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Copper Compounds Influence in Vitro Rooting of Birch Microcuttings

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Abstract. **The effects of woody plant medium (WPM) with various formulations and concentrations of Cu^{+2} on in vitro rooting and subsequent shoot growth of microcuttings of a *Betu pubescens* × *papyrzfera* (birch) clone were monitored for 28 days. Adventitious root initiation and elongation were reduced in magnitude and slowed in development by moderate to high Cu (as $CuSO_4 \cdot 5H_2O$) concentrations, with near zero root regeneration occurring at 157 µM Cu. Shoot growth was also inhibited by 157 µM Cu as cupric sulfate. Copper-toxicity symptoms (senescent leaves, necrotic stems, and bulbous and stunted roots) were significantly increased by moderate to high levels (≥ 79 µM) of Cu as cupric sulfate. Microcuttings responded differently to Cu^{+2} applied as cupric chloride ($CuCl_2 \cdot 2H_2O$). Root initiation, root elongation, and root branching were increased by moderate concentrations of Cu as cupric chloride. Shoot growth was slightly stimulated by cupric chloride at moderate levels. No significant increase in Cu-toxicity symptoms was observed at concentrations up to 157 µM Cu as cupric chloride. Cupric acetate [$Cu(CH_3COO);H_2O$] and cupric carbonate [$CuCO_3 \cdot Cu(OH)_2$] produced more severe Cu-toxicity symptoms than cupric sulfate. Root regeneration and shoot growth were inhibited and increased Cu-toxicity symptoms were apparent even with low concentrations (39 µM) of Cu as cupric acetate or cupric carbonate. There was little or no effect on root regeneration when the Cu^{+2} in cupric sulfate was replaced by different cations, i.e., magnesium sulfate ($MgSO_4 \cdot 7H_2O$), calcium sulfate ($CaSO_4 \cdot 2H_2O$), and sulfuric acid (H_2SO_4), a result suggesting that the observed responses could be attributed to the Cu^{+2} concentration. Changes in media pH did not correspond to Cu-toxicity symptoms or alterations in root or shoot growth by the Cu compounds.**

The concentrations of Cu necessary for plant growth are very low. Neales (1959) obtained normal growth of excised flax *(Linum usitatissimum* L.) roots on a medium containing 0.005 mg Cu/liter (≈ 0.08 µM). Brandenburg, in Stiles (1961), reported normal vegetative growth of *Avena sativa* L. in solution cultures containing 0.020 mg Cu/liter (≈ 0.3 µM) and normal reproductive growth with 0.050 mg Cu/liter (≈ 0.8 µM). Ozolina (1988) reported that, in solution culture, growth responses of cereal roots to cupric sulfate were concentration dependent; low concentrations stimulated root elongation, while higher concentrations changed root morphology and eventually inhibited root growth. Common tissue-culture rooting media such as Murashige and Skoog (Murashige and Skoog, 1962) and woody plant medium (WPM) (Lloyd and McCown, 1980) contain 0.064 mg Cu/liter (1.007 µM) as cupric sulfate. The effects of alternative formulations and concentrations of Cu^{+2} on in vitro rooting of woody plant microcuttings are unknown.

The objectives of this experiment were to 1) develop a dose response curve for Cu^{+2} as cupric sulfate with respect to rooting *Betula pubescens* × *papyrifera* microcuttings in WPM; 2) determine if the dose response curve of microcutting root regeneration to increased Cu levels differed among various formulations of Cu^{+2}; and 3) determine whether concentration-dependent root growth responses to cupric sulfate were attributable to the sulfate anion.

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Materials and Methods

Copper formulations (Expt. 1). This experiment was designed to determine whether alternative formulations of Cu^{+2}, i.e., cupric chloride, cupric acetate, and cupric carbonate, would induce dose response curves similar to that of cupric sulfate. Microcuttings (2.0 cm) were taken from proliferating birch shoot cultures (Mount Alto Pennsylvania State Forestry Nursery clone 247, an easily rooted clone) maintained on WPM salts and vitamins with 11.1 µM benzyladenine (BA), 7 g Difco Bacto agar/liter, and 30 g sucrose/liter. Proliferating shoot cultures were subculture to fresh medium every 3 weeks. Microcuttings were taken from actively growing single stems with four to seven partially to fully expanded leaves and no visible signs of root development. The basal 1 cm of each microcutting was inserted into 12.5 ml of the rooting medium in 25 × 150-mm clear pyrex culture tubes. The tubes were sealed with clear plastic snap-on caps. The rooting medium was the same as for shoot cultures, except BA was omitted and Cu (as cupric sulfate, cupric chloride, cupric acetate, or cupric carbonate) was added. After Cu was added, the medium pH was adjusted to 5.3 with 1 N HCl, 1 N NaOH, or both, then autoclave at 121 C for 20 min.

