Journal of Chemical Ecology, Vol. 6, No. 1, 1980

C_{10}-POLYACETYLENES AS ALLELOPATHIC SUBSTANCES IN DOMINANTS IN EARLY STAGES OF SECONDARY SUCCESSION

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(Received January 16, 1979; revised April 10, 1979)

Abstract—*cis*-Dehydromatricaria ester (*cis*-DME) in *Solidago altissima*, and *cis*-matricaria ester (*cis*-ME), *trans*-matricaria ester (*trans*-ME), and *cis*-lachnophyllum ester (*cis*-LE) in *Erigeron* spp. show strong growth inhibitory effects on other plants. The *cis*- and *trans*-DMEs were found in soil at the border of *S. altissima* communities in concentrations that were inhibitory to test plants. Among four species of *Erigeron*, the most dominant plant, *E. floribundus*, showed the highest concentrations of the esters. From the results of our experiments, we conclude that these polyacetylenes are probably allelopathic substances with ecological importance.

Key Words—*Ambrosia artemisiaefolia* L. var. *elatior* Desc., *Solidago altissima* L., *Erigeron annuus* (L.) Pers., *Erigeron philadelphicus* L., *Erigeron canadensis* L., *Erigeron floribundus* Sch.-Bip., dehydromatricaria ester, matricaria ester, lachnophyllum ester, secondary succession, growth inhibitor.

INTRODUCTION

In the urban flora of central and southern Japan, hog-weed (*Ambrosia artemisiaefolia* L. var. *elatior* Desc.) is one of a number of widespread aliens that have been naturalized in Japan. Hog-weed becomes dominant particu-

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0098-0331/80/0100-0119$03.00/0

larly on bare ground (Numata et al., 1955, 1956, 1969, 1977, Hayashi and Numata, 1967). *Solidago altissima* L., *Erigeron annuus* (L.) Pers., *Erigeron canadensis* L., *Erigeron floribundus* Sch.-Bip. and *Erigeron philadelphicus* L. are the succeeding dominants in early stages. These winter annuals are replaced by a perennial native grass, *Miscanthus sinensis* Anderss. within several years. This sequence has been widely observed on disturbed ground in the temperate zone of Japan. A secondary succession like this seems to be an autogenic one based on the ecology of seeds and seedlings of dominants, but allelopathic effects may also be instrumental (Hayashi and Numata, 1967; Rice, 1974; Numata, 1978).

The separation and identification of allelopathic substances from *S. altissima* and four species of *Erigeron* are described, and their behavior and roles in the succession are discussed.

The method by which allelopathic substances are released from plants have been summarized in four ways (Tukey, 1969): (1) Litter of leaves and stems are decomposed by physical or biological processes and the substance is released. (2) Volatile material is vaporized from plants and acts on other plants through the air. (3) Roots release the active substance to the soil directly by exudation or through the death of decay of roots. (4) Rain and fog drips transfer toxic compounds from leaves to the soil.

In the case of *S. altissima*, a preliminary test showed that only an alcohol extract of the root is active in inhibiting the growth of test plants (Kobayashi et al, 1974). Method (3) thus appeared the most probable one, and our chemical studies were initially directed at finding the active substance in the rhizome and root. On the other hand, later extracts from leaves and stems of *E. floribundus* showed the same inhibitory effect as extracts from roots, and we concluded that either methods (1) or (3) would probably produce an allelopathic effect. Therefore, both the aerial and subterranean parts of the four *Erigeron* species were chemically investigated (Figure 1).

METHODS AND MATERIALS

Plants. The rhizomes and roots of *S. altissima* (expressed as roots of *S. altissima* hereafter) were collected from a test field on the campus of Chiba University. Specimens of the plants of *Erigeron annuus, E. philadelphicus, E. canadensis,* and *E. floribundus* were collected near the campus of Tohoku University.

