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Effect of Cl-Substitution on Rooting- or Cytokinin-like Activity of Diphenylurea Derivatives

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Abstract

Twenty-eight Cl-substituted diphenylurea derivatives differing in either the number and the position of the substituents, or in the type of substitution, that is, symmetric or asymmetric, were synthesized. Their hypothetical enhancement of rooting activity was assayed using the mung bean shoot bioassay; their possible cytokinin-like activity was assessed using the betacyanin (so-called ''amaranthin'') accumulation test and the tomato regeneration test. Seven Cl-substituted diphenylurea derivatives (**2E**, **4A**, **4B**, **4E**, **4G**, **6A**, **6B**) having two substituted phenyl rings showed the capacity to enhance adventitious root formation in mung bean shoots. Furthermore the presence of a halogen substituent was not sufficient to reach the adventitious rooting activities shown by the N,N ′-bis-(2,3-methylenedioxyphenylurea) and the N,N ′-bis-(3,4-methylenedioxyphenylurea), two diphenylurea derivatives for which an interaction with auxin was the first reported in enhancing adventitious root formation. Seven compounds (**1B**, **3E**, **3D**, **4B**, **4E**, **4F**, **6B**) showed cytokinin-like activity and three of them (**4B**, **4E**, **6B**) also evidenced rooting activity, once more demonstrating the wide action spectrum of diphenylurea derivatives.

Key words: Adventitious root formation; Betacyanin accumulation test; Cytokinin-like activity; Diphenylureas; Mung bean shoots; Tomato regeneration test

Introduction

Scince Shantz and Steward (1955) showed the cytokinin-like activity of N,N′-diphenylurea (DPU), it has been hypothisized that the substituted urea derivatives could replace the cytokinin-active adenine derivatives. Although DPU was a rather weak cytokinin-like compound, this finding was followed by the synthesis of numerous analogues. In fact, these types of compounds do not occur naturally, although DPU was initially reported as a component of coconut milk (Shantz and Steward 1955). Structure-activity relationship studies on substituted ureas revealed that an intact NH-CO-NH bridge with an unsubstituted phenyl ring linked in the N-position and a substituted phenyl ring in the N′-position are required for cytokinin activity (Bruce and Zwar 1966). The presence of a heterocyclic ring in the N′-position can increase the

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N-(x-chlorophenyl)-N'-phenylureas

X	
2Cl	*N-(2-chlorophenyl)-N'-phenylurea* (**1A**)
3Cl	*N-(3-chlorophenyl)-N'-phenylurea* (**1B**)
4Cl	*N-(4-chlorophenyl)-N'-phenylurea* (**1C**)

Figure 1. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

N-(x-chlorophenyl)-N'-(x'-chlorophenyl)ureas

X	X'	
2Cl	2Cl	*N,N'-bis-(2-chlorophenyl)urea* (**2A**)
3Cl	3Cl	*N,N'-bis-(3-chlorophenyl)urea* (**2B**)
4Cl	4Cl	*N,N'-bis-(4-chlorophenyl)urea* (**2C**)
2Cl	3Cl	*N-(2-chlorophenyl)-N'-(3-chlorophenyl)urea* (**2D**)
2Cl	4Cl	*N-(2-chlorophenyl)-N'-(4-chlorophenyl)urea* (**2E**)
3Cl	4Cl	*N-(3-chlorophenyl)-N'-(4-chlorophenyl)urea* (**2F**)

Figure 2. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

cytokinin activity. In fact, either the N-phenyl-N′-(2-chloro-4-pyridyl)urea (CPPU) or the N-phenyl-N′-(1,2,3-thiadiazol-5-yl)urea (thidiazuron, TDZ) showed cytokinin activity exceeding that of the adenine type derivatives (Takahashi and others 1978; Mok and others, 1982; Mok and others 1987). This high cytokinin activity of the latter compounds demonstrated in several biological assays might be due to the extreme stability in plant tissue, as reported by Mok and Mok (1985).