A preliminary experiment (data not presented) indicated that the rooting response range of birch microcuttings to cupric sulfate was 39 to 157 µM Cu. Thus, treatments were a control WPM (1.0 µM Cu as cupric sulfate) and WPM with added Cu in the forms of cupric sulfate, cupric chloride, cupric acetate, or cupric carbonate to produce media containing 39,79, 118, and 157 µM Cu for each of the four formulations. Two blocks of 10 cuttings per formulation and concentration combination were placed in the culture room in a randomized complete-block design. Each experiment in this study was conducted twice. The culture room was maintained at 23 ± 2 C. Illumination was for 16 h/day at 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation from cool-white fluorescent lamps.

The total number of roots and the number of roots ≥ 0.5 cm long were recorded at 0, 7, 14, 21, and 28 days after the microcuttings were inserted into the culture tubes. After 28 days, cuttings were

harvested and total length of primary adventitious roots (excluding second-and higher-order roots), total shoot length, and presence of root branching (one or more second-order roots) were recorded, Measures of Cu toxicity included the presence of chlorotic, necrotic, or abscissed leaves and the presence of necrotic lesions on the stems.

Medium pH (Expt. 2). Changes in medium pH were monitored using a combination pH electrode standardized to 65C so that medium pH could be monitored in the presence of molten agar. Media were prepared as in Expt. 1 for each concentration of cupric sulfate, cupric chloride, cupric acetate, and cupric carbonate. As in the previous experiment, all media were brought to pH 5.3 before being autoclave. Medium pH was determined on four replications of each treatment combination immediately after autoclaving and 7 and 28 days thereafter.

Sulfate formulations (Expt. 3). This experiment was designed to determine whether the accompanying sulfate anion affected plant responses. Equimolar amounts of sulfate in the form of cupric sulfate, calcium sulfate, magnesium sulfate, and sulfuric acid were added to WPM to produce media containing concentrations of sulfate equal to those added for the cupric sulfate treatments in Expt. 1, i.e., 39,79,118, and 157 μM SO_4. Conditions were as described in Expt. 1. The three experiments were analyzed separately using factorial analysis of variance, polynomial regression, and full quadratic response surface modeling (SAS Institute, 1988).

Results and Discussion

There were significant ($P \leq 0.05$) interactions among the Cu formulations, Cu concentrations, and rooting times for the number of roots and number of roots 20.5 cm long. Significant ($P \leq 0.05$) interactions among the Cu formulations and Cu concentrations were found for root length, the number of cuttings with branched roots, the number of cuttings with senescent leaves, the number of cuttings with necrotic stems, and shoot length. Although treatment effects on shoot length were statistically significant ($P \leq 0.05$), differences among treatments were ≤ 0.5 cm; thus, shoot length data are not presented.

Copper formulations. Similar growth and toxicity responses were observed in Expts. 1 and 3 when Cu was supplied as cupric sulfate (Figs. 1–5, Table 1). Adventitious root initiation, as measured by the number of roots regenerated per cutting (Fig. 1A), was reduced in magnitude and developed more slowly with Cu concentrations exceeding ≈ 39 μM Cu as cupric sulfate. Root elongation rates, as measured by root length per cutting (Fig. 2A), were slowed by increased cupric sulfate concentrations and approached zero at 157 μM Cu as cupric sulfate. The number of cuttings with branched roots was reduced slightly by low to moderate concentrations of Cu as cupric sulfate, then decreasing rapidly to near zero at higher concentrations (Fig. 2B). Shoot length was slightly reduced (by 0.25 cm) by Cu as cupric sulfate, but only at the highest

