
Potential application of urea-derived herbicides as cytokinins in plant tissue culture

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Various urea-derived herbicides and different cytokinin analogues were used to determine their effects on callusing response and shoot regenerating capacity of alfalfa (*Medicago sativa* L.) and Coleus (*Coleus forskohlii* Briq.). The herbicides monuron and diuron evoked profuse callusing response from Coleus leaf segments and alfalfa petiole explants on Murashige and Skoog medium. Shoot regeneration by monuron (2.0 mg/l) showed a maximum of 3 multiple shoots both in alfalfa and Coleus with a frequency of 92% and 75%, respectively. Whereas diuron (0.5 mg/l) showed a high frequency of shoot regeneration (89%) with a mean number of 5 shoots in alfalfa, in *C. forskohlii*, the frequency of regeneration was 90% with a mean number of 6 shoots. Diuron with two chloride groups in the phenyl ring showed significantly higher cytokinin-like activity than single chloride substitution monuron. This study demonstrates the potential use of monuron and diuron as cytokinins in plant tissue culture.

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1. Introduction

There has been a surge of interest in discovering molecules that influence the regulation of plant cell growth and development (Gaspar *et al* 1996) and there have been reports on the potential application of low concentration of pesticides and herbicides as auxins and cytokinins. Among the growth regulators, cytokinins are known to affect many plant developmental processes including cell division, cell differentiation, chlorophyll senescence and apical dominance (Mok and Mok, 2001). They are structurally related to adenine and are produced by actively growing tissues, particularly roots, embryos, and fruits. Cytokinins are involved in control of cell division and differentiation and this ability of cytokinins to trigger cell division and influence differentiation is related to the fact that cytokinins stimulate RNA and protein synthesis, which may be involved in cell division.

Many urea-derived herbicides have been shown to possess cytokinin-like activity (Krikorian, 1995). A few

synthetic compounds such as N-(2-chloro-4-pyridyl)-N-phenylurea and diphenyl urea (DPU, N,N'-diphenylurea) were reported to have cytokinin-like activity (Woo and Wick 1995). Thidiazuron (N-phenyl-N',2,3-thiazol-5-yl urea), a derivative of phenylurea has been used as a cotton defoliant (Arndt *et al* 1976). This compound was later detected to have high cytokinin activity (Mok *et al* 1982) and it was used as an effective growth regulator in a number of crops (Thomas and Katterman 1986; Lu 1993). N-methyl-N-(2-thiazolyl)-N'-alkyl substituted thioureas were also found to be plant growth regulators that inhibited stem elongation of rice and kidney bean plants without phytotoxicity (Teruhisa *et al* 1972).

Plant growth regulating properties were demonstrated in substituted pyrazolyl-phenyl ureas (Creuzburg *et al* 1977). Another substituted phenylurea viz. ethylenediurea also displayed cytokinin activity as seen from tobacco callus bioassay (Lee and Chen 1982). Okamoto *et al* (1983) reported that aromatic ureas with 2-substituted and 2,6-disubstituted-4-pyridyl moiety have even higher cytokinin

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activity than trans-zeatin, while N-phenylureas containing pyridyl residues were found to be potential herbicides besides possessing growth regulator properties (Yonova *et al* 1989).

In the present study also, we surveyed various urea-derived herbicides (monuron, diuron and diphenyl urea) to develop an innovative and cost effective cytokinins for plant tissue culture. Monuron (N'-4-chlorophenyl)-N,N-dimethylurea) and diuron [N'-(3,4- dichlorophenyl)-N,N-dimethylurea] are used as pre-, and post-emergent herbicides (Bucha and Tod 1951). These compounds have also been recognized for their cytokinin-like activity in *Amaranthus* and *Raphanus* (radish) in seed germination studies (Ricci *et al* 2004; Yonova and Stoilkova 2005). The present study was carried out to understand the effects of monuron and diuron on callus formation and shoot regeneration in two-model systems viz., *Medicago sativa* (alfalfa) and *Coleus forskohlii* (Coleus). We report here the efficacy of monuron and diuron in exerting cytokinin-like response in micropropagation of these two plant models, which could be further applied in tissue culture of other economically important plant systems. Moreover, these compounds are economical over the existing cytokinins and related compounds.

