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CARBOXYATRACTYLOSIDE: A COMPOUND FROM *XANTHIUM STRUMARIUM* AND *ATRACTYLIS GUMMIFERA* WITH PLANT GROWTH INHIBITING PROPERTIES. THE PROBABLE "INHIBITOR A"

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ABSTRACT.—Potassium carboxyatractyloside, $C_{31}H_{44}O_{18}S_2K_2$, a toxin and hypoglycemic agent from *Xanthium strumarium* and *Atractylis gummifera*, exhibited plant growth regulating properties in bioassays. It significantly inhibited wheat coleoptiles at 10^{-3}, 10^{-4} and 10^{-5} M ($p<0.01$). One week after treatment, 15-day-old corn seedlings were stunted and necrotic at 10^{-2} M, and there was chlorosis within leaf whorls at 10^{-3} and 10^{-4} M. Six-week-old tobacco plants exhibited slight malformations of the leaves one week after treatment at 10^{-2} and 10^{-3} M. Week-old bean plants were unaffected by carboxyatractyloside. The compound may be the "Inhibitor A" described by Wareing and Foda.

Dormancy of *Xanthium* spp. seed has been a topic of research for almost ninety years (1-4), yet the isolation and identification of the inhibitors responsible for dormancy have not been accomplished. The presence of two plant growth inhibitors in cocklebur seed was demonstrated in 1957 by Wareing and Foda (3), using water extracts and paper chromatography in conjunction with the etiolated wheat coleoptile 'straight growth' bioassay, but no further attempts were successful in purifying and absolutely identifying the structures. The two inhibitory zones noted on paper chromatograms, by these authors, were designated Inhibitor A and Inhibitor B; the former developed at Rf 0.1-0.3, the latter at Rf 0.4-0.5. Inhibitor A was approximately 33% more inhibitory than Inhibitor B. No further physical characteristics were given in the report, and while research was initiated by Wareing to identify inhibitors A and B, the quest to isolate, structurally identify, and report the specific activity of each compound was terminated in 1958 (recent correspondence with Professor P.F. Wareing).

In 1972, Danieli *et al.* (5) isolated two important glycosides, atractyloside and carboxyatractyloside, from rhizomes of the thistle *Atractylis gummifera* L. Later, a potent hypoglycemic agent was isolated from cocklebur, *Xanthium strumarium* L., which was shown to be carboxyatractyloside (6). In 1980, Cole *et al.* (7,8) demonstrated that the toxic agent responsible for cocklebur poisoning in pigs in South Georgia was carboxyatractyloside, found in cocklebur burrs containing seed, and not, as had been previously suggested, hydroquinone (9). In fact, no hydroquinone was detected.

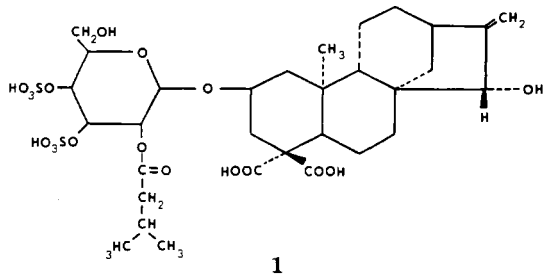
Because we are interested in identifying those natural products that exhibit plant-growth-regulating properties, especially inhibition, we tested carboxyatractyloside in the wheat coleoptile bioassay and on intact plants. These results and conclusions are reported herein.

MATERIALS AND METHODS

The initial isolation and unequivocal identification of carboxyatractyloside (I) $C_{31}H_{44}O_{18}S_2K_2$, obtained from cocklebur (*Xanthium strumarium* L. var *strumarium*) burrs containing seed, or young cotyledons, has been previously described (7).

Commercial preparations of carboxyatractyloside (as the dipotassium salt) were obtained from Sigma Chemical Company, St. Louis, MO (Lot 108C-0478 and 102F-0461).