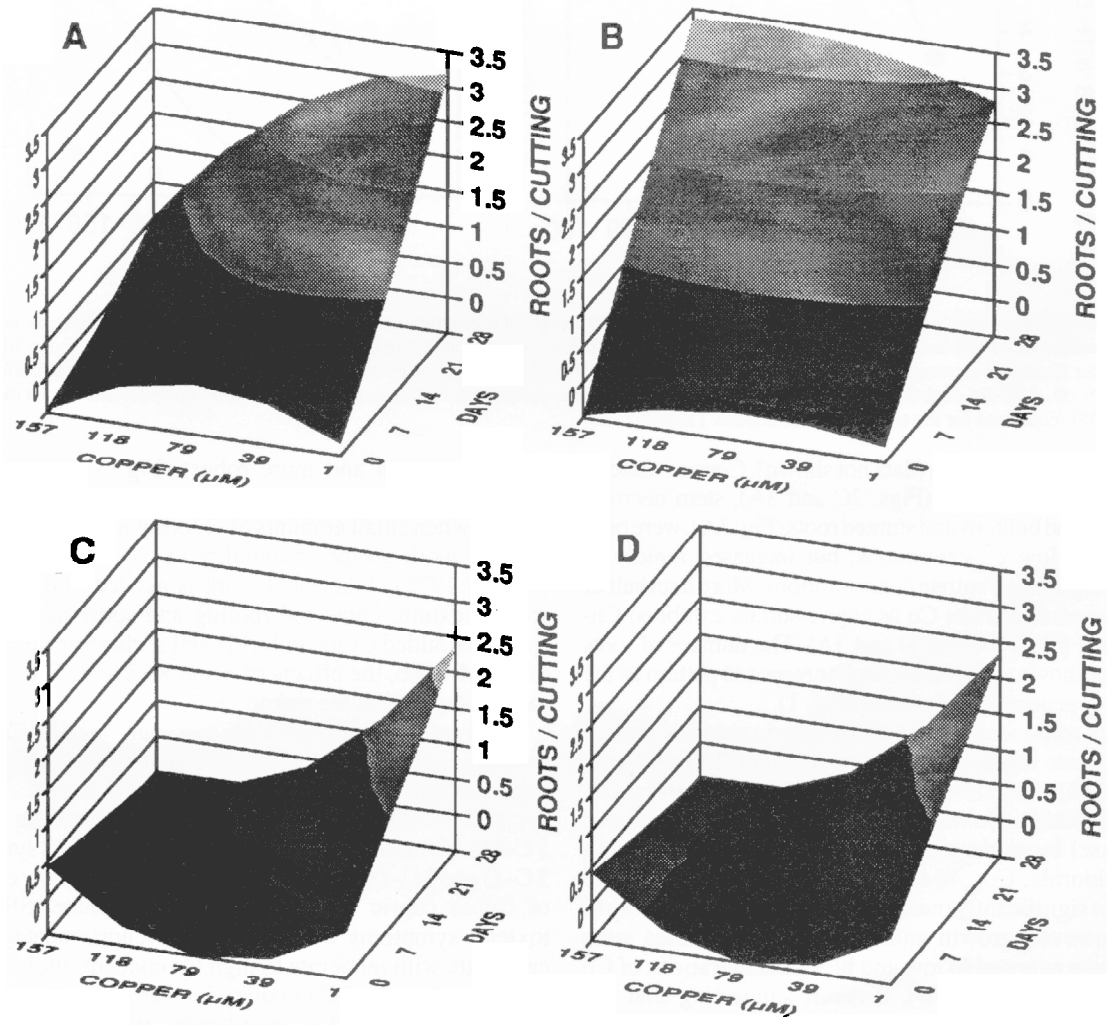

Fig. 1. Full quadratic response surfaces for the number of primary adventitious roots regenerated per birch cutting during 28 days of exposure to woody plant medium containing increased Cu concentrations as cupric sulfate **(A),** cupric chloride **(B),** cupric acetate **(C),** and cupric carbonate **(D).** Response surface equations are presented in Table 1.

