WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 8, 1305-1314. Research Article ISSN 2277– 7105

IN VITRO RESPONSE OF *CROTALARIA HEBECARPA* (DC.)RUDD.

Avula Srinivasa Rao, Shaik Babu Saheb, Luay Kadhim Hanoon and Kokkanti Mallikarjuna*

Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna nagar Guntur, 522510, A.P.

Article Received on
24 May 2017,
Revised on 14 June 2017,
Accepted on 05 July 2017
DOI: 10.20959/wjpr20178-8981

***Corresponding Author**
Kokkanti Mallikarjuna
Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna nagar Guntur, 522510, A.P.

ABSTRACT

Crotalaria hebecarpa is a small unexplored prostate herb belonging to the family Fabaceae. Though the identity and distribution of the plant is known, but its phytochemical and nutritional composition and their biological activities are not known. Since nothing is known about this plant, establishment of *in vitro* plants and bio mass enables us to use it for phytochemical and pharmacological exploration. In this paper, we for the first time are reporting the *in vitro* response of seeds, shoots and roots cultured on MS medium supplemented with different phyto hormones. The seeds cultured on MS medium supplemented with KN (4mg/l) produced shoots (Mean length, 9.23cm) while on Zeatin supplemented medium produced shoots and roots with mean length of 10cm and 7cm respectively. Maximum percentage of callus from leaf explants was observed at 3 mg/l of NAA (90%) and from roots at 2 mg/l Zeatin (95%). Maximum number of multiple shoots from callus was observed at 5mg/l BA (7.01±0.21) and at 4 mg/l of KN (5.45±0.64). The work in continuation to this preliminary report needed to be conducted to complete micro propagation process and to establish cell or cell suspension system for secondary metabolite production.

KEYWORDS: *Crotalaria hebecarpa*, phyto hormones and *in vitro* studies.

INTRODUCTION

The genus *Crotalaria* is rich with 700 species and widely used as vegetable or as edible crop, aesthetic plants and as medicinal plants. The members of genus contains starch, protein dietary fiber, oligosaccharides, phytochemicals, minerals and β-carotin (Subramanian and Pandy 2013; Devendra et al., 2013). *Crotalaria hebecarpa* is belonging to the Fabaceae

family. It is a prostate annual herb with slender branches. Leaves are alternate, size 1-15 cm, sub sessile, ovate and hairy with long cilia on margins. The availability and distribution of *Crotalaria hebecarpa* in Maharastra (Mujawar, 2012; Jagtap and Mukherjee, 2013) and in many districts of Andhra pradesh was reported (Madhava chetty et al., 2008). In Guntur district, it is available in Sattenapalli, Kondaveedu and Vinukonda areas, occasionally in open field forests and as a weed in cultivated fields (Pullaiah, 2000). The paste of whole plant is mixed with curd and orally given once in a day for two days to treat dysentery (Tiwari et al., 2012). Till to date , studies on phytochemistry, antimicrobial, pharmacological and tissue culture aspects of these plants are not being carried out. In the present work, we established the protocols for *in vitro* regeneration of *Crotalaria hebecarpa* by using seeds, leaf and root as explants. The protocols established in this work, will be useful to people working in the pharma, forestry and conservation areas.

MATERIAL AND METHODS

Collection of plant material

Explants like seeds, leaves and roots were collected from Botanical garden of Acharya Nagarjuna University.

Surface sterilization of *Crotalaria hebecarpa* seeds, mature nodal cuttings and leaf explants

Fresh, young juvenile shoots were collected and washed thoroughly under running tap water to remove dust particles. The explants were then surface disinfected by agitating gently in 5% labolene for 15 minutes. After that washed with 0.1% of bovistine for 10min and washed under running tap water. Then the explants were taken in to the inoculation chamber for further sterilization. In the laminar air flow cabinet near the flame, the materials were kept in 70% ethanol for 60 seconds, followed by repeated washings (3-4 times) in sterilized double distilled water. Then they were treated with 0.1% mercuric chloride for 1 minutes and washed with sterilized double distilled water for three to four times. The materials were kept in 70% ethanol for 60 sec. Sterilized petri plates were flamed after swabbing with ethanol. Disinfected explants were placed on the petri dishes with a sterilized forceps. After trimming the cut edges, the explants were inoculated on the medium.