More recently, we reported that the N-phenyl-N′-benzothiazol-6-ylurea, according to the above-cited favorable effect of a heterocyclic system, shows higher cytokinin-like activity than TDZ, suggesting that it could be used as a new cytokinin for shoot production *in vitro* (Ricci and others 2001a). In our continuing work on urea derivatives, we also reported that, surprisingly, two symmetric diphenylurea derivatives, the N,N′-bis-(2,3-methylenedioxyphenyl)urea and N,N′-bis-(3,4-methylenedioxyphenyl)urea, enhance adventitious root formation in microcuttings of *Malus pumila* Mill. rootstock M26 (Ricci and others 2001b). These two compounds showed no cytokinin- or auxin-like activity per se; rather we demonstrated that they cooperate with auxin in enhancing adventitious root formation (Ricci and others 2003). These intriguing results demonstrating that DPU derivatives could show a wide spectrum of action, led us to synthesize other diphenylurea derivatives, trying to better define the chemical structure related to the enhancement of rooting activity. Because other authors have shown that substitution with electron-withdrawing groups can increase the cytokinin activity (Bruce and Zwar 1966) we decided to use the chloro-substitution as a tool for investigating the possible enhancement of the rooting activity or the cytokinin-like activity. Thus, we synthesized 28 Cl-substituted diphenylurea derivatives, differing in either the number and the position of the chloro-substituent or the structural symmetry, that is, symmetric or asymmetric compounds (Figures. 1–6). Most of them are reported in the literature, but biological data were not available for the purpose of our research.

The hypothetical enhancement of rooting activity was tested by using the mung bean shoot bioassay; possible cytokinin-like activity was assessed using the betacyanin (so-called ''amaranthin'') accumulation test and the tomato regeneration test.

Materials and Methods

Synthesis

The chlorodiphenylureas under study (Fig. 1–6) were reported in the literature (Table 1), with the exception of compounds **3A**, **4A**, **4B**, and were previously prepared following different synthetic procedures. We synthetized all compounds by reacting the appropriate chloroaniline with the suitable unsubstituted or substituted phenylisocyanate. The compounds, obtained in good yields, were purified by recrystallization from ethanol /water (otherwise as specified) and characterized by IR (data not reported) and ^{1}H-NMR spectra, and by elemental analyses. The reactions were checked by TLC using a solvent system of chloroform: ethanol (95:5, v/v).

N-(x,y-dichlorophenyl)-N'-phenylureas

x	y	
2Cl	3Cl	*N-(2,3-dichlorophenyl)-N'-phenylurea* (**3A**)
2Cl	4Cl	*N-(2,4-dichlorophenyl)-N'-phenylurea* (**3B**)
2Cl	5Cl	*N-(2,5-dichlorophenyl)-N'-phenylurea* (**3C**)
2Cl	6Cl	*N-(2,6-dichlorophenyl)-N'-phenylurea* (**3D**)
3Cl	4Cl	*N-(3,4-dichlorophenyl)-N'-phenylurea* (**3E**)
3Cl	5Cl	*N-(3,5-dichlorophenyl)-N'-phenylurea* (**3F**)

Figure 3. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

Instruments and Chemicals

Melting points (mp) were determined on a Buchi apparatus (Tottoli) and are uncorrected. Infrared spectra (IR) were recorded as KBr tablets, on a Jasco FTIR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker AC 300 instrument at 298K (300 MHz), in DMSO-d_6 solutions. Chemical shifts are expressed as δ (ppm) relative to TMS as internal standard. Reactions and final compounds were checked by thin-layer chromatography (TLC) performed on aluminium-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light and using $CHCl_3$:EtOH (95:5, v/v) as eluent. Elemental analyses were performed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyzer; C H N and analytical results were within ± 0.4% of the theoretical values. Starting materials, reagents and solvents were purchased from Aldrich Chimica, Milan (Italy).

General Procedure for the Synthesis of the Chlorodiphenylureas

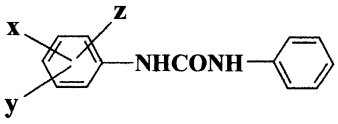
Phenylisocyanate (9.5 mmol) was added dropwise to a solution of the appropriate amine (8.5 mmol) in anhydrous benzene (30 mL). The mixture was stirred under reflux for two hours; the whitish solid formed was filtered and recrystallized. The analytical data of the compounds are reported in Table 1. ^{1}H-NMR data of known compounds (not reported) were in agreement with the proposed structures. Below we report the ^{1}H-NMR spectral data of new compounds (**3A**, **4A**, **4B**).