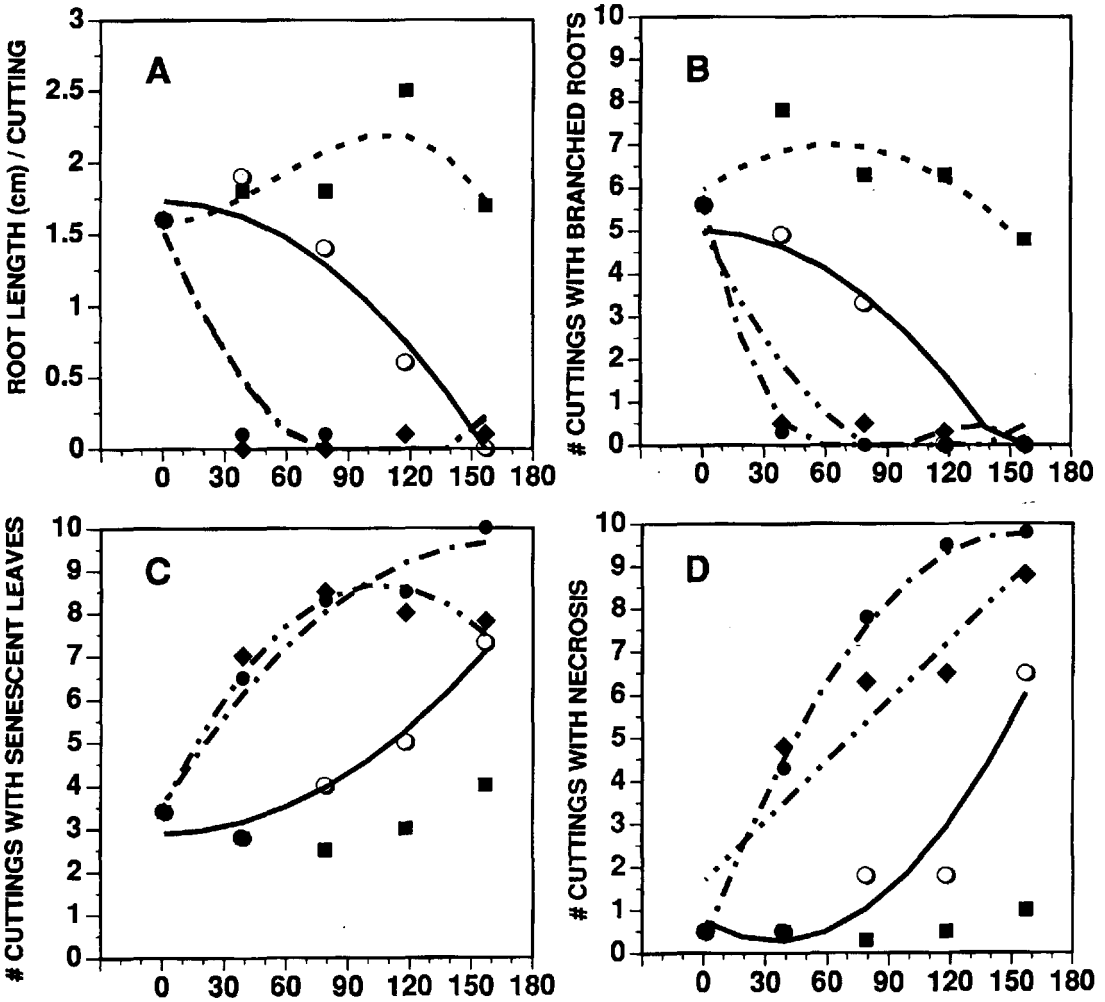

Fig. 2. Observed means (symbols) and polynomial regression curves (lines) for total length of regenerated primary lateral roots per birch cutting **(A),** the number of cuttings per block (of 10 possible) with branched roots **(B),** the number of cuttings per block with senescent leaves **(C),** and the number of cuttings per block with necrotic lesions on the stems **(D)** after 28 days of exposure to woody plant medium containing 1.0 to 157 µM Cu in the form of cupric sulfate (○ , solid lines), cupric chloride (■ , dashed lines), cupric acetate (● , dash-dot-dash lines), or cupric carbonate (◆ , dash-dot-dot-dash lines), Polynomial regression curves are presented only if the equations were significant ($P \leq 0.05$). Equations for the curves are-presented in Table 1.

concentration tested— 157 µMCu(data not shown). Copper-toxicity symptoms, i.e., leaf senescence (Figs. 2C and 3A), stem necrosis (Figs. 2D and 3A), and bulbous and stunted roots (Fig. 3A), were only slightly affected at low concentrations, but increased rapidly in occurrence with high cupric sulfate concentrations. More than half of the cuttings exposed to 157 µM Cu as cupric sulfate exhibited Cu-toxicity symptoms (Figs. 2 C and D and 3A). The number of roots 20.5 cm (data not shown) followed a similar response pattern as the number of roots regenerated per cutting (Fig, 1).

Microcuttings responded differently to cupric chloride than to cupric sulfate. Root regeneration (Figs. 1 B and 2A) and root branching (Fig. 2B) were stimulated by moderate concentrations of Cu as cupric chloride. Shoot elongation was slightly stimulated (0.25 cm increase) by moderate concentrations of Cu (79 to 118 µM) as cupric chloride. Copper-toxicity symptoms (Figs. 2 C-D and 3B) were not significantly increased with cupric chloride up to 157 µM Cu. However, growth and Cu-toxicity responses were similar for cuttings exposed to low and high concentrations of Cu as cupric chloride (Fig. 2 A–D), a result suggesting that the concentrations of cupric chloride evoking promotive effects had been exceeded. In general, microcuttings treated with low to moderate levels of cupric chloride (39 to 118 µM Cu) appeared to be darker green and more robust (Fig. 3) than those of other treatments or the control. Significant increases in root and shoot growth when small amounts of chlorine were added were reported in early work on the essentiality of chlorine as a micronutrient (Gauch, 1972). Additional work is needed to determine if other species exhibit enhanced rooting and subsequent growth in response to added cupric chloride and if chlorine levels are optimal in WPM. Also, the effects of using HCl to equilibrate pH during media preparation are unknown.

Root and shoot growth were more inhibited by Cu added to the medium in the form of cupric acetate or cupric carbonate than as cupric sulfate or cupric chloride. Root regeneration (Figs. 1 C–D, 2A, and 3 C–D), shoot growth, and root branching (Figs. 2B and 3 C–D) were virtually eliminated and Cu-toxicity symptoms (Figs. 2 C–D and 3 C–D) drastically increased even at low concentrations of Cu as cupric acetate or cupric carbonate. Slightly greater toxicity symptoms were induced by cupric acetate than cupric carbonate with moderate to high Cu concentrations (Fig. 2 C–D). Thus, the degree of Cu toxicity in vitro is affected by the accompanying anion, as it is in solution culture. Benchley, in Stiles (1961), reported that growth of *Hordeum vulgare* L. was stopped in a solution of 1 mg Cu/liter (≈ 16 µM) supplied as cupric sulfate

