Triacontanol: A New Naturally Occurring Plant Growth Regulator

Abstract. *Alfalfa meal and chloroform extracts of the meal have increased the growth and yield of several plant species. A crystalline substance isolated from the active fraction of alfalfa meal increased the dry weight and water uptake of rice seedlings when sprayed on the foliage or applied in nutrient culture. The substance was identified as triacontanol by mass spectrometry. Sprays containing this compound also increased the growth of corn, and barley grown in soil. Authentic triacontanol produced a similar response over a wide range of concentrations on rice grown in nutrient cultures and tomatoes grown in soil.*

In 1975 we demonstrated that coarsely chopped alfalfa (*Medicago sativa* L.) hay increased growth and yield when placed in a band below and to the side of crop seeds or seedlings (*1*). An application of 117 kg of alfalfa per hectare increased early tomato (*Lycopersicon esculentum* L.) yields by 10 metric tons per hectare. Cucumber (*Cucumis sativa* L.) and lettuce (*Lactuca sativa* L.) yields were also increased in the field. Several other crop species, including rice (*Oryza sativa* L.) and corn (*Zea mays* L.), were shown to accumulate dry weight more rapidly from small applications of alfalfa under various controlled environmental conditions. Our objective in this study was to isolate the compound or compounds in alfalfa that might be responsible for the observed growth increases.

We collected the first cutting of weed-free, field-dried 'Pioneer 520' alfalfa at East Lansing, Michigan. Dried hay was ground in 0.1*M* potassium phosphate buffer separately at *p*H 4 and 9 (10 g/500 ml); the mixture was centrifuged and the supernatant emulsions were extracted with 500 ml of chloroform. The chloroform extracts were yellow at *p*H 4 and yellow-green at *p*H 9. The resulting fractions were all compared to alfalfa meal at rates equivalent to 400 kg/ha by applying them in a band 2.5 cm to the side and 2.5 cm below seed of field corn ('Michigan 396'). The chloroform extracts were allowed to evaporate before planting the corn; the corn was planted in 17.5-cm clay pots containing a Spinks sandy loam soil in growth chambers at 25°C for a 16-hour day and 20°C for an 8-hour night. The untreated meal, the total water-soluble extract, and the chloroform extract at *p*H 9 significantly increased the dry weight of the 26-day-old plants. There was no significant increase from any of the fractions extracted at *p*H 4 or from the water-insoluble residue made at *p*H 9. It is notable that no activity remained in the alfalfa residues, which indicates that the factor studied in the laboratory is associated with the field observations. The chloroform extract from the water-soluble fraction of 30 g of hay yielded 111 mg of dry matter. Analysis of this by micro-Kjeldahl procedures indicated that insufficient nitrogen was present to act as a nutrient.

Similar observations were made with rice ('IR-8') seedlings growing in specimen cups wrapped in foil. The plants were suspended with a sponge rubber disk and grown in 180 ml of Hoagland solution at one-quarter strength, at 30°C for a 16-hour day and 25°C for an 8-hour night. The solution taken up by the plants was measured every 3 days. The cups were brought up to volume with Hoagland solution. The extracts, taken up in chloroform, were placed on 2-cm^2 filter papers, dried, and placed in the nutrient solutions. Rice was used for further tests because it enabled us to measure water uptake as well as growth.

In tests with rice it was found that solutions containing between 0.1 and 10.0 mg of dry matter per liter of the crude chloroform extract increased water uptake within 24 hours and increased the dry weight 18 to 42 percent in 10 days.

Gel exclusion chromatography on Sephadex LH-20 was used to further separate the components of the chloroform extract. The column was 85 by 0.8 cm; the eluent was chloroform containing 1 percent ethanol; and the flow rate was 3 ml per 20 minutes. The fractions obtained were analyzed by gas-liquid chromatography (Beckman CC-65 interfaced with a Digital PDP 8/E Pamila computer system; the glass column was 1.8 m long with a 2-mm inner diameter and contained 10 percent DC-200 on 60/80 Gas-Chrom Q; the column operated at 200°C with a helium flow rate of 40 cm^3/min). After the gel exclusion chromatography, crystals were observed in a fraction between tubes 11 and 13 following the void volume. The crystals were further purified by rinsing with hexane and then recrystallizing from chloroform. The activity of these crystals was compared with that of the crude chloroform extract.