N-(x,y-dichlorophenyl)-N'(x',y'-dichlorophenyl)ureas

x	y	x'	y'	
2Cl	3Cl	2Cl	3Cl	*N,N'-bis-(2,3-dichlorophenyl)urea* (**4A**)
2Cl	3Cl	3Cl	4Cl	*N-(2,3-dichlorophenyl)-N'-(3,4-dichlorophenyl)urea* (**4B**)
2Cl	3Cl	3Cl	5Cl	*N-(2,3-dichlorophenyl)-N'-(3,5-dichlorophenyl)urea* (**4C**)
2Cl	4Cl	2Cl	4Cl	*N,N'-bis-(2,4-dichlorophenyl)urea* (**4D**)
2Cl	5Cl	2Cl	5Cl	*N,N'-bis-(2,5-dichlorophenyl)urea* (**4E**)
2Cl	6Cl	2Cl	6Cl	*N,N'-bis-(2,6-dichlorophenyl)urea* (**4F**)
3Cl	4Cl	3Cl	4Cl	*N,N'-bis-(3,4-dichlorophenyl)urea* (**4G**)
3Cl	4Cl	3Cl	5Cl	*N-(3,4-dichlorophenyl)-N'-(3,5-dichlorophenyl)urea* (**4H**)
3Cl	5Cl	3Cl	5Cl	*N,N'-bis-(3,5-dichlorophenyl)urea* (**4I**)

Figure 4. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

N-(x,y,z-trichlorophenyl)-N'-phenylureas



x	y	z	
2Cl	4Cl	5Cl	*N-(2,4,5-trichlorophenyl)-N'-phenylurea* (**5A**)
2Cl	4Cl	6Cl	*N-(2,4,6-trichlorophenyl)-N'-phenylurea* (**5B**)

Figure 5. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

N-(2,3-dichlorophenyl)-N'-phenylurea (**3A**): δ 9.44 (s, 1H, NH); 8.44 (s, 1H, NH); 8.14 (dd, 1H, Ph-6, J = 2,1 J = 8,1); 7.44 (d, 2H, Ph-2'+6', J = 7,8); 7.34–7.25 (m, 4H, Ph-3' + 5'+ 4 + 5); 7.00 (t, 1H, Ph-4', J = 7,4)

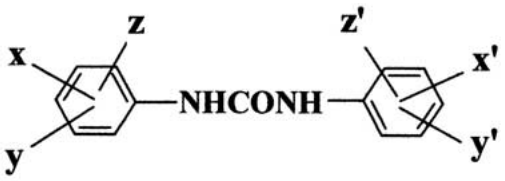
N,N'-bis-(2,3-dichlorophenylurea) (**4A**): δ 9.22 (s, 2H, NH); 8.05 (t, 2H, Ph-5 + 5', J = 8,7); 7.36–7.30 (m, 4H, Ph-4 + 6 + 4' + 6').

N-(2,3-dichlorophenyl)-N'-(3,4-dichlorophenyl) urea (**4B**): δ 9,71 (s, 1H, NH); 8,51 (s, 1H, NH); 8,10 (dd, 1H, Ph-6, J = 2,7 J = 6,6); 7,88 (d, 1H, Ph-2, J = 2,1); 7,52 (d, 1H, Ph-6' J = 8,4); 7,36–7,28 (m, 3H, Ph-4' + 5' + 5).

Biological Evaluation

All the Cl-derivatives were dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO in the aqueous solutions did not exceed 0.2% (Schmitz and Skoog 1970).

N,N'-bis-(x,y,z-trichlorophenyl)ureas



x=x'	y=y'	z=z'	
2Cl	4Cl	5Cl	*N,N'-bis-(2,4,5-trichlorophenyl)urea* (**6A**)
2Cl	4Cl	6Cl	*N,N'-bis-(2,4,6-trichlorophenyl)urea* (**6B**)

Figure 6. Molecular structure of the Cl-substituted diphenylurea derivatives used in this study.

