N,N'-Dibutylurea from n-Butyl Isocyanate, a Degradation Product of Benomyl. 2. Effects on Plant Growth and Physiology†

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Experiments were conducted to determine the effects of N,N'-dibutylurea (DBU) on plants. DBU is a product of the reaction between water and n-butyl isocyanate, which is in turn an elimination product of methyl 1-(butylcarbamoyl)-2-benzimidazole (the fungicide benomyl). Corn was unaffected by DBU regardless of rate or time of application. DBU did not affect respiration by hydrilla, seed germination, or seedling emergence of cucumber. At a rate of 2.8 kg ha^{-1}, DBU reduced cucumber root and shoot biomass between 59 and 63%, regardless of time of application. DBU caused dilation of grana and irregularities in the lamellae of cucumber chloroplasts and completely inhibited photosynthesis (measured as decreased oxygen evolution) of hydrilla after 1 h of exposure. DBU also reduced the peak-to-terminal chlorophyll a fluorescence ratio of hydrilla. DBU probably inhibits photosynthesis and consequently plant growth of susceptible plants.

INTRODUCTION

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] is the active ingredient in several fungicide formulations used for disease control in numerous crop species (*Crop Protection Chemicals Reference*, 1993; Delp, 1987; Thomson, 1991). Benomyl is translocated within plant tissues and interferes with mitosis and microtubule formation in pathogenic fungi (Davidse, 1987). Although benomyl has been used extensively for many years, several instances of phytotoxicity have been reported (Reyes, 1975; Chase, 1985; Delp, 1987). Moye et al. (1994) recently reported that N,N'-dibutylurea (DBU) was present at concentrations ranging from 0.10 to 8.85% (w/w) in sampled formulations of benomyl. Moye et al. (1994) also reported that n-butyl isocyanate (BIC), which is eliminated by benomyl, forms DBU in water or on plant surfaces. Although other substituted ureas have been shown to be phytotoxic (Stanger and Appleby, 1972; Ridley and Horton, 1984; Zbozinek, 1984), no studies have been conducted to ascertain the effects, if any, of DBU on plants.

The recent report concerning the presence of DBU in benomyl formulations and the lack of any data on possible effects of DBU on plants prompted the study reported herein. The objectives of this study were to evaluate the effects of DBU on (1) plant growth, (2) cellular integrity, photosynthesis, and respiration, and (3) chloroplast ultrastructure.

MATERIALS AND METHODS

Dose–Response Studies. DBU (Janssen Chimica, Beerse, Belgium, 99% purity) was applied to cucumber (*Cucumis sativus* L. var. Poinsett 76) and corn (*Zea mays* L. var. Hughes 5302) at two different growth stages: at the time of seeding and to the roots of 10-day-old plants. Cucumber and corn were seeded (4 seeds/pot) into pots with a surface area of 10 cm^2 filled with acid-washed silica sand and thinned to 2 plants/pot after emergence. Plants were subirrigated with half-strength Hoagland's solution throughout the study. DBU was applied at rates of 1.4 (35 μg/mL), 2.8 (70 μg/mL), 5.6 (140 μg/mL), 11.2 (280 μg/mL), and 22.4 kg ha^{-1} (560 μg/mL) using 40 mL of solution/pot. To thoroughly saturate the entire volume of sand, 40 mL of solution was applied to the sand surface. Plants were maintained in a greenhouse under the following environmental conditions: 15 h light/9 h dark photoperiod, average light intensity at noon of 1200 μmol m^{-2} s^{-1}, and average mean temperature of 29 ● 4 °C. Germination and emergence were determined at the time of thinning. Plant height and root and shoot biomass (dry weight) were determined 3 weeks after treatment.

Ion Leakage Studies. Ion leakage from hydrilla (*Hydrilla verticillata* L. f. Royle) shoot tips exposed to 1 mM DBU or diuron [N'-(3,4-dichlorophenyl)-N,N-dimethylurea] was measured (μmho/cm^{-1}) using a conductivity bridge [Yellow Springs Instrument Co., Inc., Yellow Springs, OH (Model 31)]. Deionized water was used as a control. All treatments were evaluated under both light (240 μmol m^{-2} s^{-1}) and dark conditions for periods of 24 or 48 h at 26 ± 2 °C. Hydrilla tissue used for these studies consisted of 2-cm-long apical shoots (100 mg) obtained from greenhouse-grown plants. Following the 24- and 48-h measurements, each sample was frozen and thawed twice to obtain the maximum possible conductivity. Conductivity was initially measured immediately after treatment. Ion leakage was expressed as the percent of the total possible conductivity (%C) using the formula

$$\%C = \frac{C_m - C_i}{C_t - C_i} \times 100$$

where C_m is the conductivity measured after 24 or 48 h of treatment, C_i is the initial conductivity measured, and C_t is the total conductivity measured after freezing and thawing.