Portions (3 μl, equivalent to 1 mg per liter of crude extract) of chloroform solutions of the crude extract and crystals were placed on filter papers, dried, and placed in nutrient cultures containing 16-day-old rice seedlings. Each test consisted of four replicates with four seedlings per container. After 24 hours more water had been taken up by plants growing on both the crude extract and the crystals than by the control plants. After 9 days the dry weight of the shoots and roots and the water uptake were similar for the two fractions and were greater than for the controls (Table 1). The rice seedlings treated with crystals accumulated 56 percent more dry weight than the control in 9 days.

After it had been established that the crystals increased growth and water uptake, a sufficient quantity of crystals was isolated so that an accurate weight could be obtained, and a dose-response test was conducted with rice, corn, and barley (*Hordeum vulgare* L.). Rice seedlings 15 days old were treated by adding the substance on filter paper and placing in the nutrient solution or by foliar applications. 'Michigan 396' corn seedlings 8 days old and 'Larker' barley seedlings 13

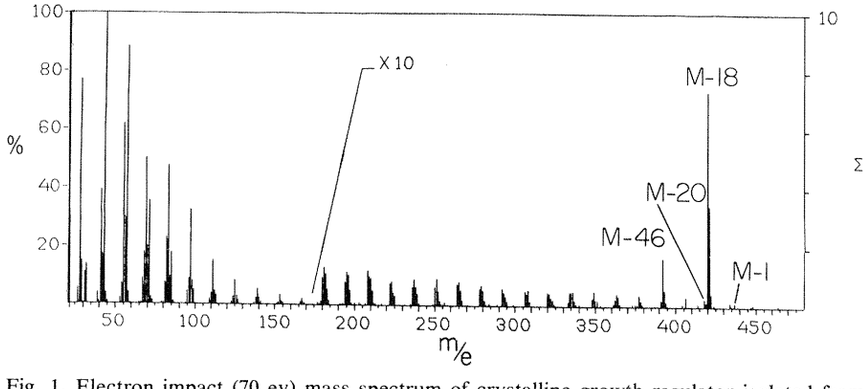

Fig. 1. Electron impact (70 ev) mass spectrum of crystalline growth regulator isolated from alfalfa. The analysis was carried out with a direct probe inlet, using a Varian CH-5 double-focusing mass spectrometer and a PDP-11/40 minicomputer system for data acquisition and reduction. The ion source temperature was 260°C and the sample gave a maximum total ion intensity at 175°C. The right ordinate shows percentage of total ionization, the left ordinate the normalized intensity.

days old grown in a fertile greenhouse potting soil also received foliar applications. Four corn seedlings and three barley plants per clay pot were replicated six times. Foliage was sprayed to the drip point with an atomizer. The spray solution consisted of 50 μl of chloroform with and without the crystals plus 50 mg of Tween-20 in 50 ml of water. The controls did not vary significantly from unsprayed treatments in previous tests with the crude extract. The rice and barley were harvested 8 days and the corn 7 days after treatment. Both the water uptake and the dry weight of the rice plants increased with increasing amounts of the crystals applied either in the nutrient solution or to the foliage (Table 2). The corn and barley grew best when sprayed with 0.01 mg/liter, whereas rice grew best at the higher concentrations. We have observed no toxic, abnormal, or atypical morphological changes at the concentration reported here.

The crystalline substance was identified by mass spectrometry to be 1-triacontanol [$CH_3(CH_2)_{28}CH_2OH$] (Fig. 1). The mass spectrum is typical of long-chain alcohols (*2*). Although the parent peak (M^+) is not observed, there is a small characteristic M^+-1 ion at mass-to-charge ratio (*m/e*) 437, a larger peak at *m/e* 420 that results from loss of water (M^+-18), and a rearrangement ion at *m/e* 392 that results from the combined loss of ethylene and water. The series of alkyl and alkene ions show no evidence for branching.

Crystals dissolved in hexane had a mass spectrum identical to that of an authentic sample of synthetic triacontanol (Analabs, North Haven, Conn.) and gas chromatographic retention times (on a 3 percent column containing SE-30 on 60/80 Gas-Chrom Q) similar to those of the authentic sample. Furthermore, the melting points of the crystals, the authentic material, and a mixture of both were similar (85°C) to that reported for triacontanol (*3*). Triacontanol is known to be the principal long-chain alcohol component of wax derived from alfalfa leaves (*3*).

The synthetic triacontanol was applied to rice (four replicates) in nutrient cultures and to 'Chico III' tomatoes (six replicates) grown in soil as previously described. The response of both rice and tomatoes to synthetic triacontanol after 7 and 6 days, respectively, was similar to that of natural triacontanol, with the optimum concentration between 0.01 and 0.1 mg/liter (Table 3). In a further test with rice, concentrations below 0.001 mg/liter did not result in an increase in water uptake or dry weight.