For germination and callus formation, observations were recorded after 30 days of incubation and 45 days in shoot multiplication experiments. For each experiment, minimum of 5 tubes were maintained and all experiments were repeated at least thrice

Culture Conditions

All the cultures were incubated in culture room at 25±2^{0}C with a relative humidity of 60% at 50 to 1200µ mol m^{-2} sec^{-1}. Light intensity for 16h photo period (Preece and Sutter, 1991). To provide this light intensity, two 4 feet (1.2 m) cool- white fluorescent lamps, space 50cm apart were provided for each shelf.

Direct organogenesis of *Crotalaria hebecarpa*

Explants like nodal cuttings, leaf segments and roots were excised aseptically from 40-60 days old plants. These explants were cultured on MS medium supplemented with 3% sucrose with different phyto hormones concentrations & combinations. The morphogenic responses of the explants were recorded. Best explants for shoot regeneration were identified based on the number of shoots, shoot length and percentage of response.

Medium evaluation

After selecting the best explants shoot regenerations, it was cultured on MS agarified media viz, MS supplemented with different types and concentrations of growth hormones (BA, KN, NAA and Zeatin). Best suitable medium was determined by frequency and morphology of response.

Indirect organogenesis or caulogenesis of *Crotalaria hebecarpa*

Callus culture of *Crotalaria hebecarpa*

Different explants such as nodal explants and leaf were excised aseptically and cultured on MS medium supplemented with different concentrations of Auxins alone and in combinations with Cytokinins. Considering the quantity and quality of callus and percentage of response, best explants were selected. Further callus studies were continued with that explants only. Medium on which high percentage of response was noticed and the same was subjected to various experimental manipulations to assess the morpho genic influence of different hormonal combinations, organic supplements, and different concentrations of sucrose.

RESULTS

Table: 1 Divergent responses of seed culture on different media. Observations were recorded after 8 weeks of inoculation. 1 cm bar = 4.2mm

No explants for treatment	Phyto hormone			Type of response	Mean length of shoot (±)	Mean length of root (±)
	KN	Zeatin	NAA			
20	1.0	-	-	Shoot	5.1±0.26	1.1±0.31
20	2.0	-	-	Shoot	5.2±0.31	1.0±0.56

20	3.0	-	-	Shoot	4.0±0.61	2.4±0.83
20	4.0	-	-	Shoot	9.23±0.52	2.5±0.71
20	-	1.0	-	Shoot + root	5.0±0.24	7.0±0.6
20	-	2.0	-	Shoot+ root	4.0±0.36	2.5±0.62
20	-	3.0	-	Shoot + root	8.11±0.05	7.1±0.5
20	-	4.0	-	Shoot + root	10.0±0.02	7.0±0.18
20	-		1.0	Shoot + callus	0.5±0.53	0.9±0.21
20	-		2.0	Shoot + callus	2.0±0.05	1.0±0.13
20	-	-	3.0	Shoot+ callus	2.1±0.17	1.3±0.04
20	-	-	4.0	Shoot +callus	5.07±0.76	1.7±0.72