2. Materials and methods

2.1 Materials

Benzyladenine (BAP), N,N'-diphenyl urea (DPU), monuron, diuron and thidiazuron (TDZ) were obtained from Sigma Chemical Company (St. Louis, USA). First and second leaves from apical bud were collected from greenhouse grown *C. forskohlii* Briq. plants and were soaked in 3% (v/v) Tween-20 for 10 min. The leaves were disinfected by immersing in 0.1% (w/v) HgCl₂ containing 2 drops of Tween-20 for 5 min. After four rinses in sterile distilled water, leaves were cut into pieces of about 1 cm² and then inoculated onto culture medium as explants (Reddy *et al* 2001). Alfalfa (*M. sativa* L.) seeds were surface-sterilized as described above and were allowed to germinate in the culture medium. Young petioles were dissected aseptically from these seedlings and used as explants.

2.2 Medium preparation and composition

Culture medium consisted of Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962), 100 mg/l myo-inositol, and 3% sucrose. The medium was solidified with 0.8% agar and autoclaved at 1.06 kg cm⁻² for 20 min. Each tube containing 15 ml of medium was inoculated with the explants and cultures were maintained at 25°C with 16 h light/8 h dark photoperiods (light intensity 50 μE m⁻² s⁻¹).

2.3 Callus formation

Various concentrations of monuron, diuron, TDZ and BAP (0.05 to 1 mg/l) were tested for their effects on callus formation in *Coleus* leaf and alfalfa petiole explants. MS medium lacking growth regulator served as control. One explant per tube was inoculated on slanted media surface in 150 x 25 mm culture tubes (Borosil, India) and data on callus formation was recorded and expressed as percentage of frequency formed. Fresh and dry weight of callus was determined after 30 days of inoculation. Twenty explants were used per treatment and the experiment was repeated three times (Reddy *et al* 2001).

2.4 Shoot morphogenesis

Callus was cultured in 250 ml conical flasks containing 50 ml of solid basal MS medium. Light-green compact calli were transferred onto MS medium supplemented with different concentrations of various cytokinins (monuron, diuron, DPU, TDZ and BAP) and the shoot differentiation efficiency of the callus was evaluated. MS medium lacking growth regulators served as control. For all the experiments, 20 callus pieces (of approximately 150 mg fresh weight per piece) were used for each treatment and all the experiments were repeated thrice. Data on number of explants responded to the treatment represented as percentage of frequency of shoot regeneration and the mean numbers of multiple shoots formed per treatment were determined at the end of 30 days of transfer into cytokinin-supplemented medium for alfalfa and *Coleus*.

3. Results

Many compounds with cytokinin-like activity and their effects on a variety of plant genera have already been reported (Mok and Mok 2001; Takahasi *et al* 1978). Cytokinins are found to be useful for regeneration of shoots from seedlings, shoot tip and callus cultures (Hassanein and Mazen 2001). Initially, we examined the effect of various urea herbicides on seed germination responses in alfalfa, brassica, cucumber and wheat. We found significantly higher germination responses in the presence of monuron and diuron than commonly used cytokinins like BAP and kinetin (data not shown).