There have been numerous reports that many naturally occurring aliphatic compounds possess growth inhibiting or promoting activities (*4*). Fatty alcohols with chain lengths of 9, 10, and 11 carbon atoms are active inhibitors of axillary and terminal bud growth (*5*). The brassins, a group of unidentified compounds that induce elongation of plants, probably have a glyceride structure (*6*). The primary alcohol, 1-docosanol, isolated from 'Maryland Mammoth' tobacco (*Nicotiana tobacum* L.) was shown to increase growth by the oat first-internode method (*7*). Other synthetic alcohols with 17 to 22 carbons and their esters also showed activity. Triacontanol was tested but was not found active (*7*).

The rapid increase in water uptake indicates that triacontanol affects transpiration, but perhaps not directly. The increased dry weight accumulation of several species of plants with both foliar and root applications at low concentrations ($2.3 \times 10^{-8}M$, or 0.45 μg per rice plant) of triacontanol suggests that this naturally occurring compound may be involved in growth processes. We postulate that this lipoidal substance with a terminal polar group may have specific effects on membranes. Whether the growth response is primarily associated with altered water uptake, with carbon dioxide fixation, or with respiration cannot be determined from these data.

It has been suggested (*8*) that the molecular length of lipids is an important parameter in the regulation of lipid membranes. Regardless of the mode of action of triacontanol, it appears to be determined by a specific chain length since synthetic octacosanol, the analogous C_{28}

Table 1. Growth and water uptake 9 days after 16-day-old rice plants were treated with crude extract and crystals isolated from alfalfa. The LSD values are least significant differences derived from analysis of variance.

Alfalfa fraction (1.0 mg/liter)	Dry weight (mg)			Water uptake per plant (g)
	Shoots	Roots	Total	
Control	44	25	69	25.3
Crude extract	57	29	86	30.0
Crystals	59	30	89	31.5
LSD at .05 level	8	3	11	3.1
Initial weight	16	18	34	

Table 2. Response of rice grown in nutrient cultures and of corn and barley grown in soil to application of crystals isolated from alfalfa.

Alfalfa crystals (mg/liter)	Rice grown in nutrient solution				Crops grown in soil and sprayed	
	Filter paper		Foliar spray			
	Water uptake (g/plant)	Dry weight (mg/plant)	Water uptake (g/plant)	Dry weight (mg/plant)	Barley (mg/shoot)	Corn (mg/shoot)
0.00	36.5	109	35.4	110	58	355
0.01	44.3	132	38.8	118	88	466
0.10	44.5	135	40.8	123	65	405
1.00	46.1	139	43.0	132	71	429
LSD at .05 level	5.6	18	4.4	15	17	66

Table 3. Response of rice and tomatoes to authentic triacontanol 1 week after application. For rice (including the control) treatments were applied on filter paper and placed in nutrient solution. The solution was changed after 4 days. Seedlings weighed 57 mg at the initiation of the test. Tomatoes (including the control) were grown in greenhouse soil and the foliage was sprayed.

Triacontanol (mg/liter)	Rice		Tomatoes dry weight (mg/shoot)
	Water uptake (g/plant)	Dry weight (mg/plant)	
0.000	32.7	81	190
0.001	37.0	103	227
0.010	38.8	107	251
0.100	39.0	106	245
1.000	33.4	91	234
LSD at .05 level	2.4	10	33
LSD at .01 level	3.4	14	44

straight-chain primary alcohol, did not affect rice growth in three experiments. Compounds similar to triacontanol are present in other species (*9*) and may be active as plant growth regulators.

The response of crops to applications of triacontanol, alfalfa hay, and crude chloroform extracts of alfalfa must be compared in the field to directly associate the data reported here with the increased yields from small quantities of alfalfa that we have measured in the field.

STANLEY K. RIES, VIOLET WERT
Department of Horticulture, Michigan State University, East Lansing 48824
CHARLES C. SWEELEY
Department of Biochemistry, Michigan State University
RICHARD A. LEAVITT
Department of Entomology, Michigan State University

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Sarcocystosis: A Clinical Outbreak in Dairy Calves

Abstract. *Death and illness in a pen of eight yearling dairy heifers was caused by the protozoan parasite* Sarcocystis. *All animals had weight loss, weakness, marginal anemia, and elevated serum enzymes. Affected animals had high hemagglutinating antibody titers to* Sarcocystis *antigen. Affected tissues of the two animals that died demonstrated schizonts and young cysts during pathologic examination. The resident farm dog was shedding* Sarcocystis *sporocysts and was incriminated as the source of infection.*