Fluorescence Studies. Cucumber cotyledon disks (1-cm diameter) from 10-day-old plants were vacuum infiltrated for 70 s in 1 mM solutions of DBU, diuron, or deionized water and were equilibrated in darkness for 20 min. Chlorophyll a fluorescence was then measured for 50 s (Model SF-20 plant productivity fluorometer, Richard Brancker Research Ltd., Ottawa, Canada), and the key parameters of the fluorescence induction curve, initial (I), peak (P), and terminal (T) fluorescence, were recorded. Preliminary experiments were conducted to determine the amount of time (50 s) required to establish steady-state fluorescence (i.e., terminal fluorescence) after initial induction. An excitation wavelength of 670 nm was used, and fluorescence was measured at 710 nm and above. Fluorescence measurements

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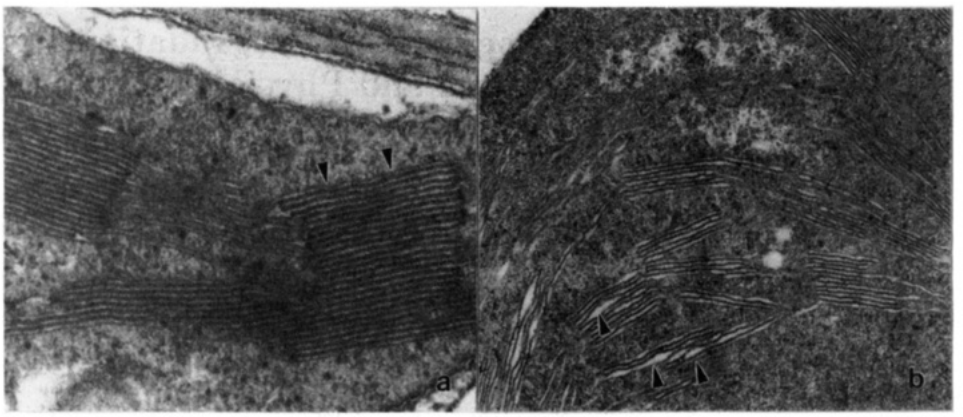

Figure 1. Transmission electron micrographs of cucumber chloroplasts: (a) control [note regular parallel spacing of granallamellae (arrows), ×72000; (b) 10 days after treatment with 5.6 kg ha^{-1} DBU [note disruption of orderly parallel arrangement of granallamellae (arrows), ×37500]. (Figure is reproduced here at 70% of the original.)

were taken 1, 2, and 6 h after treatment. Fluorescence (F: peak-to-terminal ratio) was expressed as a ratio using the following formula:

$$F = P - I/T - I$$

Photosynthesis. The effect of 1 mM DBU and diuron on photosynthesis was evaluated by measuring photosynthetic oxygen evolution (Orion Model 820 oxygen meter, Orion Research Inc., Boston, MA) from 2-cm hydrilla shoot tips (100 mg). Hydrilla shoot tips were incubated in sealed glass vials filled with 18 mL of solution containing either of the two compounds and 2 mM $NaHCO_3$ or 2 mM $NaHCO_3$ alone. Sodium bicarbonate was provided as a carbon source for photosynthesis. The treated tissue was maintained in a shaking water bath at 28 °C and exposed to 240 μmol m^{-2} s^{-1} of light. The net amount of oxygen evolved (micromoles) over a 10-min period was measured after 0.17, 1.0, 2.0, and 24.0 h of exposure.

Dark Respiration. Dark respiration was estimated by measuring the oxygen consumed by 2-cm hydrilla shoot tips (100 mg) over a 10-minute period after 2, 4, 24, and 48 h of exposure to 1 mM DBU or diuron. Hydrilla shoot tips were incubated in the dark at 28 °C in sealed glass vials with 18 mL of solution containing the two compounds or deionized water.