Ascending paper chromatography was used to compare Rf values of carboxyatractyloside with the re-



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ported Rfs of inhibitors A and B, which were detected and located using bioassays, but which were not isolated or identified by Wareing and Foda (3). Approximately 75 μg of carboxytractyloside was loaded on Whatman 4 filter paper, and chromatograms were developed in isopropanol-1% NH_4OH in H_2O (4:1 v/v) by the ascending method. On completion of development, the paper strips were dried, sprayed with anisaldehyde reagent (10), and gently heated with a hair dryer. Similarly, 250 μg of (±) abscisic acid (R.J. Reynolds Tobacco Co.) was loaded onto Whatman 4 filter paper, developed, and sprayed with anisaldehyde.

ETIOLATED WHEAT COLEOPTILE BIOASSAY.—Wheat seeds (*Triticum aestivum* L. cv Wakeland) were sown on moist sand and grown in the dark for 4 d at 22± 1° (11). The apical 2 mm of each etiolated coleoptile were removed in a Van der Weij guillotine and discarded, and the next 4 mm were retained for bioassay. Solutions of carboxyactractyloside were made in phosphate-citrate buffer containing 2% sucrose at pH 5.6 (12) at concentrations of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M. Of each concentration, 2 ml was placed in a test tube with ten coleoptile sections; the tubes were rotated at 0.25 rpm for 24 h at 22° in the dark. All manipulations were carried out under a green safelight (12). Coleoptiles were then measured using a photographic enlarger to produce ×3 images. Data were analyzed statistically (13).

CORN SEEDLING BIOASSAY.—Solutions of carboxyatractyloside were tested on 15-day-old, greenhouse-grown corn seedlings (*Zea mays* L. cv Norfolk Market White). Of the test solutions, 100 μl were pipetted into the leaf whorls of individual corn plants at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M (847, 84.7, and 8.47 μg, respectively, per plant). Four corn seedlings were used per treatment, and each treatment was replicated nine times.

TOBACCO SEEDLING BIOASSAY.—Six-week-old, greenhouse-grown tobacco seedlings (*Nicotiana tabacum* L. cv Hicks) were each treated with 1 ml of carboxyactractyloside solution at 10^{-2}, 10^{-3} and 10^{-4} M (8470, 847, and 84.7 μg, respectively). Solutions were applied in aerosol. There was one plant per treatment, and treatments were replicated nine times.

BEAN SEEDLING BIOASSAY.—Seven-day-old, greenhouse-grown bean plants (*Phaseolus vulgaris* L. cv Black Valentine) were treated with 1 ml of carboxyatractyloside solution applied as an aerosol at 10^{-2}, 10^{-3} and 10^{-4} M (8470, 847, and 84.7 μg, respectively, per pot). Seedlings were at the first true-leaf stage of growth. There were four plants per pot, and treatments were triplicated.

BIOASSAY OF PAPER CHROMATOGRAMS.—Developed paper chromatograms were dried, then divided longitudinally in halves. One half was sprayed with anisaldehyde reagent and gently heated. The other was cut into ten equal parts, from the origin to the front, and each paper segment was put into individual test-tubes with 2 ml of phosphate-citrate buffer containing 2% sucrose. Ten etiolated coleoptiles were introduced into each tube and assayed (see above).

RESULTS AND DISCUSSION

Carboxyatractyloside significantly inhibited ($p<0.01$) the growth of etiolated wheat coleoptiles at 10^{-3}, 10^{-4} and 10^{-5} M; they were inhibited 100, 91, and 48% relative to controls (figure 1). In comparison, (±) abscisic acid has been shown to inhibit wheat coleoptiles 100, 90, and 69% at 10^{-3}, 10^{-4} and 10^{-5} M (14).