Historically, the finding of *Sarcocystis* cysts in a high percentage of slaughtered domestic animals has been regarded with interest, but little concern has been shown because the parasite appeared nonpathogenic (*1*) and its life cycle was unknown except for the intramuscular cyst stage. *Sarcocystis* is a coccidian parasite (*2*); schizonts and cysts develop in domestic food animals (intermediate hosts) (*3, 4*) and gametes and sporocysts develop in carnivores (final hosts) (*5*). There have been several reports on the pathogenicity of some species of *Sarcocystis* for the intermediate host. Under experimental conditions the feeding of sporocysts from dogs to calves resulted in generalized clinical signs, including inappetence, weight loss, fever, anemia, and death (*3, 4, 6*). Schizonts or young cysts were seen in histologic sections from affected animals (*3, 4, 6*). Serum enzymes and antibody titers to *Sarcocystis* antigen were elevated (*7, 8*). Similarities were recognized between experimentally induced acute sarcocystosis and Dalmeny disease (*9–13*), an outbreak involving an unidentified protozoan organism in a herd of dairy cattle in Canada in 1961 (*14*). Recently it was postulated that numerous field cases of sarcocystosis may be unrecognized, resulting in considerable economic loss (*6*). Until now no documented field cases have been reported as acute sarcocystosis. Documentation of such a field case is presented in this report.

A dead heifer, from a pen containing eight yearling Holstein heifers on a dairy farm in Seneca County, central New York State, was presented to the Necropsy Service at the New York State College of Veterinary Medicine. The animal had been weak for 2 days and unable to rise the day before death. At necropsy, the carcass was emaciated and icteric, with an excessive amount of yellow fluid in the peritoneal and pleural cavities. The histologic findings included pneumonitis and mild leptomeningitis, with schizonts present in the vascular endothelium of many soft tissues (Fig. 1A). Death was attributed to a diffuse infection by a *Sarcocystis*-like organism.

A second heifer from the same pen was clinically examined at the Large Animal Hospital of the College. It was significantly underweight for its age, depressed, weak, and had a poor appetite. Temperature, pulse, and respiratory rates were within normal limits. Palpable lymph nodes were enlarged and there was obvious pallor of the visible mucous membranes. Hematologic evaluation and a bone marrow biopsy indicated the presence of a macrocytic hypochromic responsive anemia (hematocrit, 16 percent). Serum enzymes were elevated [lactate dehydrogenase (LDH), 451 I.U. and glutamic-oxaloacetic transaminase (GOT), 215 I.U.]. The serum albumin level was below normal (1.8 g/dl). Young cysts found in a cervical muscle biopsy provided strong presumptive evidence that the clinical signs were attributable to acute sarcocystosis. Serologically, the heifer was negative by the indirect hemagglutination (IHA) test for *Toxoplasma gondii*, a protozoan parasite related to *Sarcocystis*.

The heifer became moribund after 3 days of hospitalization, was killed, and a complete postmortem examination was performed. The carcass was emaciated and the mucous membranes were pale. All lymph nodes were moderately enlarged and edematous. Histologic findings included pneumonitis, diffuse degenerative myositis, and lymphoid hyperplasia. Young *Sarcocystis* cysts were found in all striated muscles examined.

An epidemiologic evaluation provided additional circumstantial evidence that the outbreak was acute sarcocystosis. Adjacent to the pen in which the sick heifers were housed, a dog had been tied for 3 weeks while it was in estrus. A fecal sample from this dog contained typical *Sarcocystis* sporocysts with four sporozoites and a granular residual body (Fig. 1B). Four weeks later, old dog feces found in the barn contained sporocysts, although fresh fecal material from the dog was negative. The dog had been fed three cow heads over the preceding 2 months; the last feeding was approximately 3 weeks before the death of the first heifer.

Intramuscular cysts found in the moribund heifer were examined by electron microscopy and contained only metrocytes (*15*). (Fig. 1C). After the death of this heifer, refrigerated meat from the carcass was fed to a dog and a cat for four consecutive days. Fecal samples were negative for *Sarcocystis* sporocysts from 2 days before feeding until 40 days after feeding. The electron microscopy and feeding trials suggest that the cysts in this heifer were immature and noninfectious.

The remaining six heifers on the farm were evaluated clinically, hematologically, and serologically. One heifer was obviously underweight for its age and had enlarged lymph nodes and pale mucous membranes. Two other heifers also had peripheral lymphadenopathy. The six animals had marginal anemia (mean hematocrit, 28 percent; range, 25 to 30 percent)