Rooting of Mung Bean Shoots

Seeds of mung bean (*Vigna radiata* L.) were surface sterilized with 20% commercial bleach for 10 min, washed 3 times with sterile distilled water and soaked in sterile distilled water for 24 hours. Then seeds were planted in sterile moist sawdust, germinated and grown in a greenhouse at 24 ± 1°C, 16 h photoperiod, at a light intensity of 27 μmol m^{-2} s^{-1} for 1 week (after 4 days the plantlets were sprayed with 16 mM H_3BO_3). The shoots were harvested by cutting 3 cm under the cotyledonary node and deprived of cotyledons when present. Then they were cultured separately in darkened shell vials containing 15 ml of aqueous solution of the Cl-substituted diphenylurea derivatives at 0.01, 0.05, 0.1, 0.5, 1 and 5 μM, and incubated at a light intensity of 27 μmol m^{-2} s^{-1} at 26°C under a 16 h photoperiod (rooting incubation conditions) for the initial 24 hr. The shoots were then transferred to distilled water and incubated under the same culture conditions described above. Controls were performed with mung bean shoots cultured in distilled water (HF) under the rooting incubation conditions.

All the experiments were carried out using 10 shoots, repeated three times and the number of roots was counted after 1 week. The results were expressed as a percentage of the control (H_2O alone) by the formula (Tested-Control/Control) × 100 [(T-C/C) × 100].

Tomato Regeneration Test

Twelve cotyledon explants, obtained from seedlings cultivated *in vitro*, were plated on MS medium (Murashige and Skoog 1962) containing the Cl-substituted diphenylurea derivatives instead of traditional cytokinins, at 1, 5 and 10 μM, both alone and in the presence of 20 μM 1,2-benzisoxazole-3-acetic acid (BOAA), as auxin (Branca and others 1990). The results were compared with that of TDZ under the same culture conditions.

After 2 weeks of incubation at a light intensity of 27 μmol m^{-2} s^{-1} at 26°C under a 16 h photoperiod, the cotyledon explants were transferred to a hormone-free medium and the number of explants that regenerated shoots was checked 2 weeks later. The experiments were carried out in triplicate, and repeated three times.

Betacyanin Accumulation Test

The *Amaranthus* bioassay was performed as described previously by Romanov and others (2000) with minor modifications: seed germination was carried on for 4 days; the phosphate buffer was supplemented with 0.45 mg/ml L-tyrosine (Sigma); multiwell dishes (12 wells/dish, Costar) were used; the explants were incubated for 20 hr in the dark at 23°C. Four different concentrations (10 nM, 100 nM, 1 μM and 10 μM) of the Cl-substituted diphenylurea derivatives were assayed. Controls were performed with phosphate buffer alone (Hormone Free, HF). For each experiment the same concentrations of 6-benzylaminopurine (BAP) were also tested and the experiment was considered ''valid'' only in the presence of a classical BAP concentration-course variation, as reported by Romanov and others 2000 (data not shown). The experiments were carried out in triplicate, and repeated three times. The results were expressed as a percentage of the control (phosphate buffer alone) by the formula (T-C/C)×100.

Results (Table 1)

N-(x-chIorophenyl)-N′-phenylureas (Figure 1)

Rooting of Mung Bean Shoots. All three N-(x-chlorophenyl)-N′-phenylureas were ineffective at all the concentrations tested.

Tomato Regeneration Test. Among the three N-(x-chlorophenyl)-N′-phenylureas, only the (**1B**) at 10 μM showed a moderate activity (20% of the explants formed shoots) in the presence of 20 μM BOAA as auxin, that was, however, significantly lower than that obtained by the same concentration of TDZ (55% of the explants formed shoots) in the presence of 20 μM BOAA as auxin. All other compounds were ineffective at all the concentrations tested either in the presence or in the absence of 20 μM BOAA.

Betacyanin Accumulation Test. Among the three N-(x-chlorophenyl)-N′-phenylureas, only the N-(3-chlorophenyl)-N′-phenylurea (**1B**) at the higher concentration tested (10 μM) was able to induce betacyanin accumulation in *Amaranthus caudatus* explants (98% over the control). All the other compounds were ineffective.

