins	*Crotalaria retusa*	12.9 ± 0.31e	9.14 ± 0.32 c	6.82 ± 0.09d
		Crotalaria prostrate	2.36 ± 0.21g	1.94 ± 0.24h	1.09 ± 0.95h
		Crotalaria medicaginea	1.69 ± 0.02g	1.24 ± 0.31h	0.45 ± 0.67i

The data collected were statistically analyzed by one-way ANOVA and the means were compared using Tukey's test (HSD) at p = 0.05 using SPSS version 17.0.

Similar protocol with efficient and comparable results of pharmacologically active metabolites extraction and their estimation was reported for *Strychnos potatorum* and *Pluchea lanceolata* (**Mallikharjuna *et al.*, 2007; Deepika *et al.*, 2008**).

Table 5 Qualitative separation of secondary metabolites from *Crotalaria* species

Sl. No.	Type of metabolite	Type of species	Color of the spot	hRf values	Samples from *Crotalaria* species tested		
					In vitro propagated	Callus	Wild
1	Alkaloids	*Crotalaria retusa*	Olive Green	56.00	+	+	+
		Crotalaria prostrate	Light Green	49.12	+	+	-
		Crotalaria medicaginea	Light Green	48.62	+	+	-
2	Flavonoids	*Crotalaria retusa*	Lemon	42.56	+	+	+
		Crotalaria prostrate	Pale Yellow	50.42	+	+	+
		Crotalaria medicaginea	Yellow	48.63	+	+	-
3	Phenols	*Crotalaria retusa*	Light Blue	64.67	+	+	-
		Crotalaria prostrate	Light Blue	62.95	+	+	+
		Crotalaria medicaginea	Light Blue	67.29	+	+	+
4	Tannins	*Crotalaria retusa*	Yellowish Green	71.23	-	-	+
		Crotalaria prostrate	Light Green	69.56	+	+	+
		Crotalaria medicaginea	Light Green	70.45	-	+	-
5	Saponins	*Crotalaria retusa*	Dark Yellow	84.74	+	+	+
		Crotalaria prostrate	Dark yellow	79.21	+	+	+
		Crotalaria medicaginea	Dark Yellow	72.32	+	+	+

"+" indicates the presence & "-" indicates the absence of the specific compound

The data of qualitative determination of secondary metabolites is tabulated (Table 5). Among the five groups of phytochemicals determined for wild, *in vitro* regenerated and callus cultures of *Crotalaria* species, alkaloids were found to be an abundant and pharmaceutically

important metabolite followed by flavonoids, whereas phenols, tannins and saponins were found as lower abundance in *Crotalaria* species.

CONCLUSION

Tissue culture technology offers a sole source for the conservation of germplasm and mass propagation of medicinally important plant resources like *Crotalaria* species. The standardization of protocol for rapid *in vitro* propagation of *Crotalaria* species provides a raw material for comparative estimation of secondary metabolites for wild, callus and *in vitro* propagated *Crotalaria* species. The high quantity of medicinally important phytochemicals was accumulated in *in vitro* propagated plant species over field grown (wild) plants was observed in present investigation, provides resource for preparation of drugs against various diseases by the pharmaceutical industries.

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REFERENCES

AKHARAIYI, F. C. – BOLATITO, B. 2010. Antibacterial and phytochemical evaluation of three medicinal plants. In *Journal of Natural Products,* vol. 3, 2010, p. 27-34.

ARUMUGAM, S. – CHU, F. H. – WANG, S. Y. – CHANG, S. T. 2009. *In vitro* plant regeneration from immature leaflets derived callus of *Acacia confusa* Merr via organogenesis. In *Journal of Plant Biochemistry and Biotechnology*, vol. 18, 2009, no. 2, p. 197-201.

BASKARAN, P. – VELAYUTHAM, P. – JAYABALAN, N. 2009. *In vitro* regeneration of *Melothria maderaspatana* via indirect organogenesis. In *In Vitro Cellular and Developmental Biology - Plant*, vol. 45, 2009, no. 4, p. 407-413.

CASTILLO, P. J. – MARQUEZ, A. – RUBLUO, G. 2000. Plant regeneration from callus and suspension cultures of *Valeriana edulis* sps. Procera via simultaneous organogenesis and somatic embryogenesis. In *Plant Science,* vol. 151, 2000, no. 2, p. 115-119.

VIJAYA CHITRA, D. S. – PADMAJA, G. 1999. Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through *in vitro* culture of nodal explants. In *Scientia Horticulturae,* vol. 80, 1999, no. 3-4, p. 289–298.