3.1 Effect of monuron and diuron on callus induction

Experiments conducted with *Coleus* and alfalfa in MS medium without growth regulators showed no callusing response. Petiole explant of alfalfa showed a high frequency of callus formation (92%) in MS media supplemented

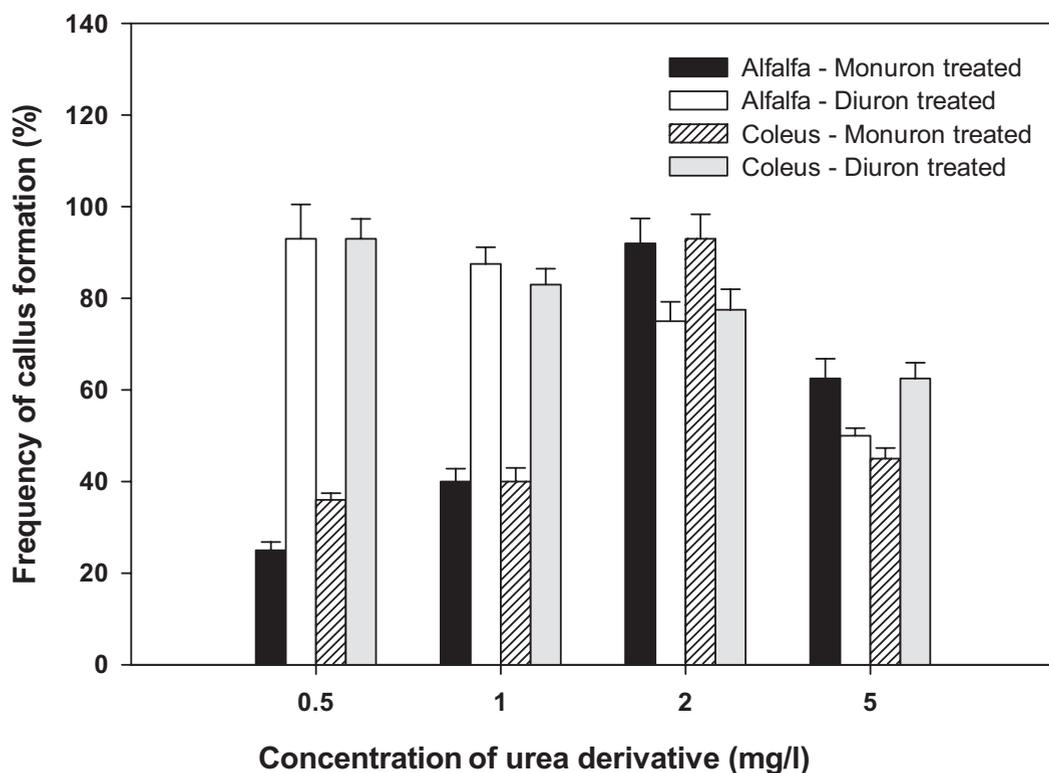


Figure 1. Effect of monuron and diuron on induction of callus from alfalfa petiole and Coleus leaf. Explants were used in MS media supplemented with various concentrations of monuron and diuron. Frequency of callusing was recorded after 30 days of culture. Data on number of explants that responded to the treatments are represented as percentage of frequency of callusing. Error bars represent the deviation from three independent experiments and each one performed in 20 callus pieces per treatment.

with 2.0 mg/l monuron (figure 1). A similar response was observed with lower concentrations (0.5 mg/l) of diuron (figure 1). High concentrations of diuron hindered the frequency of callusing and biomass content.

The leaf segments of Coleus showed high frequency of callusing (93%) with both monuron (2.0 mg/l) and 0.5 mg/l diuron (figure 1). Frequency of callus formation with monuron was found to be concentration dependent (0.5 to 2.0 mg/l) and there was a linear increase in fresh weight and dry weight (figure 2B). At high concentration of monuron (5.0 mg/l), there was a ~2-fold decrease in callus biomass content in both alfalfa and Coleus. On the contrary, diuron showed concentration dependent decrease of callus biomass content even at lower concentrations (figure 2).