N-(x-chlorophenyl)-N′-(x′-chlorophenyl)ureas (Figure 2)

Rooting of Mung Bean Shoots. Among the six N-(x-chlorophenyl)-N′-(x′-chlorophenyl)ureas, the N-(2-chlorophenyl)-N′-(4-chlorophenyl)urea (**2E**) enhanced the adventitious root formation in mung bean plantlets at all the concentrations tested. The root amount increased with increasing the concentration used (93% over the control at 10 μM). Other compounds showed weak enhancement of the adventitious root formation, but their activities were not significantly different from that of HF.

Tomato Regeneration Test. All the six N-(x-chlorophenyl)-N′-(x′-chlorophenyl)ureas were ineffective at all the concentrations tested either in the presence or absence of 20 μM BOAA.

Betacyanin Accumulation Test. Among the six N-(x-chlorophenyl)-N′-(x′-chlorophenyl)ureas, the N-(2-chlorophenyl)-N′-(4-chlorophenyl)urea (**2E**) lowered the betacyanin accumulation in *Amaranthus caudatus* explants. The maximum reduction rate (−35%) was reached at 10 μM. All the other compounds were ineffective.

N-(x,y-dichIorophenyl)-N′-phenylureas (Figure 3)

Rooting of Mung Bean Shoots. All six N-(x,y-dichlorophenyl)-N′-phenylureas were ineffective at all the concentrations tested.

Tomato Regeneration Test. Among the six N-(x,y-chlorophenyl)-N′-phenylureas, only the N-(3,4-dichlorophenyl)-N′-phenylurea (**3E**) at 5 μM and 10 μM showed a moderate activity (33 and 31% of the explants formed shoots) in the presence of 20 μM BOAA. Once again these results were significantly lower than those obtained by the same concentrations of TDZ (40% and 55% of the explants formed shoots, respectively) in the presence of 20 μM BOAA as auxin. All the other compounds were ineffective at all the concentrations tested either in the presence or absence of 20 μM BOAA.

Betacyanin Accumulation Test. Among the six N-(x,y-chlorophenyl)-N′-phenylureas, the N-(2,6-dichlorophenyl)-N′-phenylurea (**3D**) was able to induce betacyanin accumulation in *Amaranthus caudatus* explants. At 0.1 μM, the pigment amount was 102% over the HF control and decreased (30% over the control) at the highest concentration tested (10 μM). Otherwise the N-(3,4-chlorophenyl)-N′-phenylurea (**3E**) lowered the betacyanin accumulation in *Amaranthus caudatus* explants at all the concentrations tested. At 1 μM, the maximum reduction rate, −62% of the HF control condition, was reached. All the other compounds were ineffective.

N-(x,y-dichlorophenyl)-N′-(x′,y′-dichlorophenyl)ureas (Figure 4)

Rooting of Mung Bean Shoots. Among the nine N-(x,y-dichlorophenyl)-N′-(x′,y′-dichlorophenyl)ureas, the N,N′-bis-(2,3-dichlorophenyl)urea (**4A**) enhanced adventitious root formation in mung bean plantlets. At 1 μM the amount was 65% higher than that obtained by the HF control condition. The N-(2,3-dichlorophenyl)-N′-(3,4-dichlorophenyl) urea (**4B**) moderately enhanced adventitious root formation at 0.1 μM (36% over the control). The N,N′-bis-(2,5-dichlorophenyl) urea (**4E**) also enhanced adventitious root formation (78% over the control) at 1 μM. Finally, the N,N′-bis-(3,4-dichlorophenyl) urea (**4G**) enhanced adventitious root formation (51% over the control) at 0.1 μM.