DAIMON, H. – OHNO, H. – AKASAKA, Y. – MII, M. 2002. A histological evaluation of adventitious bud formation in *Crotalaria juncea* L. In *Plant Production Science*, vol. 5, 2002, no. 4, p. 301-304.

DEEPIKA, A. – VIDYA, P. – UMA, K. 2008. *In vitro* propagation and quercetin quantification in callus cultures of Rasna (*Pluchea lanceolata* Oliver & Hiern). In *Indian Journal of Biotechnology*, vol. 7, 2008, no. 3, p. 383-387.

ESAM, A. H. 2011. *In vitro* versus *in vivo*: A comparative study of *Solanum villosum* (Mill) plant leaves. In *International journal of Integrative Biology*, vol. 11, 2011, no. 3, p. 140-144.

HARBORNE, J. B. 1998. Phytochemical methods: A guide to modern techniques of plant analysis, 3rd Ed., Chapman and Hall Co.: New York, 1998, p. 1-302.

IKAN, R. 1981. Natural Products: A laboratory guide, Academic Press, London, 1981, p. 101.

MALLIKHARJUNA, P. B. – RAJANNA, L. N. – SEETHARAM, Y. N. – SHARANABASAPPA, G. K. 2007. Phytochemical studies of *Strychnos potatorum* L.f.- A medicinal plant. In *E-Journal of Chemistry*, vol. 4, 2007, no. 4, p. 510-518.

MEETHALEY, V. J. – SUBRAMANIAM, P. 2011. Evaluation of certain flavonoids of medicinal importance in the wild and micropropagated plants of the endangered medicinal species, *Exacum bicolor* Roxb. In *Journal of Applied Pharmaceutical Science*, vol.1, 2011, no. 5, p. 99-102.

MERCIER, H. – SOUZA, B. M. – KRAUS, J. E. – HAMSAKI, R. M. – SOTTA, B. 2003. Endogenous auxin and cytokinin contents associated with shoot formation in leaves of pineapple cultured *in vitro*. In *Brazilian Journal of Plant Physiology*, vol. 15, 2003, no. 2, p. 107-112.

MURASHIGE, T. – SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. In *Physiologia Plantarum*, vol. 15, 1962, no. 3, p. 473-497.

NISHANTHI, M. – MOHANAMBAL, E. – NARENDIRAN, S. – SHANKAR, M. – VIJEY AANANDHI M. – VIJAYAKUMAR, B. 2012. Extraction and preliminary phytochemical investigation on whole plant of *Peperomia tetraphylla* (g.forst.hook & arn). In *International Journal of Phytopharmacology*, vol. 3, 2012, no. 2, p. 173-177.

NUHU, H. –ABDURRAHMAN, E. M. – SHOK, M. 2009. Ethnomedical studies of *Crotalaria* species found in zaria, Northern Nigeria. In *Nigerian Journal of Pharmaceutical Sciences,* vol. 8, 2009, no. 2, p. 46 – 53.

NWUDE, N. 1986. The state of medicinal plants research in Nigeria. Proceedings of workshop, Ife (Edited by Sofowara, A.). Ibadan University Press, Nigeria, 1986.

PICKENS, K. A. – CHENG, Z. M. –TRIGIANO. R. N. 2005. Axillary bud proliferation and organogenesis of *Euphorbia pulchurrima* winter rose. In *In Vitro Cellular and Developmental Biology - Plant*, vol. 41, 2005, no. 6, p. 770–774.

PORTER, L. J. – HRSTICH, L.N. – CHAN, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. In *Phytochemistry*, vol. 25, 1986, no. 1, p. 223-230.

PRABHU, K. – KARAR, P. K. – HEMALATHA, S. – PONNUDURAI, K. 2011. A comparative preliminary phytochemical screening on the leaves, stem and the roots of three *Viburnum* Linn. species. In *Der Pharmacia Sinica*, vol. 2, 2011, no. 3, p. 81-93.

PULLAIAH, T. – CHANDRASEKHAR NAIDU, K. 2003. Antidiabetic plants in India and herbal based antidiabetic research: Ed. 1, Regency Publication, New Delhi, 2003, p. 125.

RAMAR, P. S. – PETER, N. P. – GOPALAKRISHNAKONE, P. 2008. A compilation of bioactive compounds from ayurveda. In *Bioinformation,* vol. 3, 2008, no. 3, p. 100-110.