A) KN 1mg/l **B) KN 2mg/l** **C) KN 4mg/l**

D) Zeatin 1 mg/l **E) Zeatin 3mg/l** **F) Zeatin4 mg/l**

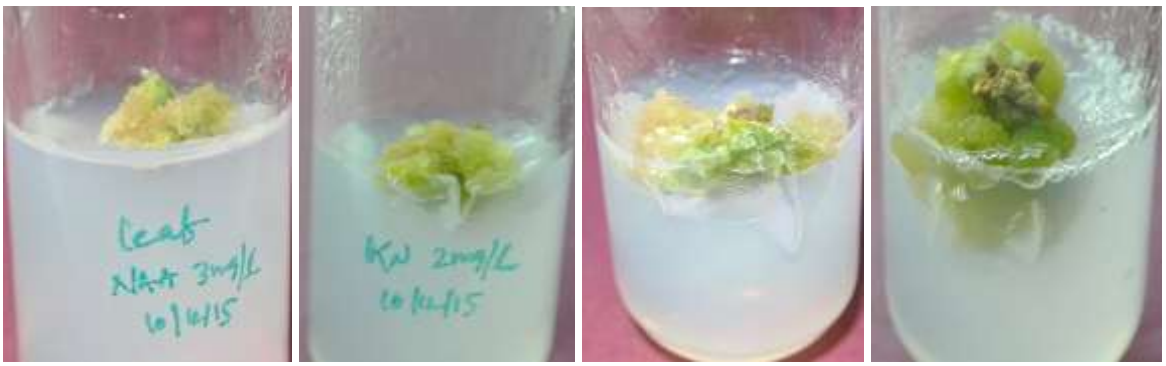
G) NAA 1mg/l **H) NAA 2mg/l** **I) NAA 4mg/l**

Fig.1 Effect on different concentrations of KN, Zeatin and NAA on induction of plantlets from seeds of *Crotalaria hebecarpa*.

When seeds were cultured on MS medium supplemented with KN 1, 2, 3 and 4mg/l, shoot formation was observed. Maximum shoot height was observed at 4 mg/l KN, and on Zeatin 1, 2, 3 and 4mg/l, shoot and root formation was observed. Maximum shoot and root length at 4 mg/l were 10.0 ± 0.02 and 7.0 ± 0.18 respectively. The shoot and callus induction was observed at NAA 1, 2, 3 and at 4 mg/l with maximum shoot length at 4mg/l (5.07±0.76) (Table.1) (fig1).

Table: 2 Effect of different concentrations of Auxins and Cytokinins on induction of callus from leaf explants of *Crotalaria hebecarpa.* Observations were recorded after 8 weeks of culture. 1cm bar =4.2mm

S.No	No explants for treatment	Explant	Phyto hormone (mg/l)			Type of response	Percentage of callus (%) formation
			KN	Zeatin	NAA		
1	20	Leaf	1.0	-	-	Callus	60
2	20	Leaf	2.0	-	-	Callus	85
3	20	Leaf	3.0	-	-	Callus	70
3	20	Leaf	4.0	-	-	Callus	75
4	20	Leaf	5.0	-	-	Callus	65
6	20	Leaf	-	1.0	-	Callus	70
6	20	Leaf	-	2.0	-	Callus	80
7	20	Leaf	-	3.0	-	Callus	65
8	20	Leaf	-	4.0	-	Callus	60
9	20	Leaf	-	5.0	-	Callus	65
10	20	Leaf	-	-	1.0	Callus	60
9	20	Leaf	-		2.0	Callus	85
10	20	Leaf	-	-	3.0	Callus	90
11	20	Leaf	-	-	4.0	Callus	75
12	20	Leaf	-	-	5.0	Callus	65