Table 1. Analytical Data and Biological Activities of Compounds

	M.p.(°C) found/literature	Yield %	Ref. no	[c]*Mung bean rooting*	[c]*Tomato regeneration*	[c]*Betacyanin accumulation*
1A	180–183 / 180–181	62	13			
1B	188–189 / 187–188	60	8		+	+
1C	250–253 / 250–251	64	1			
2A	252–253 / 244–245	62	20			
2B	257–258 / 250–251	63	35			
2C	314–316 / 308–310	60	34			
2D	195–197 / 200–201	64	33			
2E	215–216 / 212–213	60	9	+ +		–
2F	223–224 / 229–230	59	5			
3A	191–192	60	—			
3B	232–233 / 227–228	63	5			
3C	227–228 / 234–235	70	12			
3D	258–259 / 240–241	60	30			+ +
3E	210–211 / 214–216	62	12		+	– –
3F	202–203 / 198–199	61	12			
4A	225–226	60	—	+ +		–
4B	243–244	67	—	+		+ +
4C	244–245 / 271–272	64	6			
4D	270–274 / 272–273	61	2			
4E	300–305 / 296–297	66	31	+ +		+ +
4F	325–328 / 293–295	60	11			+
4G	250–251 / 255–256	64	4	+ +		– –
4H	248–249 / 264–265	65	6			
4I	298–300 / 299–300	60	27			
5A	218–220 / 216–217	65	25			
5B	278–280 / 274–275	63	15			
6A	303–304 / 303–304[a]	60	15	+ +		
6B	297–298 / 295–296[b]	64	10	+ +		+ +

[a]*Recrystallized from THF- water.*
[b]*Sublimated 0.1 mm/HgT: 240°C.*
[c]*Empty cells: no activity.*
+Biological activity ranging from 0 to 50% ; + + biological activity over 50%.
–Reduced biological activity from 0 to −50% ; – – reduced biological activity over −50%.

All the other N-(x,y-dichlorophenyl)-N′-(x′,y′-dichlorophenyl)ureas were ineffective at all the concentrations tested.

Tomato Regeneration Test. All the compounds were ineffective at all the concentrations tested either in the presence or absence of 20 μM BOAA.

Betacyanin Accumulation Test. Among the nine N-(x,y-dichlorophenyl)-N′-(x′,y′-dichlorophenyl)ureas, the (**4B**) was able to induce betacyanin accumulation in *Amaranthus caudatus* explants. The pigment amount increased with increasing concentration (106% over the control at 10 μM). The N,N′-bis-(2,5-dichlorophenyl) urea (**4E**) was able to induce betacyanin accumulation. The amount reached its maximum at 1 μM (91% over the control) and then decreased. Also the N,N′-bis-(2,6-dichlorophenyl) urea (**4F**) was able to induce betacyanin accumulation. The pigment amount was 40% over the control at the highest concentration tested (10 μM). Otherwise the N,N′-bis-(2,3-dichlorophenyl) urea (**4A**) and the N,N′-bis-(3,4-dichlorophenyl) urea (**4G**) lowered the betacyanin accumulation. The maximum reduction rate (−12% and −64% of the control, respectively) was reached at 10 μM. All the other compounds were ineffective.

N-(x,y,z-trichlorophenyl)-N′-phenylureas (Figure 5)

Rooting of Mung Bean Shoots. The two N-(x,y,z-trichlorophenyl)-N′-phenylureas were ineffective at all the concentrations tested.

Tomato Regeneration Test. The two N-(x,y,z-trichlorophenyl)-N′-phenylureas were ineffective at all the concentrations tested either in the presence or absence of 20 μM BOAA, as auxin.

Betacyanin Accumulation Test. The two N-(x,y,z-trichlorophenyl)-N′-phenylureas were ineffective at all the concentrations tested.

N,N′-bis-(x,y,z-trichlorophenyl)ureas (Figure 6)

Rooting of Mung Bean Shoots. From the two N,N′-bis-(x,y,z-trichlorophenyl)ureas, the N,N′-bis-(2,4,6-trichlorophenyl)urea (**6B**) enhanced the adventitious root formation in mung bean plantlets (73% over the control) at 1 μM. The N,N′-bis-(2,4,5-trichlorophenyl) urea (**6A**) was also able to enhance adventitious root formation (58% over the control at 0.1 μM).

Tomato Regeneration Test. The two N,N′-bis-(x,y,z-trichlorophenyl)ureas were ineffective at all the concentrations tested either in the presence or absence of 20 μM BOAA.