RAJA NAIKA, H. – KRISHNA, V. 2008a. Plant regeneration from callus culture of *Clematis gouriana* Roxb. – A rare medicinal plant. In *Turkish Journal of Biology*, vol. 32, 2008a, no. 2, p. 99-103.

RAJA NAIKA, H. – KRISHNA, V. 2008b. Micropropagation, isolation and characterization of berberine from the leaves of *Naravelia zeylanica* (L.) DC. In *Research Journal of Medicinal Plant*, vol. 2, 2008b, no. 1, p. 1-9.

ROEDER, E. – WIEDENFELD, H. 2009. Pyrrolizidine alkaloids in medicinal plants of Mongolia, Nepal and Tibet. In *Pharmazie,* vol. 64, 2009, no. 11, p. 699–716.

ROUT, G. R. 2005. Micropropagation of *Clitoria ternatea* Linn. (Fabaceae)- an endangered medicinal plant. In *In Vitro Cellular and Developmental Biology - Plant,* vol. 41, 2005, no. 4, p. 516-519.

SAURABH, C. – SUNITA, F. – ASHOK, K.S. – VIRENDRA, S. B. 2002. Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. In *Biotechnology and Bioprocess Engineering*, vol. 7, 2002, p. 138-149.

SAVITHRAMMA, N. – LINGA RAO, M. – SUHRULATHA, D. 2011. Screening of medicinal plants for secondary metabolites. In *Middle-East Journal of Scientific Research,* vol. 8, 2011, no. 3, p. 579-584.

SCHANDERI, S.H. 1970. Methods in food analysis: Academic Press, New York, 1970, p. 709.

SCHMEDA-HIRSCHMANN, G. – JORDAN, M. – GERTH, A. – WILKEN, D. 2005. Secondary metabolite content in rhizomes, callus cultures and *in vitro* regenerated plantlets of *Solidago chilensis*. In *Zeitschrift fur Naturforschung*, vol. 60, 2005, no. 1-2, p. 5-10.

SELVARAJ, N. – VASUDEVAN, A. – MANICKAVASAGAM, M. – GANAPATHI, A. 2006. *In vitro* organogenesis and plant formation in *Cucumber*. In *Biologia Plantarum,* vol. 50, 2006, no. 1, p. 123-126.

SHU, Y. – YING-CAI, Y. – HONG-HUI, L. 2005. Plant regeneration through somatic embryogenesis from callus cultures of *Dioscorea zingiberensis*. In *Plant Cell Tissue and Organ Culture*, vol. 80, 2005, no. 2, p. 157-161.

SRIDHAR, K. R. – BHAGYA, B. 2007. Coastal sand dune vegetation: a potential source of food, fodder and pharmaceuticals. In *Livestock Research for Rural Development.* Vol. 19, 2007, no. 6, p. 84.

STEGLICH, W. – FUGMANN, B. – LANG-FUGMANN, S. 1997. Ro¨mpp Lexikon, Naturstoffe. Georg Thieme, Stuttgart, New York, 1997, p. 43. ISBN 3-13-749901-1.

SWAIN, T. – HILLIS, W. E. 1959. The phenolic constituents of *Prunus domestica* . I. The quantitative analysis of phenolic constituents. In *Journal of the Science of Food and Agriculture*, vol. 10, 1959, no. 1, p. 63-68.

THAKUR, R. C. – KARNOSKY, D. F. 2007. Micropropagation and germplasm conservation of Central Park Splendor Chinese elm (Ulmus parvifolia Jacq. 'A/Ross Central Park') trees. In *Plant Cell Reports,* vol. 26, 2007, no. 8, p. 1171-1177.

THOMAS, T. D. – SURABHI, S. 2009. Multiple shoot induction and callus regeneration in *Sarcostemma brevistigma* Wight & Arnott, a rare medicinal plant. In *Plant Biotechnology Reports*, vol. 3, 2009, no. 1, p. 67-74.

VARDHANA, R. 2008. Direct uses of medicinal plants and their identification, 1st Ed: Sarup & Sons publication, New Delhi, 2008, p. 208, 375. ISBN: 81-7625-833-4.

VIDYA, S. M. – KRISHNA, V. – MANJUNATHA, B. K. 2005. Micropropagation of *Clerodendrum serratum* L. from leaf explants. In *Journal of Non-Timber forest Products*, vol. 12, 2005, no. 1, p. 57-60.

WAGNER, R. – BLADT, S. 1996. Plant drug analysis, A Thin Layer Chromatography Atlas,2nd Ed: Springer Verlag; Berlin, 1996, p. 53-86. ISBN: 978-3-642-00574-9 (Online).