3.2 Effect of TDZ, monuron and diuron on multiple shoot formation

Different cytokinins were used in the present study to determine their shoot regenerating capacity in alfalfa and Coleus. Thidiazuron showed a maximum response in terms of frequency and mean number of shoots (table 1). In alfalfa,

thidiazuron (0.5 mg/l) induced a maximum frequency of 92% with a mean number of 7.0 shoots after 30 days of culture, while with Coleus a maximum frequency of 84.2% with a mean number of 8.0 shoots were obtained. However, the shoots obtained in the presence of TDZ were stunted and showed symptoms of hyperhydricity. In the case of alfalfa, the transfer of TDZ grown embryonal axes to NAA-fortified medium (0.2 mg/l) promoted elongation of shoots in the second stage. The shoot regeneration response was also found to be high with diuron with a frequency of 89% for alfalfa explants, while with Coleus, a maximum of 6.0 shoots with a frequency of 76% was obtained (table 1). Diuron showed better shoot regeneration response than monuron and other urea derivatives. Diuron with two chloride groups in the phenyl ring showed significantly higher cytokinin-like activity than monuron, which has a single chloride substitution.

4. Discussion

Urea-derived herbicides are known to possess cytokinin-like activity. In our study we have used monuron and

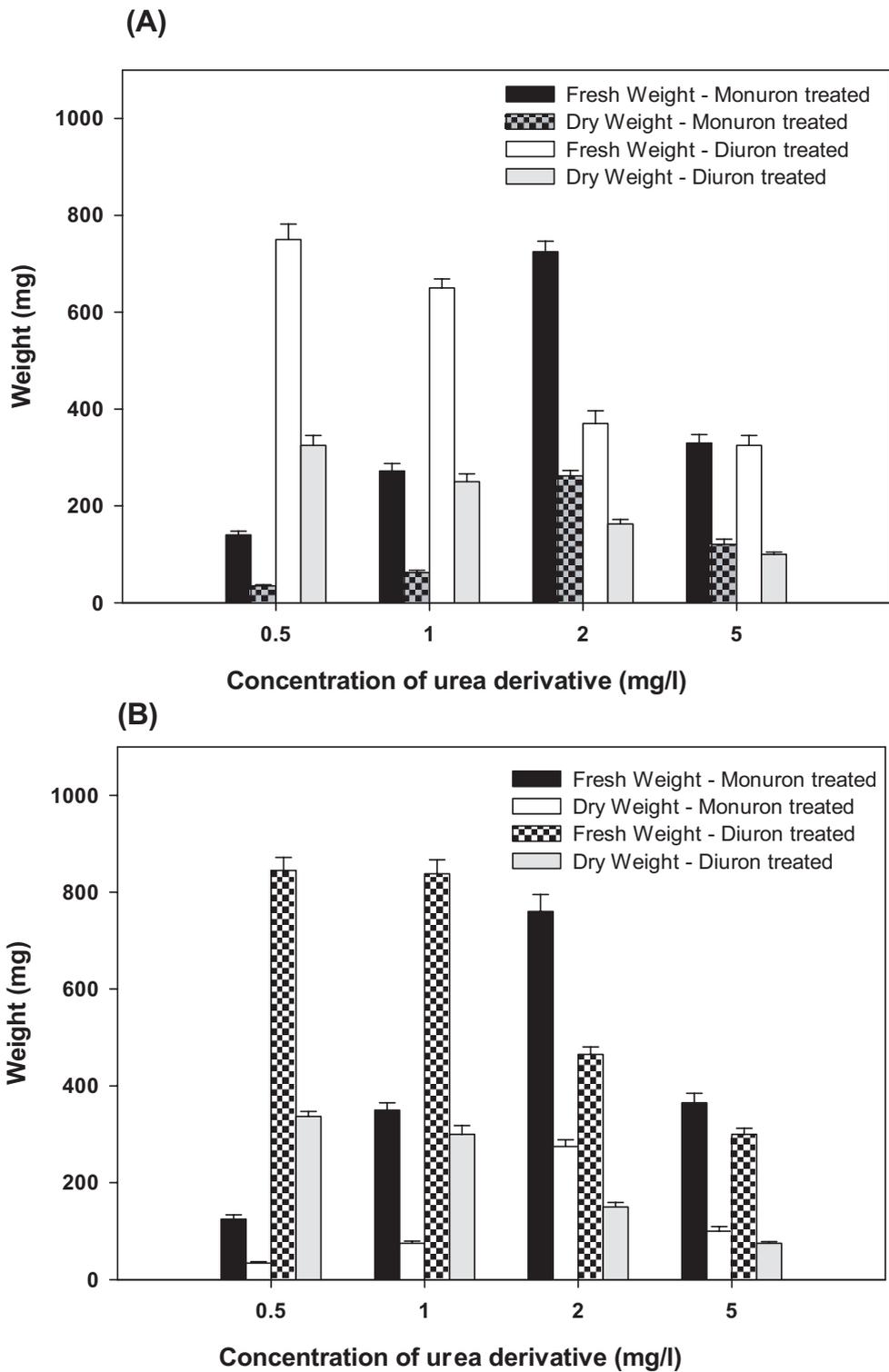
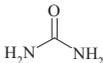
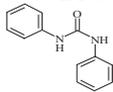
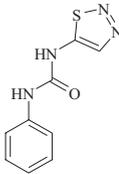
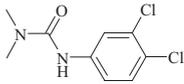
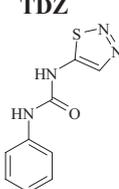
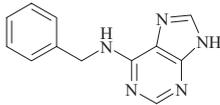


