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EFFECT OF SOME CYCLIC UREA COMPOUNDS ON PLANT REGENERATION OF *BACOPA MONNIERI* (L.) PENNELLE

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ABSTRACT

This paper highlights the study of effects of various urea derived herbicides *viz*., monuron, diuron, thidiazuron and classical cytokinin i.e., BAP on shoot morphogenetic behaviour of medicinal plant *Bacopa monnieri*. The studies were carried out on different cultivars. Thidiazuron (0.02µM) induced highest shoot morphogenetic response from axillary buds of Chuharpur cultivar with a maximum number of 13.36 shoots per explant followed by 8.33 and 5.55 in Lucknow and Hisar cultivar. Diuron(0.10 µM) showed shoot morphogenesis highest in Chuharpur cultivar 11.20 per explant. Monuron (0.10 µM) also showed 11.53 in Chuharpur cultivar. Also, the shoot regeneration response of shoot tip explant found to be high with a mean no. of 9.17 shoots per explant with Thidiazuron(0.02µM) . For rooting, different concentrations of NAA, IBA and IAA were used and highest rooting was observed on MS medium supplemented with NAA(1.5mg/l)+IBA(1.5mg/l) with 96% response. The *in vitro* regenerated plantlets were transferred to greenhouse for hardening. The soil mixture having sand:soil:vermicompost (1:1:1) showed the best response in terms of 97% survival rate. Results of the present study supports the potential of thidiazuron and monuron in exerting unusual cytokinin like activity that are useful for micropropagation of endangered medicinal plants.

KEYWORDS: Thidiazuron, Monuron, Diuron, *Bacopa monnieri,* BAP

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INTRODUCTION

Plants are an important source of medicines and play a key role in world health, as approximately 80% of traditional medicinal preparation involve the use of plant or plant extracts [1].The increasing demand for herbal medicines in recent years due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. *Bacopa monnieri* (L.) Pennelle (Scrophulariaceae), popularly known as 'Brahmi' or 'Neer Brahmi' in India is one of the sources of the Medhya rasayan drugs (that counteract stress and improve intelligence and memory) of Ayurveda[2]. It is a small glabrous, sprawling succulent herb that grows in damp and marshy places and on the banks of slow flowing rivers and lakes, ascending up to an altitude of 1,320 m.[3] It has been used for centuries in folklore and traditional system of medicine as a memory enhancer, anti- inflammatory, analgesic, antipyretic, sedative and anti- epileptic agent[4]. *B. monnieri* was placed second in the list of most important Indian medicinal plants evaluated on the basis of medicinal importance, commercial value and potential for further research and development[5]. The memory enhancing effects of *B. monnieri* have been attributed to the active constituent's bacoside A and B[6]. This property of these bacosides has increased the international demand for this plant for its extensive use in several commercial preparations[7]. The continuous exploitation of *B. monnieri* from the natural habitat has now resulted in the depletion of natural population which has resulted in listing of this plant as a threatened species[8]. Propagation of this species through seed is unreliable due to poor seed setting, low seed viability and frequent seedling death at two leaf- stage. There is thus an immediate need for assessing the natural populations, developing protocols for micropropagation, regeneration and agronomical practices. There has been a surge of interest in discovering molecules that influence the regulation of plant cell growth and development[9]. There have been reports on the potential application of low concentration of pesticides and herbicides as auxins and cytokinins. Cytokinins produce various effects when applied to intact plants. They particularly stimulate protein synthesis and participate in cell cycle control and thus, can promote the maturation of chloroplasts and delay the senescence of detached leaves. Cytokinin application to a single site in the plant (e.g. on one leaf) causes the treated organ to become an active sink for amino acids, which then migrate to the organ from surrounding sites. Along with auxins they stimulate cell division and control morphogenesis[10]. Various urea-derived herbicides have been shown to possess cytokinin– like activity[11]. Diphenylureas are not commonly used in tissue cultures, but there are a few reports in legumes. In sugar beet callus cultures, it has been found that 2mg/l 1,3- diphenylurea facilitated organogenesis. Some N- pyridyl- N' phenylureas are more active than N^6 – substituted purines such as BA and Zeatin in promoting callus growth and morphogenesis in tobacco and several other kinds of plants[12,13]. The thidiazole- substituted phenylurea: thidiazuron (TDZ) (N- phenyl- N'- 1,2,3- thiadiazol-5-ylurea) which was registered as a cotton defoliant[14] and given the product name 'Dropp', has high cytokinin activity[15]. In some plants it is more effective than adenine-based compounds for inducing adventitious shoot regeneration, N'- alkyl substituted thioureas were also found to be plant growth regulators that inhibited stem elongation of rice and kidney bean plants without phytotoxicity[13]. In this study, we surveyed the effects of various concentrations of urea derived herbicides/ cyclic urea compounds (Monuron, diuron, TDZ) and BAP on shoot regeneration in *Bacopa monnieri*. Monuron (N'-4- chlorophenyl)-N, N- dimethylurea and diuron [N'-(3, 4- dichlorophenyl)- N, N- dimethyl urea] are used as pre and post emergent herbicides[16]. These compounds have also been recognized for their cytokinin like activity in *Amaranthus* and *Raphanus* (radish) in seed germination studies[17,18]. Here we report the ability of urea derived herbicides in exerting cytokinin- like response in micropropagation of *Bacopa monnieri*, which