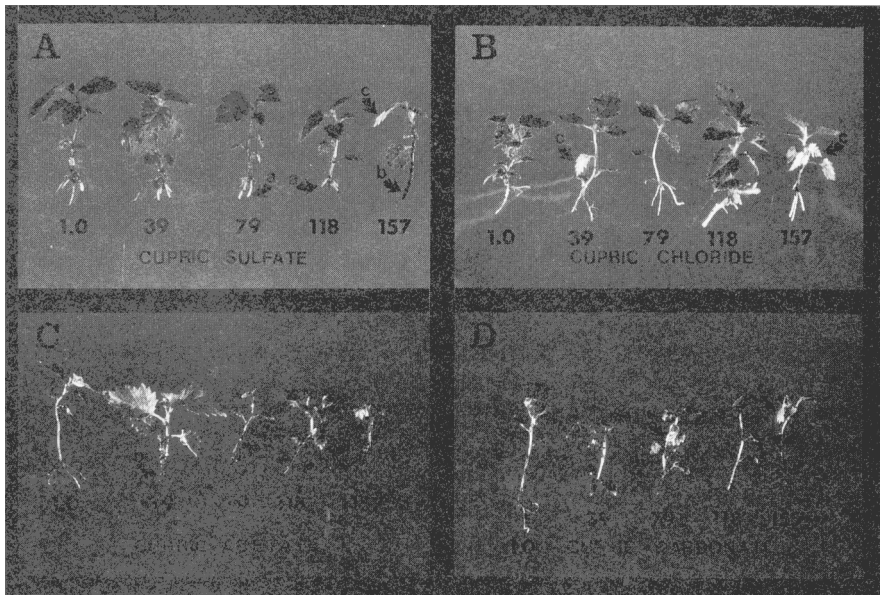

Fig. 3. Birch microcuttings after 28 days of exposure to woody plant medium containing 1.0 (control), 39,79,118, or 157 μM in the form of cupric sulfate **(A),** cupric chloride **(B),** cupric acetate **(C),** or cupric carbonate **(D)**. Black arrows indicate possible Cu-toxicity symptoms such as thickened bulbous roots **(a),** necrotic stem tissue **(b),** and senescent leaves **(c).**