Figure 2. Effect of monuron and diuron on callus biomass from petiole of *M. sativa* and leaf of *C. forskohlii*. Alfalfa petiole (A) and Coleus leaf (B) were used as explants in MS media supplemented with various concentrations of monuron and diuron. Callus biomass was recorded after 30 days of culture. Error bars represent the deviation from three independent experiments and each one performed in twenty replicates per treatment in three independent experiments.

Table 1. Effect of various urea herbicides and classical cytokinins on shooting efficiency in Coleus and alfalfa.

Cytokinin and structure	Concentration (mg/l)	Frequency of regeneration (%)		Mean number of shoots	
		Alfalfa	Coleus	Alfalfa	Coleus
Control	0.00	10.0 ± 4.9	13.0 ± 4.2	1.0 ± 0.3	1.4 ± 0.7
Urea 	0.05	14.0 ± 2.3	17.4 ± 2.8	1.0 ± 0.2	1.2 ± 0.2
	0.20	16.0 ± 4.0	21.2 ± 4.8	1.0 ± 0.4	1.4 ± 0.3
	0.50	19.0 ± 4.0	27.8 ± 6.3	2.0 ± 1.1	2.1 ± 0.3
	1.00	17.0 ± 3.0	24.3 ± 3.4	1.5 ± 0.7	1.0 ± 0.4
DPU 	0.05	20.0 ± 5.1	29.6 ± 4.2	1.0 ± 0.3	2.0 ± 0.5
	0.20	21.6 ± 4.6	35.5 ± 5.2	1.0 ± 0.4	2.4 ± 0.2
	0.50	25.6 ± 5.7	42.0 ± 5.6	1.5 ± 0.7	2.7 ± 0.5
	1.00	22.9 ± 4.3	31.0 ± 4.3	2.0 ± 0.9	1.8 ± 0.7
Monuron 	0.05	28.9 ± 5.9	26.7 ± 3.9	1.0 ± 0.2	1.0 ± 0.4
	0.20	44.5 ± 6.2	39.5 ± 6.1	1.0 ± 0.4	1.0 ± 0.3
	0.50	75.0 ± 8.2	65.6 ± 5.2	3.0 ± 0.6	3.0 ± 0.5
	1.00	52.8 ± 5.9	42.5 ± 4.7	1.0 ± 0.2	1.5 ± 0.6
Diuron 	0.05	39.4 ± 4.2	36.2 ± 5.7	2.0 ± 0.3	2.7 ± 0.5
	0.20	47.8 ± 5.1	42.4 ± 5.2	2.0 ± 0.6	3.9 ± 0.4
	0.50	89.0 ± 7.6	76.2 ± 6.4	5.0 ± 0.6	6.0 ± 0.7
	1.00	66.4 ± 8.2	59.9 ± 8.2	2.0 ± 0.8	3.8 ± 0.9
TDZ 	0.05	29.6 ± 2.4	32.1 ± 7.8	2.0 ± 0.6	2.4 ± 0.5
	0.20	39.7 ± 3.7	45.5 ± 4.9	3.4 ± 0.8	3.7 ± 1.0
	0.50	92.0 ± 9.8	84.2 ± 6.8	7.0 ± 1.2	8.0 ± 1.9
	1.00	70.3 ± 9.2	69.8 ± 7.1	3.0 ± 1.1	3.4 ± 0.7
BAP 	0.05	37.4 ± 5.4	32.1 ± 4.8	1.2 ± 0.5	3.0 ± 0.6
	0.20	55.6 ± 6.2	44.8 ± 5.2	1.6 ± 0.4	4.7 ± 0.4
	0.50	40.0 ± 4.7	36.7 ± 4.3	1.0 ± 0.3	2.0 ± 0.6
	1.00	31.2 ± 5.1	30.8 ± 5.6	1.7 ± 0.6	1.0 ± 0.5