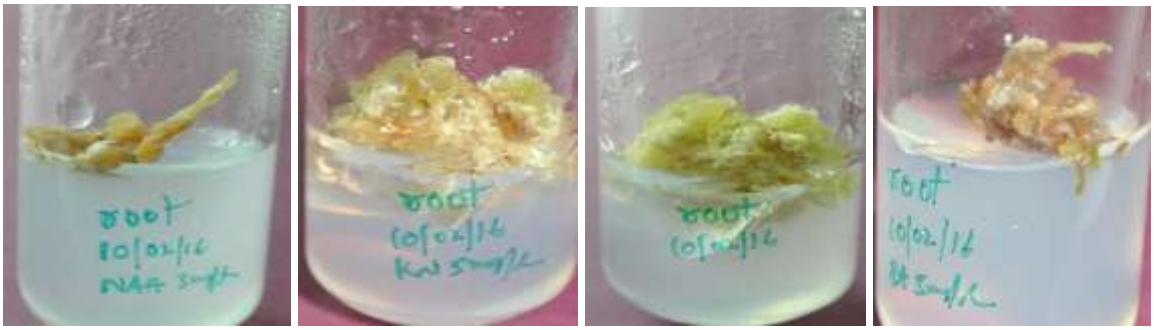


A) NAA 3 mg/l B) KN 2 mg/l C) Zeatin 1mg/l D) Zeatin 2 mg/l

Fig.2 Callus induction from leaf explant of *Crotalaria hebecarpa*

Table.3 Effect of different concentrations of Auxins and Cytokinins on induction of callus from root explants of *Crotalaria hebecarpa.* Observations were recorded after 8 weeks of cultures. 1cm bar =4.2mm.

S.No	No explants for treatment	Explant	Phyto hormone(mg/l)				Type of response	Percentage of callus (%) formation
			BA	KN	Zeatin	NAA		
1	**20**	**Root**	**-**	1.0	**-**	**-**	Callus	**60**
2	20	Root	-	2.0	-	-	Callus	65
3	20	Root	-	3.0	-	-	Callus	70
3	20	Root	-	4.0	-	-	Callus	70
4	20	Root	-	5.0	-	-	Callus	90
6	20	Root	-	-	1.0	-	Callus	70
6	20	Root	-	-	2.0	-	Callus	95
7	20	Root	-	-	3.0	-	Callus	65
8	20	Root	-	-	4.0	-	Callus	60
9	20	Root	-	-	5.0	-	Callus	55
10	20	Root	-	-	-	1.0	Callus	65
9	20	Root	-	-		2.0	Callus	70
10	20	Root	-	-	-	3.0	Callus	85
11	20	Root	-	-	-	4.0	Callus	75
12	20	Root	-	-	-	5.0	Callus	60
13	20	Root	1.0	-	-	-	Callus	65
14	20	Root	2.0	-	-	-	Callus	65
15	20	Root	3.0	-	-	-	Callus	70
16	20	Root	4.0	-	-	-	Callus	75
17	20	Root	5.0	-	-	-	Callus	90



A) NAA 3mg/l B) KN 5mg/l C) Zeatin 2mg/l D) BA 5mg/l

Fig.3 Callus induction from root explants of *Crotalaria hebecarpa*

The above data indicated that, leaf cultured on MS medium supplemented with KN 2 and 4mg/l, Zeatin 1 and 2mg/l/ and NAA 2 and 3mg/l caused callus formation. Maximum Percentage of callus was observed at 3 mg/l of NAA (90%) (Table.2) (Fig.2). And roots cultured on MS medium supplemented with KN 5mg/l, Zeatin 2 mg/l, NAA 3mg/l and BA 5 mg/l induced callus formation. Maximum percentage of callus from roots was observed at 2 mg/l Zeatin (95%) (Table.3)(Fig.3).

Table.4 Effect of different concentrations of cytokinins on direct regeneration of multiple shoots from callus of *Crotalaria hebecarpa.* Observations were recorded 8 weeks of cultures 1cm=4.2mm.

S.no	Treatment of explants	Type of explants	Phyto hormones		Mean no. of shoots (±)	Mean length of shoots (±)
			BA	KN		
1	20	Callus	1.0	-	1.54±0.043	0.81±0.04
2	20	Callus	2.0	-	2.0±0.67	2.5±0.41
3	20	Callus	3.0	-	3.75±0.57	4.24±0.22
4	20	Callus	4.0	-	1.59±0.73	1.93±0.11
5	20	Callus	5.0	-	7.01±0.21	5.28±0.46
6	20	Callus	-	1.0	0.73±0.91	0.89±0.18
7	20	Callus	-	2.0	1.2±0.81	2.0±0.15
8	20	Callus	-	3.0	2.13±0.53	3.12±0.14
9	20	Callus	-	4.0	5.45±0.64	6.29±0.56
10	20	Callus	-	5.0	3.42±0.24	4.12±0.18

A) BA 5mg/l **B) KN 4mg/l**

Fig.4 Effect on different concentrations of BA and KN on induction of multiple shoots from callus explants of *Crotalaria hebecarpa*.