Ultrastructure. Small segments from the margins of treated (with 5.6 kg ha^{-1} DBU) and untreated cucumber secondary leaves (plants maintained and treated as described previously) were excised at various times after treatment and immediately immersed in cacodylate buffer containing 2.5% glutaraldehyde, 2.5% formaldehyde, and a trace of Aerosol OT to aid wetting. Samples were immediately placed under vacuum for 1–2 min to remove air from the spongy mesophyll layers. Fixation continued at atmospheric pressure for 2–4 h. After washing, samples were fixed in buffered (potassium phosphate) 1% OsO_4 for 1 h, dehydrated in ethanol, and then embedded in low-viscosity epoxy resin. Thin sections were cut and affixed to grids. Multiple samples were examined, and the cellular anatomy of each sample was evaluated. The micrographs (Zeiss EM-10, Carl Zeiss Inc., Oberkochen, Germany) presented (Figure 1) are representative of both control and treated leaves.

Statistics. Experiments for each study were conducted twice with treatments replicated at least three times. A completely randomized block design was used for all experiments. Data from each study were evaluated using analysis of variance and treatment means separated from the control using Dunnett's t-test ($P < 0.05$) (Helwig and Council, 1985). Average mean values from both experiments within each study (no experiment by treatment interaction) are presented with standard errors.

RESULTS

DBU did not affect the germination or emergence of either corn or cucumber at concentrations up to 22.4 kg ha^{-1} (data not shown). Corn was unaffected by all rates of DBU applied at seeding or to the roots of established plants (data not shown).

Table 1. Effects of Root-Applied DBU on the Growth of 10-Day-Old Cucumber Plants[a]

rate (kg ha^{-1})	biomass (g)		shoot height (cm)
	root	shoot	
0.0	0.40 ± 0.1	0.95 ± 0.28	11.67 ± 3.86
1.4	0.28 ± 0.04	0.64 ± 0.17	11.37 ± 3.86
2.8	0.15 ± 0.03*	0.39 ± 0.39*	9.67 ± 2.40
5.6	0.05 ± 0.01*	0.21 ± 0.05*	7.17 ± 2.36
11.2	0.04 ± 0.02*	0.14 ± 0.05*	3.67 ± 1.41*
22.4	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*

[a] Mean values are followed by standard errors. Mean values followed by an asterisk are significantly different from the control (Dunnett's t-test, $P < 0.05$) within a growth parameter.

Table 2. Effects of DBU on Cucumber Growth When Applied at the Time of Planting[a]

rate (kg ha^{-1})	biomass (g)		shoot height (cm)
	root	shoot	
0.0	0.13 ± 0.02	0.43 ± 0.04	8.00 ± 0.63
1.4	0.15 ± 0.04	0.38 ± 0.05	8.83 ± 0.60
2.8	0.05 ± 0.02*	0.16 ± 0.04*	6.83 ± 1.04
5.6	0.06 ± 0.02*	0.15 ± 0.08*	4.33 ± 1.40*
11.2	0.02 ± 0.00*	0.03 ± 0.02*	1.33 ± 0.88*
22.4	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*

[a] Mean values are followed by standard errors. Mean values followed by an asterisk are significantly different from the control (Dunnett's t-test, $P < 0.05$) within a growth parameter.

DBU reduced root and shoot growth of cucumber when applied to the root zone of established plants (Table 1). Root biomass was reduced by 63, 88, 90, and 100% at 2.8, 5.6, 11.2, and 22.4 kg ha^{-1}, respectively. Shoot growth was inhibited by 59, 78, 85, and 100% at 2.8, 5.6, 11.2, and 22.4 kg ha^{-1}, respectively. Cucumber shoot height was not affected by DBU when applied at 5.6 kg ha^{-1} or less but was inhibited by 69 and 100% at 11.2 and 22.4 kg ha^{-1}, respectively.

Both root growth and shoot growth of cucumber were affected by DBU applied at seeding (Table 2). DBU reduced cucumber root biomass by 62, 54, 85, and 100% at rates of 2.8, 5.6, 11.2, and 22.4 kg ha^{-1}, respectively. Shoot biomass was reduced 63, 65, 93, and 100% at rates of 2.8, 5.6, 11.2, and 22.4 kg ha^{-1}, respectively, and shoot height was reduced by 46, 83, and 100% at 5.6, 11.2, and 22.4 kg ha^{-1}, respectively.