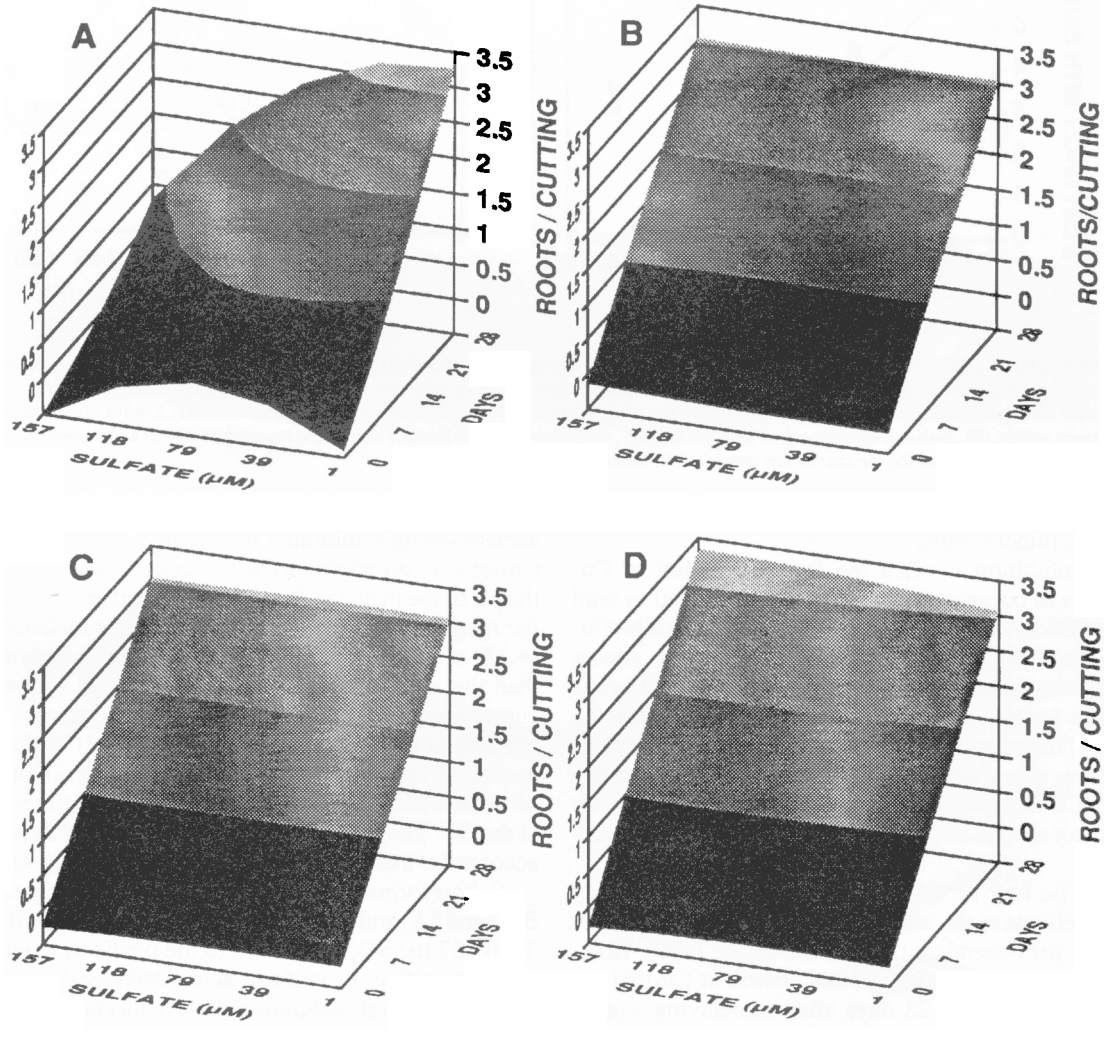

Fig. 4. Full quadratic response surfaces for the number of primary adventitious roots regenerated per birch cutting during 28 days of exposure to woody plant medium containing increased SO_4 concentrations as cupric sulfate **(A),** magnesium sulfate **(B),** calcium sulfate **(C),** and sulfuric acid **(D).** Response surface equations are presented in Table 1.