Corn seedlings treated with carboxyatractyloside responded within 72 h. At 10^{-2} M there were necrotic lesions on the leaves, and at 10^{-3} and 10^{-4} there was marked chlorosis within the leaf whorls (figure 2a, 2b, 2c) and stunting. At one week there was massive necrosis of the leaves and stunting at 10^{-2} M; plants were inhibited approximately 50% relative to controls (figure 3). The 10^{-3} M treatments exhibited chlorosis inside the whorls and chlorosis of the leaf margins; plants were inhibited approximately

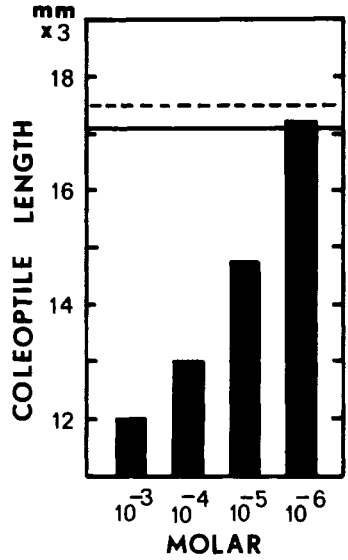

FIG. 1. Effects on the growth of etiolated wheat coleoptiles (*Triticum aestivum* L., cv. Wakeland) by carboxyatractyloside. Significant inhibition ($p<0.01$): below solid line. Control: dashed line.

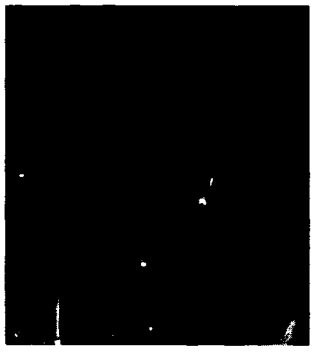

FIG. 2a.

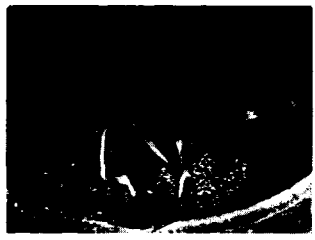

FIG. 2b.

FIG. 2c.

FIG. 2. Corn Seedlings (*Zea mays* L., cv. Norfolk Market White) treated with carboxyatractyloside at 10^{-2} and 10^{-3} M; effects after 72 h:
a. 10^{-2} M treatments showing necrosis and lesions along vein and margins; b. 10^{-3} M treatments showing chlorosis within leaf whorls; c. controls.

25% less than the controls (figure 3). At 10^{-4} M plants were slightly chlorotic, but were as tall as the controls. Two weeks after treatment, all plants at 10^{-2} and 10^{-3} M were inhibited (43% and 14%, respectively, less than the controls).

Tobacco plants treated with carboxyatractyloside exhibited unusual characteristics. Within 24 h all treatments (10^{-2}, 10^{-3}, and 10^{-4} M) showed cupping of the leaves and resembled tobacco that had been treated with cold night temperatures (2-7°), even though the greenhouse had been maintained at 18.5° at night. During the daytime, when temperatures reached 35°, the leaves flattened and resumed their normal position. However, at night the cupping effect reappeared as the temperature dipped to 18.5°. This phenomenon continued for more than 72 h, then the plants appeared to resume normal growth habits, with one exception. Treatments with 10^{-2} and 10^{-3} M concentrations exhibited slight distortion of the leaves nine days after treatment. These distortions, though relatively mild, were apparent and were not accompanied by any necrotic lesions. But the consistent recurrence of the distortion, from test to test, indi-