could be further used in tissue culture of other economically important plant.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade. The chemicals were purchased from Sigma Chemical company (St. Louis, USA).

Collection of material

Bacopa monnieri L. Penn. plant material was purchased from three different places *viz.,* Devi Lal Herbal Park, Chuharpur, Yamunanagar, (Haryana); CIMAP Lucknow (UP) and CCS HAU, Hisar, (Haryana). It was established in field during the months of July-August 2013. The axillary buds and shoot tips were used as explants for study.

Methods

The explants first were washed thoroughly in running tap water to remove dust/contaminants and placed in another container. They were washed with tap water containing 4-5 drops of Tween-20 for 5 minutes with gentle shaking followed by washing three times with sterile distilled water to remove any traces of detergent. Under laminar hood, these explants were disinfected with 0.1% (w/v) $HgCl_2$ for one minute and then rinsed thrice with sterile distilled water for about 5 minutes each time. After drying on sterilized filter paper explants were cultured upon MS basal media[19] supplemented with various concentrations of BAP, TDZ, monuron and diuron to study their effects on shoot regeneration in *Bacopa monnieri* explants. The experiments were carried out in three replications and the percentage of frequency was calculated. At the end of 30 days of inoculation of *B. monnieri* explants into the cytokinin supplemented medium, the mean numbers of multiple shoots formed per treatment were determined. About two to three centimetre long, surgically separated individual shoots were rooted on MS basal medium supplemented with various concentrations of NAA, IBA and IAA (Table 3). And the *in vitro* regenerated plantlets were transferred to various sand, soil, Fym mixture combinations in polybags after 30 days. In green house the plantlets were hardened for one month under high humidity conditions (80%) and then finally transferred to experimental field.

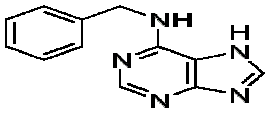
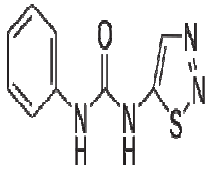
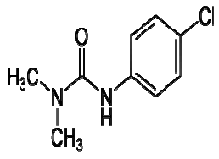
RESULTS