Neither DBU nor diuron caused ion leakage from treated hydrilla shoot tips (data not shown). DBU and diuron affected chlorophyll a fluorescence by hydrilla, reducing the peak-to-terminal fluorescence ratio (Table 3). DBU reduced this ratio by 48, 52, and 48% after 1, 2, and 6 h of exposure, respectively, similar to that observed with

Table 3. Effects of DBU and Diuron (1 mM) on Chlorophyll *a* Fluorescence by Hydrilla[a]

	peak:terminal ratio		
treatment	1 h	2 h	6 h
none (control)	2.3 ± 0.3	2.5 ± 0.2	2.1 ± 0.2
DBU	1.2 ± 0.3*	1.2 ± 0.03*	1.1 ± 0.02*
diuron	1.3 ± 0.1*	1.4 ± 0.1*	1.0 ± 0.9*

[a] Mean peak:terminal ratio values are followed by standard errors. Mean values followed by an asterisk are significantly different from the control (Dunnett's *t*-test, $P < 0.05$) within a time period.

Table 4. Effect of DBU and Diuron (1 mM) on Photosynthetic Oxygen Evolution over 10 min by 100 mg of Hydrilla Shoot Tips[a]

	O_2 (μmol)			
treatment	0.17 h	1.0 h	2.0 h	24 h
control	0.96 ± 0.08	1.30 ± 0.15	1.08 ± 0.13	1.12 ± 0.18
DBU	0.14 ± 0.10*	0.00*	0.00*	0.00*
diuron	0.00*	0.00*	0.00*	0.00*

[a] Mean values are followed by standard errors. Mean values followed by an asterisk are significantly different from the control (Dunnett's *t*-test, $P < 0.05$) within a time period.

diuron. DBU and diuron inhibited photosynthetic O_2 evolution (Table 4). DBU at 1 mM reduced O_2 evolution by 87, 100, 100, and 100% following 0.17, 1, 2, and 24 h of exposure, while diuron completely inhibited O_2 evolution at all evaluation times. Neither of the two compounds affected dark respiration by hydrilla shoot tips over 48 h (data not shown).

DBU at 5.6 kg ha^{-1} caused several abnormalities in the mesophyll cells of treated cucumber plants (Figure 1). DBU caused dilation of chloroplast granal and stromal lamellae of treated leaf tissue. In addition, the granal lamellae were disorganized, with irregular stacking. These changes occurred in both green and chlorotic regions of treated plants.

DISCUSSION

DBU produced a dose–response relationship characteristic of phytotoxic compounds when applied either to the root zone of emerged cucumber plants or to sand planted with cucumber seed prior to germination. Although DBU did not affect germination or emergence of cucumber or corn, leaf margins of 10-day-old cucumber plants became chlorotic within 8 h of treatment. Chlorosis was rapidly followed by necrosis. DBU did not affect corn regardless of rate or time of application. The symptoms observed on cucumber plants treated with DBU were similar to those reported for diuron—a commercial substituted urea herbicide that inhibits photosynthesis (Stanger and Appleby, 1972). Symptoms caused by compounds with this mode of action include rapid chlorosis followed by necrosis when applied to the roots of rapidly transpiring plants. Generally, substituted urea herbicides do not inhibit germination or emergence, because they inhibit photosynthesis. The similarities in symptomology of treated plants and structure of DBU and diuron provided the impetus for the physiological studies reported herein which confirmed the mode of action of DBU.

Hydrilla was used as a model system for the physiological studies for two reasons. First, hydrilla is an aquatic macrophyte adapted to water and would not be adversely affected by submergence in solutions. Second, hydrilla has leaves two-cells thick with no cuticle, which allows rapid penetration of tested compounds (Yeo et al., 1984).

Neither DBU nor diuron had any effect on ion leakage. This observation might be misleading unless properly interpreted. Diuron is a herbicide known to block photosynthetic electron transport (Stanger and Appleby, 1972) and ultimately cause cellular disruption (Ridley and Horton, 1984). However, diuron does not cause a rapid increase in ion leakage under low light conditions. Without sufficient light intensity, the effects of electron transport inhibitors are slowed and dramatically reduced.

The similar symptomology between DBU and herbicides known to inhibit photosynthesis by blocking electron transport led to a series of studies on the possible effects of DBU on photosynthesis. Diuron was included in these studies for comparative purposes. Photosynthetic O_2 evolution was affected by DBU in a manner characteristic of compounds known to block photosynthetic electron transport (Voss et al., 1984). DBU also reduced the ratio of peak-to-terminal chlorophyll *a* fluorescence, similar to that observed for diuron.