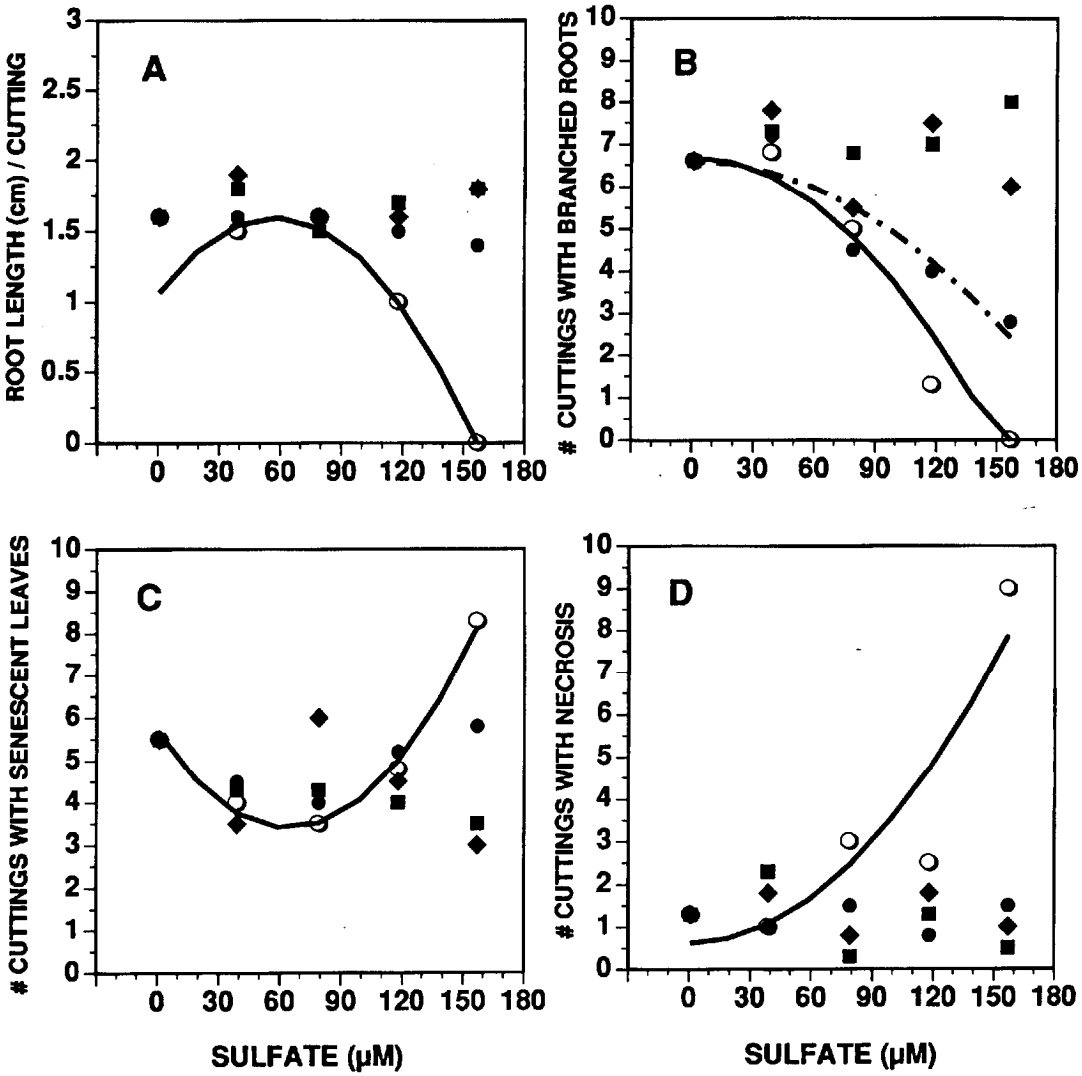

Fig. 5. Observed means (symbols) and polynomial regression curves (lines) for total length of regenerated primary lateral roots per birch cutting **(A),** the number of cuttings per block (of 10 possible) with branched roots **(B),** the number of cuttings per block with senescent leaves **(C),** and the number of cuttings per block with necrotic lesions on the stems **(D)** after 28 days of exposure to woody plant medium containing 1.0 to 157 μM SO_4 in the form of cupric sulfate (○ , solid lines), magnesium sulfate (■ , dashed lines), calcium sulfate (● , dash-dot-dash lines), or sulfuric acid (◆ , dash-dot-dot-dash lines). Polynomial regression curves are presented only if the equations were significant ($P \leq 0.05$). Equations for the curves are presented in Table 1.

alone, but not in a solution containing 4 mg Cu/liter (≈ 63 μM) as cupric sulfate plus mineral salts.

Root growth inhibition is often the first expression of Cu-toxicity symptoms to occur, probably because roots tend to bind Cu (Mengel and Kirkby, 1982), while leaves and stems exhibit Cu-toxicity symptoms at higher concentrations. In this study, shoots exhibited Cu-toxicity symptoms at exposure to the same or lower Cu concentrations as those that inhibited root growth (Figs. 1-3). Direct exposure of the stems to the Cu compounds in this study (vs. in-solution cultures where roots are often the only tissues directly exposed to the Cu compounds) may explain why Cu-toxicity symptoms were not always manifested in the roots before appearing in the shoots.

Medium pH. The four formulations of Cu used in this study elicited different effects at the same Cu^{+2} concentrations. Copper uptake is strongly pH dependent (Allan and Jarrell, 1989). However, no consistent patterns among pH and growth or Cu-toxicity symptoms were apparent. By 28 days after autoclaving, cupric chloride treatments had the highest pH (4.61 at 39 μM Cu) and the lowest pH (4.19 at 157 μM Cu) of any treatment. Yet no significant increases in Cu-toxicity symptoms were noted over this pH range for these treatments (Fig. 2). While cupric carbonate and cupric acetate strongly inhibited root growth and induced considerable Cu-toxicity symptoms at Cu concentrations ≥ 39 μM (Figs. 1 and 2), the pH of the media of the two compounds did not correspond over the range of Cu concentrations tested. For instance, while the pH (4.33) of medium with 79 μM Cu as cupric carbonate was lower than that of many other treatments, the pH of media containing cupric acetate was closer to that of either cupric sulfate or cupric chloride at most Cu concentrations. The pH of the various media declined over the 28 days (from pH 5.3 before autoclaving), but the pH values of all media were within 0.5 or fewer units of each other at day 28. These results suggest that changes in pH alone did not account for treatment effects on growth or Cu-toxicity symptoms.

Sulfate formulations. Little change in root regeneration (Figs. 4 B–D and 5A) and shoot growth (data not presented) occurred when 39 to 157 μM SO_4 was added to the media as magnesium sulfate, calcium sulfate, or sulfuric acid. Decreased root branching occurred with high calcium sulfate concentrations (Fig. 5B), although the effects were not as severe as with cupric sulfate. Copper-toxicity symptoms were not induced by increased concentrations of magnesium sulfate, calcium sulfate, or sulfuric acid

Table 1. Formulas for quadratic response surfaces and polynomial regression equations used in Figs. 1, 2,4, and 5. In equations, C = concentration (in μM) of Cu (Figs. 1 and 2) or sulfate (Figs. 4 and 5), D = days of exposure. All equations are significant at P 0.05.