A time period of 5 minutes with concentration of $HgCl_2$ 0.1% found suitable for disinfection of axillary bud and shoot tip explants of *B. monnieri* followed by washing thrice with sterilized water to remove traces of $HgCl_2$ and drying on sterilized filter paper before culturing over medium. When axillary buds were cultured over MS basal medium (with 2% agar and 3% sucrose) supplemented with various concentrations of cyclic urea compounds and BAP (Table 1), the response of Chuharpur cultivar was best (13.36 number of shoots per explant) on TDZ concentration of 0.02 µM followed by Lucknow and Hisar cultivars (8.33 and 5.5 respectively). Cyclic urea compounds monuron and diuron showed the same pattern (Table 1) but at different concentration of 0.10µM BAP. The traditionally used cytokinin showed lesser response i.e. 10.5 average number of shoot per explant in Chuharpur cultivar followed by Lucknow and Hisar (7.33 and 6.2 respectively) at a concentration of 4.44 µM (Table 1). Shoot morphogenesis responses of shoot tip explants on the same media and same cultivars were recorded (Table 2). And the Chuharpur cultivar responded better on medium supplemented with TDZ (0.02 µM) showing 9.17 average number of shoots per explant followed by Lucknow and Hisar (7.27 and 3.77 respectively). A concentration of 0.10 µM of monuron and diuron was found effective to elicit shoot morphogenetic response from shoot tips of *B. monnieri* where Chuharpur cultivar showed 7.8 average number of shoots per explant followed by Lucknow (5.6) and Hisar (3.66) in case of monuron and 6.8, 4.47 and 2.7 in case of diuron (Table 2). Shoot tips showed the best shoot morphogenesis results where at a concentration of BAP 4.44 µM the average number of shoots were 11.7 in Chuharpur cultivar, 8.57 in Lucknow cultivar and 6.0 in Hisar cultivar. MS basal medium was used for rooting of surgically separated individual shoots supplemented with different

concentrations and combinations of auxins (Table 3). In the Chuharpur cultivar maximum number of shoots which induced roots were on MS medium supplemented with NAA and IBA (1.5 mgl^{-1} each, 96.00%) and they also showed highest survival in greenhouse after one month (98.00%) (Table 3) followed by Lucknow cultivar (root induction 86.41%; field survival 88.40%) and Hisar cultivar (root induction 76.80%; field survival 80.50%) (Table 3). After one month in rooting medium, the plants were taken out gently from flasks and washed with tap water to remove traces of medium to avoid growth of microorganisms before transferring to pots containing mixtures of sand, soil, FYM and vermicompost in different proportions (Fig.1,2,3). Here, cultivar Chuharpur showed better survival in sand:soil:vermicompost (1:1:1) mixture (97%) followed by Lucknow (87.3%) and Hisar(78.5%) as compared to sand:soil:FYM mixture (1:1:1) where the survival rate was 94%, 84.6% and 76.5% respectively (Fig.1,2,3).

Table 1

Effect of various cyclic urea compounds and BAP on shooting morphogenesis of axillary buds in Bacopa monnieri cultivars viz. Chuharpur , Lucknow, Hisar

Growth Regulators	Concentration	Average No. of Shoots/Explant Chuharpur	Average No. of Shoots/Explant Lucknow	Average No.of Shoots/Explant Hisar
BAP 	4.44µM	10.5±0.68	7.73±0.55	6.2±0.31
	8.88 µM	9.53±0.39	8.13±0.35	5.77±0.15
	13.32 µM	9.23±0.32	7.66±0.68	4.93±0.27
TDZ 	0.01µM	0.96±0.55	6.13±0.24	5.4±0.46
	0.02µM	13.36±0.29	8.33±0.74	5.5±0.35
	0.05 µM	6.27±0.38	7.9±0.32	5.23±0.32
Monuron 	0.05µM	7.17±0.23	8.2±0.31	4.9±0.55
	0.10µM	11.53±0.43	7.13±0.24	5.47±0.37
	0.20 µM	8.4±0.29	7.96±0.29	5.3±0.26
Diuron	0.05µM	7.97±0.33	8.3±0.32	5.23±0.26
	0.10 µM	11.2±0.35	7.57±0.79	4.0±0.12

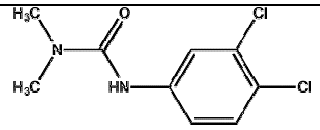
	0.20 µM	7.5±0.35	8.2±0.31	5.27±0.59

Table 2

Effect of various cyclic urea compounds and classical cytokinins on shooting efficiency from shoot tips in Bacopa monnieri cultivar viz. Chuharpur, Lucknow, Hisar