The above data indicated that, callus cultured on MS supplemented with KN 3 and 4mg/l, BA 3 and 5mg/l resulted in multiple shoot formation. Maximum number of multiple Shoots was observed at 4mg/l of KN (5.45+0.64) and maximum number of shoots from Callus were observed at 5mg/l BA (7.01+0.21) (Table.4) (Fig.4).

DISCUSSION

Due to large scale and unrestricted exploitation to meet increasing demands by the pharmaceutical industries, coupled with limited cultivation and in sufficient attempts for its reforestation, the wild stocks of this important plant species has been markedly decresed (Jayasree et al., 2005). Tissue culture techniques have been reported for conservation and multiplication of several medicinal plants (Naomita et al., 2000: Hussain et al., 2008). *In vitro* of regeneration medicinal plants could be help in producing disease free health clones in large

scale for extraction of various drugs (Rajender et al., 2012). *Crotalaria*, one of the medicinally recognized genuses belonging to the Fabaceae family is mostly found in the Tropical regions (Daimon et al., 2002). The different species of *Crotalaria* and their applications are described below. *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 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).

Tissue culture protocols have been extensively used for in vitro propagation, germplasm conservation and production of pharmaceutically important bioactive compounds (Nalawade, et. al., (2003); Phatak and Heble (2002). Plant tissue culture provides the possibility of applying different growing conditions including phytoregulators to induce a response of germination in seeds. Tissues obtained from *in vitro* seed germination are frequently used in micropropagation because they are usually easy to establish in culture due to soft tissue without endogenous microbes and secondary metabolites (Aitken-Christie and Thorpe, 1984).

Hence *Crotalaria hebecarpa* micro propagation system also need to be established to discover the secondary metabolites and as well as bioactive compounds from the *in vitro* regenerated plants and somatic embryos. Explant selection and usage of appropriate plant growth regulators are extremely important for rapid clonal multiplication which has been well documented in other studies (Koleva., 2012). In the case of *Crotalaria hebecarpa*, seeds, leaf and root explants are used for achieving high multiplication rate, because each explant has different age, physiology, genetics and developmental back ground and many respond differently to *in vitro* conditions. In the present study, *Crotalaria hebecarpa* was cultured directly and indirectly from the seed, leaf and root explants. Nodal explants as such did not respond well when compared with other explants. Shoot formation on Kinetin was found

better then BAP when explants were treated with individual phytohormones. Plant regeneration from epicotyl explants have been reported earlier by researchers (Siddique et. al., 2013; Barik et. al., 2005; Costa et al., 2004). Seeds were cultured on MS medium supplemented with KN 1, 2 and 4mg/l caused shoot formation. Maximum shoot length was observed at 4mg/l KN, and supplemented with Zeatin 1,3 and 4 mg/l for shoot and root formation was observed, maximum shoot length and root length at 4 mg/l 10.0±0.02 and 7.0±0.18 respectively. The *in vitro* sown seeds developed into proto corms within 18 weeks of culture in 0.5 mg/l NAA containing medium and are differentiated into seedlings after 26 weeks (Paudel et al., 2012). Similar findings were also reported in previous studies on seeds germination of *Aerides odorata* (Pant and Gurung, 2005) and in seed germination of *Cymbidium* sp., *Dendrobium nobile* and *D. primulinum*(Luan et al., 2006). Similar results were observed for shoot and callus induction at NAA 1, 2, 3 and 4 mg/l, with maximum shoot length at 4mg/l (5.07±0.76) (Table.1).