Fig.	Treatment	Equation	r^2
1A	Cupric sulfate	$\text{Roots/cutting} = -0.332 + 0.0148 \times C + 0.102 \times D - 0.0000990 \times C^2 - 0.0000434 \times C \times D + 0.000823 \times D^2$	0.60
1B	Cupric chloride	$\text{Roots/cutting} = -0.288 + 0.00564 \times C + 0.118 \times D - 0.0000427 \times C^2 + 0.000169 \times C \times D - 0.000292 \times D^2$	0.59
1C	Cupric acetate	$\text{Roots/cutting} = 0.147 - 0.0178 \times C + 0.0725 \times D + 0.000126 \times C^2 - 0.000631 \times C \times D + 0.000328 \times D^2$	0.58
1D	Cupric carbonate	$\text{Roots/cutting} = 0.151 - 0.0186 \times C + 0.0763 \times D + 0.000130 \times C^2 - 0.000613 \times C \times D + 0.000121 \times D^2$	0.57
2A	Cupric sulfate	$\text{Root length (cm)/cutting} = 1.64 + 0.00482 \times C - 0.000100 \times C^2$	0.45
	Cupric chloride	$\text{Root length (cm)/cutting} = 1.64 - 0.153 \times C + 0.000313 \times C^2 - 0.00000158 \times C^3$	0.06
	Cupric acetate	$\text{Root length (cm)/cutting} = 1.66 - 0.970 \times C + 0.000674 \times C^2 - 0.00000221 \times C^3$	0.70
	Cupric carbonate	$\text{Root length (cm)/cutting} = 1.66 - 0.989 \times C + 0.000690 \times C^2 - 0.00000225 \times C^3$	0.71
2B	Cupric sulfate	$\text{Cuttings with branched roots} = 5.00 - 0.000242 \times C^2$	0.71
	Cupric chloride	$\text{Cuttings with branched roots} = 5.93 + 0.0340 \times C - 0.000268 \times C^2$	0.29
	Cupric acetate	$\text{Cuttings with branched roots} = 5.12 - 0.111 \times C + 0.000517 \times C^2$	0.83
	Cupric carbonate	$\text{Cuttings with branched roots} = 5.10 - 0.0997 \times C + 0.000446 \times C^2$	0.80
2C	Cupric sulfate	$\text{Cuttings with senescent leaves} = 2.90 + 0.000170 \times C^2$	0.35
	Cupric acetate	$\text{Cuttings with senescent leaves} = 3.56 + 0.0752 \times C - 0.000230 \times C^2$	0.78
	Cupric carbonate	$\text{Cuttings with senescent leaves} = 3.51 + 0.0975 \times C - 0.000460 \times C^2$	0.60
2D	Cupric sulfate	$\text{Cuttings with necrotic stems} = 0.759 - 0.0273 \times C + 0.000388 \times C^2$	0.75
	Cupric acetate	$\text{Cuttings with necrotic stems} = 0.244 + 0.126 \times C - 0.000416 \times C^2$	0.88
	Cupric carbonate	$\text{Cuttings with necrotic stems} = 1.68 + 0.0466 \times C$	0.65
4A	Cupric sulfate	$\text{Roots/cutting} = -0.425 + 0.0179 \times C + 0.109 \times D - 0.000118 \times C^2 - 0.000540 \times C \times D + 0.000805 \times D^2$	0.57
4B	Magnesium sulfate	$\text{Roots/cutting} = -0.207 - 0.000340 \times C + 0.119 \times D + 0.00000545 \times C^2 - 0.0000195 \times C \times D - 0.000102 \times D^2$	0.56
4C	Calcium sulfate	$\text{Roots/cutting} = -0.188 + 0.000156 \times C + 0.111 \times D - 0.00000320 \times C^2 + 0.00000734 \times C \times D + 0.000170 \times D^2$	0.61
4D	Sulfuric acid	$\text{Roots/cutting} = -0.247 + 0.00120 \times C + 0.128 \times D - 0.00000880 \times C^2 + 0.0000707 \times D \times C - 0.000364 \times D^2$	0.58
5A	Cupric sulfate	$\text{Root length (cm)/cutting} = 1.05 + 0.0191 \times C - 0.000165 \times C^2$	0.45
5B	Cupric sulfate	$\text{Cuttings with branched roots} = 6.68 - 0.000298 \times C^2$	0.69
	Calcium sulfate	$\text{Cuttings with branched roots} = 6.58 - 0.000169 \times C^2$	0.49
5C	Cupric sulfate	$\text{Cuttings with senescent leaves} = 5.70 - 0.0714 \times C + 0.000554 \times C^2$	0.57
5D	Cupric sulfate	$\text{Cuttings with necrotic stems} = 0.622 + 0.000294 \times C^2$	0.71

(Fig. 5 C–D). Thus, we conclude that the cupric sulfate effects discussed above were caused by the Cu cation and not the SO_4 anion. We do not know what portion of the growth and toxicity effects were due to Cu or the anions in the cupric chloride, cupric acetate, and cupric carbonate treatments in Expt. 1.

Conclusions

We have ‘demonstrated that root growth and morphology responses of birch microcuttings depend on the formulation and concentration of Cu^{+2} used in the rooting medium. The stimulator effects of moderate concentrations of Cu added as cupric chloride suggest that cupric chloride may be a better formulation of Cu for use in rooting medium than cupric sulfate. Similar root growth and phytotoxicity symptoms were elicited with cupric acetate and cupric carbonate. Growth and phytotoxicity effects from cupric sulfate treatments were due to the Cu cation and not the sulfate anion. Consistent patterns among media pH and growth or phytoxicity responses to the applied treatments were not apparent.

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