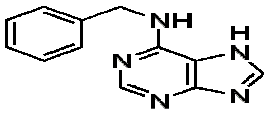
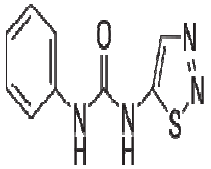
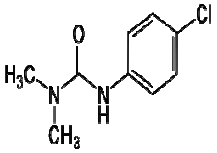
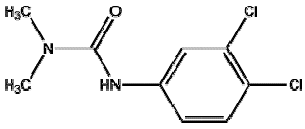
Growth Regulators	Concentration	Average No. of Shoots/Explant Chuharpur	Average No. of Shoots/Explant Lucknow	Average No. of Shoots/Explant Hisar
BAP 	4.44µM	11.7±0.26	8.57±0.35	6.0±0.47
	8.88 µM	10.1±0.32	8.13±0.32	5.27±0.27
	13.32 µM	5.97±0.27	7.63±0.33	5.56±0.09
TDZ 	0.01µM	3.77±0.2	2.96±0.09	2.63±0.19
	0.02µM	9.17±0.38	7.27±0.39	3.77±0.18
	0.05 µM	3.87±0.38	2.6±0.38	2.6±0.31
Monuron 	0.05µM	3.83±0.2	3.73±0.24	3.4±0.5
	0.10µM	7.8±0.15	5.6±0.17	3.66±0.33
	0.20 µM	5.37±0.32	5.33±0.18	2.57±0.28
Diuron 	0.05µM	4.87±0.35	4.37±0.19	3.43±0.27
	0.10 µM	6.8±0.46	4.47±0.34	2.7±0.17
	0.20 µM	3.77±0.15	3.2±0.23	2.63±0.26

Table 3

Effect of auxins on root induction on surgically separated in vitro generated individual shoot of Bacopa monnieri cultivars viz. Chuharpur, Lucknow, Hisar

Growth Regulator (mg/l) MS Basal Medium	Chuharpur cultivar		Lucknow cultivar		Hisar cultivar	
	Percent Shoots Exhibiting Roots	Percent Plantlets Survived in Greenhouse (30 days)	Percent Shoots Exhibiting Roots	Percent Plantlets Survived in Greenhouse (30 days)	Percent Shoots Exhibiting Roots	Percent Plantlets Survived in Greenhouse (30 days)
NAA + IBA						
0.5 + 0.5	63.50	90.00	56.95	81.10	50.80	72.00
1.0 + 1.0	75.00	92.50	67.20	83.25	60.50	75.50
1.5 + 1.5	96.00	98.00	86.41	88.40	76.80	80.50
2.5 + 2.5	67.50	80.00	60.75	72.00	54.50	63.50
IAA						
1.5	45.50	65.50	40.50	58.92	36.40	52.40
2.5	55.00	73.00	49.20	65.70	42.50	57.50
3.5	68.90	74.00	63.01	66.40	55.12	59.20