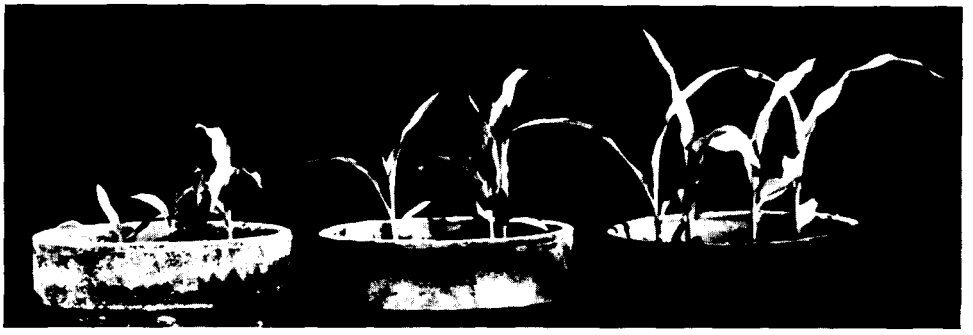

FIG. 3. Effects of carboxyatractyloside on the growth of corn (*Zea mays* L. cv Norfolk Market White) one week after treatment. Left to right: 10^{-2} M, 10^{-3} M, control.

cates that selective cells within the leaf tissue were subtly influenced by carboxyatractyloside. And whether carboxyatractyloside altered the biochemistry of the plants in such a way as to make them more sensitive to cooler temperatures, or whether the cupping is a true growth-regulator response remains to be determined. Carboxyatractyloside induced no apparent effects in bean plants

When paper chromotograms of carboxyatractyloside (developed in isopropanol, 1% ammonia, and water) were sprayed with anisaldehyde reagent and gently heated, a bright magenta area appeared at Rf 0.00-0.28 and a salmon-pink area appeared at Rf 0.46-0.56. In the coleoptile bioassay, the first three segments of the paper chromatogram representing Rfs 0.00-0.10, 0.10-0.20, 0.20-0.30 were inhibitory. Coleoptiles were inhibited 88.4, 76.7, and 86.4%, respectively, relative to controls. This inhibitory area corresponds to Inhibitor A as described by Wareing and Foda (3) using the same solvent system and descending paper chromatography. In their study, Inhibitor A inhibited 'Atle' wheat coleoptiles approximately 58%. Even though two atoms of potassium replace the two atoms of hydrogen on the HO_3SO groups, leaving the two dicarboxylate groups free, it is more probable that Inhibitor A and carboxyatractloside behave identically in paper chromatography and that the Rfs are vitually identical.

Paper chromatographs of (±) abscisic acid, developed under identical conditions, yielded a brown area on treatment with anisaldehyde and heat at Rf 0.64-0.86. Wheat coleoptiles were inhibited 100% with the three sequential segments cut from the paper chromatogram at Rf 0.60-0.90. No other chromogenic areas or areas inhibitory to the growth of coleoptiles were noted. But it is evident from comparison of the Rf values reported for Inhibitors A and B that neither of them is abscisic acid. However, the possibility that abscisic acid also occurs naturally in cocklebur seed has yet to be proved.

Inhibitor B remains unidentified at this time. Because atractyloside is often extracted in conjunction with carboxyatractyloside, we thought that the former, differing from carboxyatractyloside by one less carboxyl group, might be the Inhibitor B. However, atractyloside was inactive in the coleoptile bioassay. It is also interesting to note that atractyloside is nontoxic to animals (R.J. Cole, B.P. Stuart, and H.S. Gosser, unpublished data), whereas carboxyatractyloside is highly toxic (7). Thus, the presence of two carboxyl groups is necessary for biological activity in both plants and animals.

Of further interest is that within each burr of *Xanthium* spp. there is a pair of seeds. One is borne slightly above the other, and the two seeds are commonly referred to as the "upper" and "lower." The lower of the pair generally germinates rapidly, but the upper may take months or years to germinate, even under favorable conditions (15). A selective study to determine the amounts of carboxyatractyloside in the upper *versus* the lower seed is suggested. The polar nature of carboxyatractyloside (5,6) indicates that under natural conditions the inhibitor is probably leached out of the seed in wet wea-

ther, assuming that the normally impermeable seed coat (3) is naturally cracked or broken to allow passage of water.

The mode of action of carboxytractyloside is such that it strongly inhibits translocation of adenine nucleotides across the mitochondrial membrane (16). Thus, the unique activity and structure of the molecule indicate that it may be a useful basic template for further synthetic work in the production of new plant-growth regulators.

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