Leaf cultured on MS medium supplemented with KN 2 and 4mg/l, Zeatin 1 and 2 mg/l/ and NAA 2 and 3 mg/l resulted in callus formation. Maximum percentage of callus was observed At 3 mg/l of NAA (90%) (Table.2). And roots cultured on MS medium supplemented with KN 5mg/l, Zeatin 2mg/l, NAA 3mg/l and BA 5.0mg/l produced callus. Maximum percentage of callus from roots was observed at 2 mg/l Zeatin (95%)(Table.3).

In all of the earlier reports, BA and KN increased shoot proliferation but in this study KN was more effective than BA and produced highest number of shoots as registered by (Bonyanpour et al., 2013). Similarly, callus cultured on MS medium supplemented with KN 3 and 4mg/l, BA 3 and 5mg/l induced multiple shoots. Maximum number of multiple shoots was observed at 5 mg/l of BA (7.01+0.21) (Table.4).

CONCLUSION

In this preliminary report, it can be concluded that *Crotalaria hebecarpa* can be used to establish in vitro biomass for micro propagation and cell culture studies.

REFERENCES

1. Daimon, H., Ohno, H., Akasaka, Y. and Mii, M., A histological evaluation of adventitious bud formation in cotyledons in *Crotalaria juncea* L. *Plant production science*, 2002; *5*(4): 301-304.

2. Hussain, T.M. and Gopal, G.R., In vitro propagation of Crotalaria verrucosa L. an important ethnobotanical plant. *Journal of Medicinal Plants Research*, 2013; *2*(9): 242-245.
3. Koleva Gudeva, L., Mitrev, S., Trajkova, F. and Ilievski, M., Micropropagation of Potato Solanum tuberosum L. *Electronic journal of biology*, 2012; *8*(3): 45-49.
4. Karuppusamy, S., Aruna, V., Kiranmai, C. and Pullaiah, T., In vitro propagation of an endemic umbellifer, Hydrocotyle conferta, 2007.
5. Naomita, V.D. and Rai, V.R., In vitro regeneration of Crotalaria lutescens (Dalz.), an endemic and rare species of Western Ghats. *Phytomorphology*, 2000; *50*(3/4): 291-295.
6. Nuhu, H., Abdurrahman, E.M. and Shok, M., Comparative analysis of the alkaloids of three Crotalaria species. *Niger J Pharma Sci.*, 2009; *8*(2): 54-58.
7. Nwude, N., Veterinary aspects of medicinal plant research in Nigeria. *state of medicinal plant Res. Nig. Ed. Sofowora, A*, 1986; *197*.
8. Pullaiah, T. and Naidu, K.C., *Antidiabetic plants in India and herbal based antidiabetic research*. Daya Books, 2003.
9. Paudel, M., Pradhan, S. and Pant, B., In vitro seed germination and seedling development of Esmeralda clarkei Rchb. f.(Orchidaceae). *Plant Tissue Culture and Biotechnology*, 2013; *22*(2): 107-111.
10. Rajender, K., Thirupathi, M., Srinivas, D., Raju, D. and Reddy, K.J., Micropropagation of Crotalaria laburnifolia L.–An ethnomedicinally important herbal species. *Journal of Phytology*, 2012; *4*(4).
11. Samy, R.P., Pushparaj, P.N. and Gopalakrishnakone, P., A compilation of bioactive compounds from Ayurveda. *Bioinformation*, 2008; *3*(3): 100.
12. Roeder, E. and Wiedenfeld, H., Pyrrolizidine alkaloids in medicinal plants of Mongolia, Nepal and Tibet. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 2009; *64*(11): 699-716.
13. Sridhar, K.R. and Bhat, R., Agrobotanical, nutritional and bioactive potential of unconventional legume–Mucuna. *Livestock Research for Rural Development*, 2007; *19*(9): 126-130.
14. Steglich, W., Fugmann, B. and Lang-Fugmann, S., 1997. Römpp Lexikon Naturstoffe. *Stuttgart: Georg Thieme*.
15. Vardhana, R., *Direct uses of medicinal plants and their identification*. Sarup & Sons, 2008.