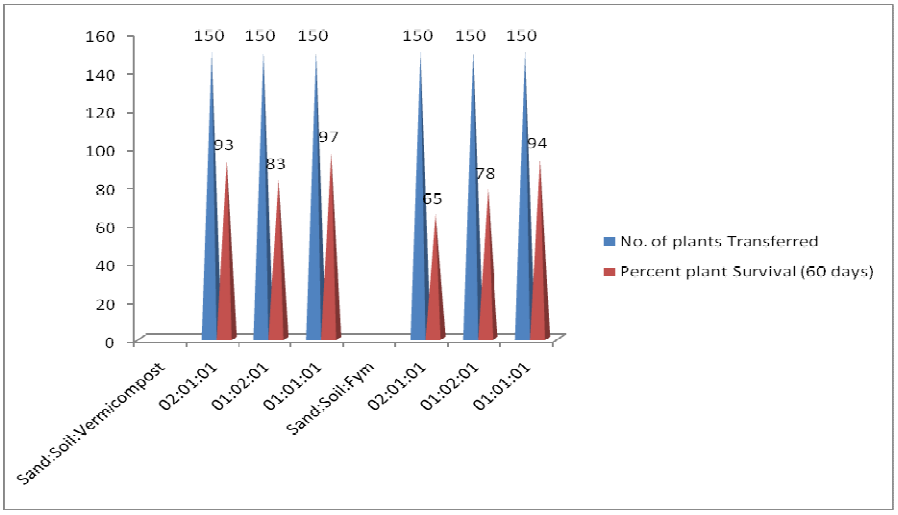


Figure1

Field performance of in vitro regenerated Bacopa monnieri plants Chuharpur cultivar

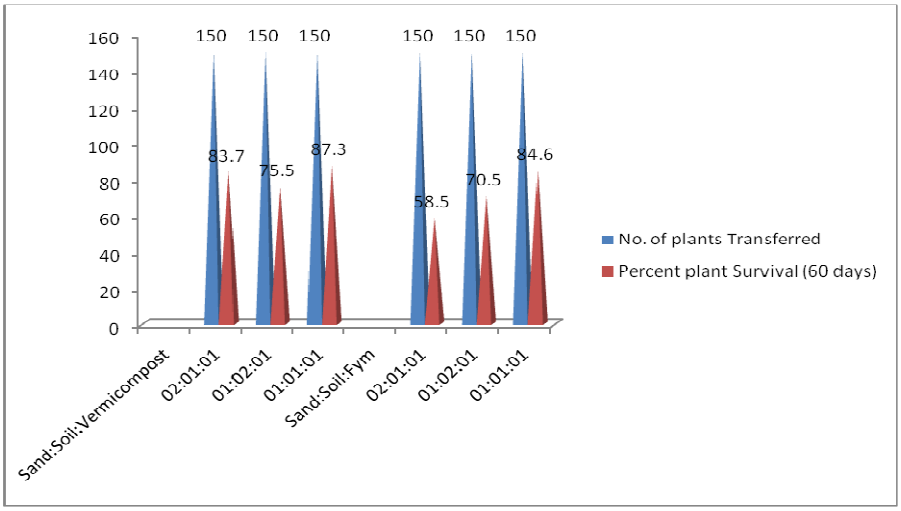


Figure2

Field performance of in vitro regenerated Bacopa monnieri plants Lucknow cultivar

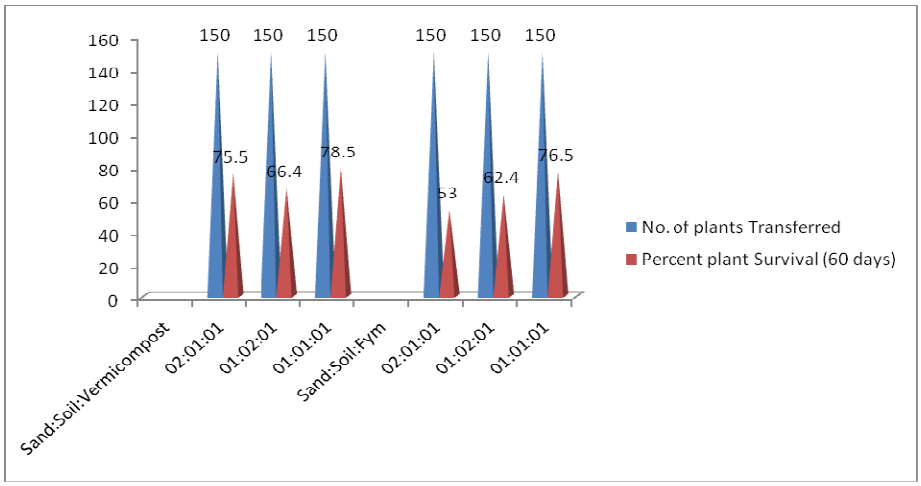


Figure3

Field performance of in vitro regenerated Bacopa monnieri plants Hisar cultivar

Plate 1

Plant regeneration in Bacopa monnieri (Chuharpur cultivar) axillary bud explant 1. One week after culture on medium (MS+ TDZ 0.02µM) 2. Two weeks after culture 3. Shoot multiplication 4. Shoots ready for rooting 5. Induced roots 6. Transfer to pots in green house 7. Hardening in polybags 8. Field transfer