Data on number of explants that responded to the treatment is represented as percentage of frequency of shoot regeneration and the mean numbers of multiple shoots formed per treatment were determined at the end of 30 days of transfer into cytokinin-supplemented MS medium. Values represent mean ± SE of 20 replicates per treatment in three independent experiments

diuron to demonstrate their role in micropropagation. Petiole explants and leaf bit explants of alfalfa and Coleus showed differences in their response to monuron and diuron treatments in terms of both callus formation and multiple shoot formation. The differences noted among the tested synthetic cytokinins in inducing callusing response could be due to their uptake, metabolism and structural requirement for activity as was reported by Leonard *et al* (1969) with zeatin like compounds.

The response in terms of callus formation in alfalfa and Coleus was dose dependent. Such dose dependent increase in callus growth was also observed with many other cytokinins like BAP, kinetin and TDZ (Nobre *et al* 2001; Reddy *et al* 2001). The increased frequency of callus formation observed in Coleus leaf explants with monuron and diuron was suggestive of high morphogenetic capacity. A similar response was observed in Coleus explants with kinetin (Sharma *et al* 1991). It was observed from the

present study that the optimal concentrations of monuron and diuron are 2.0 mg/l and 0.5 mg/l, respectively for alfalfa and *Coleus* explants.

The response of both plants to the urea-derived herbicides in terms of multiple shoot formation has been encouraging. Treatment with TDZ also showed a good result in shoot formation, but it was observed that there was a stunted growth and hyperhydricity, which could be due to the inability to degrade TDZ leading to its accumulation (Mok and Mok 2001). Results in this study have shown that further treatment with naphthalene acetic acid substituted medium can help overcome this condition, which is similar to the report of Briggs *et al* (1988) who observed improved shoot quality with TDZ in combination with BAP.

The present study shows unusual cytokinin-like property for monuron and diuron. This could be due to the structural similarities of these urea compounds with cytokinins. However the efficiency of these compounds is more than cytokinins as they have chloride substitutions in their phenyl rings. BAP, a cytokinin with purine structure had an effect on alfalfa embryonal axes. DPU has two phenyl group substitutions, which has a weak cytokinin like activity and elicits more shooting response at a concentration of 0.5 mg/l in alfalfa and *Coleus* than urea (table 1). Similarly, Miller (1960) has reported sporadic activity of DPU with tobacco pith callus bioassay. Hence, both urea-derived herbicides as well as substituted adenine derivatives demonstrated cytokinin-like property in *Coleus* and alfalfa. Long exposure of multiple shoot cultures to monuron and diuron are not recommended as this may cause abnormal leaf morphology, stunted growth and difficulty in rooting. In summary, monuron and diuron induced more multiple shoots than adenine type cytokinins in *Coleus* and alfalfa. Results of the present study validate the potential of monuron and diuron in exerting unusual cytokinin-like activity that are useful for micropropagation.

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