Other workers have shown that substituted urea herbicides bind to protein D-1 of the light-harvesting complex of photosystem II, located in the granal lamellae (Trebst, 1984; Bowyer et al., 1990). In our studies the granal membranes of cucumbers were dilated and disorganized after treatment with DBU, suggesting a similar mode of action to substituted urea herbicides. The chloroplast disruption observed was indicative of severe damage to the photosynthetic apparatus. This is also consistent with the effects of other herbicides that influence photosynthesis (Moreland, 1980).

The objectives of these studies were to determine the phytotoxicity and physiological effects of DBU. On the basis of these results, DBU appears to affect cucumber growth by inhibiting photosynthetic electron transport. The phytotoxicity of DBU was evaluated using corn and cucumber grown in sand culture. Cucumber, a sensitive plant species, and an inert growth medium were used to minimize any confounding factors and therefore optimize the effects of DBU on plant growth. Therefore, the use of a somewhat artificial system was appropriate. Corn was included to compare the responses of two vastly different plant species to DBU. Prudence should be used when drawing generalizations from these data because the phytotoxic properties of DBU were determined using an artificial growth medium and a sensitive plant species. In addition, DBU did not affect corn. On the basis of the findings reported herein and those of Moye et al. (1994), determination of the practical implications of DBU phytotoxicity as related to use of the fungicide benomyl is warranted.

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LITERATURE CITED

Bowyer, S.; Hilton, M.; Whitelegge, S.; Sewess, P. Molecular modeling studies on the binding of phenylurea inhibitors to the D1 protein of photosystem II. *Z. Naturforsch.* **1990**, *45C*, 379–87.

Chase, A. R. Phytotoxicity of fungicides and bactericides used on foliage plants—1985. *Foliage Dig.* **1985**, *8*, 2–3.

Crop Protection Chemicals Reference; Chemical and Pharmaceutical Corp., OVP, Paris; Wiley: New York, 1993; p 1812.

Davidse, L. C. Biochemical aspects of benzimidazole fungicides—Action and Resistance. In *Modern Selective Fungicides—Properties, Applications, Methods of Action*; Lyr, H., Ed.; Longman: New York, 1987; p 245–257.

Delp, C. J. Benzimidazole and related fungicides. In *Modern Selective Fungicides—Properties, Applications, Methods of Action*; Lyr, H., Ed.; Longman: New York, 1987; p 233–244.

Helwig, J. T.; Council, K. A. *Statistical Analysis Systems User's Guide*; SAS Institute: Cary, NC, 1985; p 584.

Moreland, D. E. Mechanisms of action of herbicides. *Annu. Rev. Plant Physiol.* **1980**, *31*, 597–638.

Moye, H. A.; Shilling, D. G.; Aldrich, H. C.; Gander, J. E.; Toth, J. P.; Brey, W. S.; Tolson, J. K. Formation of *N,N'*-Dibutylurea from *n*-butyl isocyanate, a degradation product of benomyl. 1. Formation in benlate formulations and on plants. *J. Agric. Food Chem.* **1994**, preceding paper in this issue.

Reyes, A. A. Phytotoxicity of benomyl to crucifers. *Phytopathology* **1975**, *65*, 535–539.

Ridley, S. M.; Horton, P. DCMU-induced fluorescence changes and photodestruction of pigments associated with an inhibition of photosystem I cyclic electron flow. *Z. Naturforsch.* **1984**, *39C*, 351–353.

Stanger, C. E.; Appleby, A. P. A proposed mechanism for diuron-induced phytotoxicity. *Weed Sci.* **1972**, *20*, 357–363.

Thomson, W. T. *Agricultural Chemicals, Book IV—Fungicides*. Thomson Publications: Fresno, CA, 1991; p 198.

Trebst, A. Structure activity correlation of herbicides affecting plastoquinone reduction by photosystem II. *Z. Naturforsch.* **1984**, *39C*, 405–411.

Voss, M.; Renger, G.; Graber, P. Measurements of penetration and detoxification of PS II herbicides in whole leaves by a fluorometric method. *Z. Naturforsch.* **1984**, *39C*, 359–361.

Yeo, R. R.; Falk, R. H.; Thurston, J. R. The morphology of hydrilla. *J. Aquat. Plant Manage.* **1984**, *22*, 1–17.

Zbozinek, J. V. Environmental transformations of DPA, SOPP, Benomyl and TBZ. *Residue Rev.* **1984**, *92*, 114–155.

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