Betacyanin Accumulation Test. Among the two N,N′-bis-(x,y,z-trichlorophenyl)ureas, the N,N′-bis-(2,4,6-trichlorophenyl)urea (**6B**) was able to induce betacyanin accumulation in *Amaranthus caudatus* explants. The amount was 66% over the control at the highest concentration tested (10 μM). The N,N′-bis-(2,4,5-trichlorophenyl)urea (**6A**) was ineffective at all the concentrations tested.

Discussion

The hypothetical enhancement of rooting activity or cytokinin-like activity of 28 Cl-substituted diphenylurea derivatives was assayed. As for the rooting activity, from our data we can infer that no Cl-substituted diphenylurea derivative possessing one substituted phenyl ring (**1A**, **1B**, **1C**, **3A**, **3B**, **3C**, **3D**, **3E**, **3F**, **5A**, **5B**) is able to enhance adventitious root formation. Instead, some of the Cl-substituted diphenylurea derivatives possessing two substituted phenyl rings enhanced adventitious root formation as shown by the compounds **2E**, **4A**, **4B**, **4E**, **4G**, **6A** and **6B**. However, the physiological action mechanism by which they enhanced rooting seems to be different depending on the compounds. In fact, three of the above-cited compounds, namely, **4B**, **4E** and **6B**, were also able to induce betacyanin accumulation in the *Amaranthus caudatus* test. Therefore they could be considered weak cytokinins able to initiate cell division, which is essential for adventitious root formation, according to De Klerk and others (2001). One other Cl-substituted diphenylurea derivative **6A** was unable to induce tomato shoot regeneration or betacyanin accumulation; in this case the enhancement of rooting activity could be due to a cooperation with endogenous auxin. Thus, its action mechanism might be similar to that shown by two methylenedioxy-substituted diphenylureas, previously described by us (Ricci and others 2001b). Three other Cl-substituted diphenylurea derivatives (**2E**, **4A** and **4G**) lower betacyanin accumulation, thus confirming the idea that the root formation mechanism could be the result of a changed endogenous auxin/cytokinin ratio (Kevers and others 1997). However, the Cl-substitution of the two phenyl rings was not a necessary and sufficient condition to show rooting activity. In fact, no other Cl-substituted diphenylurea derivatives possessing two substituted phenyl rings (**2A**, **2B**, **2C**, **2D**, **2F**, **4C**, **4D**, **4F**, **4H**, **4I**) was able to enhance adventitious root formation.

The cytokinin-like activity was assessed by two different bioassays, the tomato cotyledon regeneration test and the betacyanin accumulation test. The former is suitable for extremely stable cytokinins because it requires a long assay time (4 weeks) and the latter allows for detection of weakly active compounds because it is highly specific with a relatively fast response (hours).

Using the tomato regeneration test, all the Cl-substituted diphenylurea derivatives possessing two substituted phenyl rings, were ineffective. Among the diphenylurea derivatives possessing one substituted phenyl ring, **1B** and **3E** showed regeneration activity, but this moderate activity was lower than that shown by the same concentration of TDZ.

The results from the betacyanin accumulation test appear inconsistent and therefore are difficult to evaluate. In fact, we detected an enhancement over the control (**1B**, **3D**, **4B**, **4E**, **4F**, **6B**) and a reduction of the betacyanin accumulation (**2E**, **3E**, **4A**, **4G**) by heterogeneous compounds, and it is impossible to define a common structural trend linked to the specific biological activity.

In conclusion, the diphenylurea derivatives seem to be active in enhancing adventitious root formation. The rooting activity seems to be exclusively linked to the substituted diphenylurea derivatives possessing two substituted phenyl rings, but this structural rule is not a sufficient condition. Indeed none of the tested compounds enhanced adventitious rooting at a level similar to that shown by the N,N′-bis-(2,3-methylenedioxyphenyl) urea and the N,N′-bis-(3,4-methylenedioxyphenyl) urea (Ricci

and others 2001b). Work is in progress to identify the substituents of the phenyl rings that better cooperate with auxin in the enhancement of adventitious rooting. The low cytokinin-like activity of diphenylurea derivatives, already reported by Shudo (1994), is confirmed by our data and it seems inconsistent with any structure-activity relationships.

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