DISCUSSION

Axillary buds and shoot tip explants were found to be suitable for *in vitro* propagation of *B. monnieri* and their sterilization method is more or less standardised[8,20] although for different time and varying concentration on different cultivars. Use of cyclic urea compounds such as thidiazuron having cytokinin like activity is not reported for *Bacopa* but is reported for legumes[10,11] . In the present study, different cyclic urea compounds were used to determine their shoot morphogenetic ability on different cultivars of *Bacopa monnieri*. With axillary buds as explants, a maximum response in terms of frequency and mean number of shoots was observed with thidiazuron (TDZ) in cultivar from Chuharpur. TDZ (0.02µM) induced a maximum average number of shoots per plant 13.36 ± 0.29 in Chuharpur cultivar followed by Lucknow having 7.9 after 30 days of culture (Table 1). However, the shoots obtained in the presence of TDZ were stunted. These results relate to the findings of Ceasar *et al*[22] who worked with TDZ. With diuron at a concentration of 0.10 µM the maximum average number of shoots were obtained 11.2 ± 0.32 when axillary bud was used as explant source and 6.8 ± 0.46 when shoot tip was used as explant source of Chuharpur variety (Table1). The difference in response to shoot morphogenesis under *in vitro* conditions by different explants is explained to endogenous level of hormones in explants and also shown by Jain *et al.*[23] , Asha *et al.*[24] *and Tiwari et al*.[25] (Table 1). It has been observed that monuron (0.10 µM) gives good shoot morphogenetic average response with axillary buds of Chuharpur variety (11.53± 0.43) and when shoot tips were used as explant source the average response was 7.8 ± 0.15 shoots per explant. Monuron and diuron has not been tried on this plant and this is the first report in this regard. The best results of shoot morphogenesis in shoot tip explants have been observed with BAP (2.0mg/l) average number of shoots per explant 11.7 ± 0.26 shoots (Table 2). Use of BAP for shoot morphogenesis in this medicinal plant has been reported by several workers although with different cultivars[23,24,25]. Various concentrations of auxins induced rooting when added into MS medium 2% agar. The best rooting response, was observed on medium supplemented with NAA (1.5 mg/l) + IBA (1.5mg/l) where 96.00% explants showed good root formation Chuharpur cultivar followed by 86.41% response from same hormone combination with Lucknow cultivar (Table 3). In *Bacopa monnieri* the use of IBA as root inducer is also reported by other workers[6,21,23] although with different concentrations. The medium supplemented with IAA (3.5mg/l) showed 68.90% rooting response (Table 3). Initially the *in vitro* rooted plantlets were hardened in culture room and after 30 days, the plantlets were transferred to green-house. The plantlets generated from MS medium supplemented with NAA (1.5 mg/l) + IBA (1.5mg/l) reported best results with 98.00% survival rate. Among the various mixtures of sand:soil:vermicompost tried for field performance , the mixture having equal amount of all showed best response in terms of 97.00% survival rate in Chuharpur cultivar followed by Lucknow (87.30%) (Fig.2) and Hisar (78.50%) (Fig.3). Use of vermicompost for better survival of *in vitro* regenerated plants of brahmi is also reported by Kashyap[26]. The Chuharpur cultivar again showed maximum response and this argues well because the plant is natural habitat of muddy shores and wetlands.

CONCLUSION

In the present study it has been observed that urea-derived herbicides shows unusual cytokinin property like thidiazuron (TDZ) showed best response as compared to monuron and diuron. This could be due to the structural similarities of these urea compounds with cytokinins. It has been observed that, the efficiency of these compounds is due to chloride substitutions in their phenyl rings. Results of the present study supports the potential of thidiazuron and monuron in exerting unusual cytokinin like activity that is useful for micropropagation of various endangered medicinal plants.

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