The test plants used for growth and germination were rice (*Oryza sativa* L.), hog-weed (*Ambrosia artemisiaefolia* var. *elatior*), and eularia (*Miscanthus sinensis*). The last two were chosen as typical dominants in early stages of secondary succession.

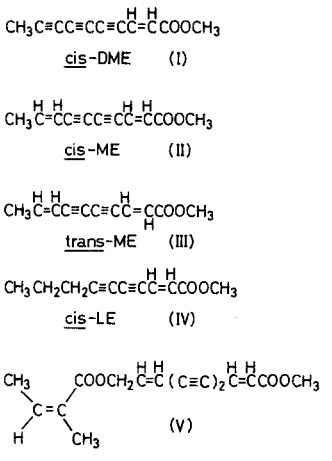

FIG. 1. Chemical structures of C_{10}-polyacetylenes.

Rice seeds were sterilized and germinated at 28° C in water. Hog-weed seeds were stratified for 10 days at 5° C before use.

Test of Inhibitory Effect. Growth test: In a 9-cm-diam. petri dish 8 germinated rice seeds were placed on 0.5% agar gel with a definite concentration of the test sample. After growing for 6 days at 29° C under 2000 lux, the length of the second leaf sheath and the longest root were compared to the standard ones grown on 0.5% agar gel only.

Germination Test. Thirty seeds were placed on the 0.5% agar gel containing the test substance. The number of germinated seeds were counted after several days at 25° C.

Single Pot Experiment. The experiment was conducted in a greenhouse. Forty hog-weed seeds were placed in 17-cm-diam. pots containing 4 kg of river sand with clay. For the first 6 days, each pot was watered daily with 100 ml of 30 ppm aqueous solution of the test substance and then fresh water was given to maintain normal growing conditions. After 30 days, the number of growing plants was calculated. Differences in the length and fresh weight were compared to the control by harvesting after 90 days.

Test of Antibacterial Activity. The standard disk pulp method used for screening of antibiotics was applied to six typical bacteria: *Arthrobacter simplex, Bacillus megaterium, Rhizobium japonicum, Corynebacterium sp., Pseudomonas sp.,* and a *coryneform* bacteria.

Separation of Active Substances from Plants. The plant body was cut into 3- to 5-cm fragments after washing and drying at room temperature overnight and dipped in 3 times its weight of methanol for 3 weeks. Methanol extraction was repeated twice, and the combined extracts were concentrated

in a flash evaporator to one twentieth of the volume. The condensate was a solution of methanol–water mixture which was extracted with hexane. The active substance is present in the hexane solution, which was washed with water and dried on Na_2SO_4, and then hexane was distilled off in vacuo. The residual oil was called "crude extract." Recrystallization and column chromatography with 10 times its weight of silica gel column packed with hexane were performed for further purification. The scheme of the separation is shown in Figure 2.

Extraction of Active Substance from Soil. Soil samples were collected from the edge of *S. altissima* community to a depth of 10 cm. Litters and other organic materials were removed as completely as possible, and the soil was dried at room temperature overnight and passed through a 1-mm sieve. The soil was submerged in twice its weight of methanol for 2 weeks. Extraction and fractionation of the active substance were done in the manner illustrated in Figure 2.

Synthesis of trans-*DME.* Cadiot-Chodkiewicz coupling reactions of 5-bromo-(*E*)-2-penten-4-ynoic acid with pentadiyne yielded *trans*-dehydromatricaria acid, which was methyl esterfied with diazomethane in ether to yield *trans* DME. This condensation procedure for the series of polyacetylenes has been described by the authors (Kobayashi, 1975).

Chromatographies. For thin-layer chromatography a 20- × 5-cm glass plate was coated with silica gel H at 0.25 mm thickness. The samples were developed with a hexane–ether (5:1 vol/vol) mixture and detected by spraying 2% alkaline aq. $KMnO_4$ sol. (yellow spot). For gas chromatography

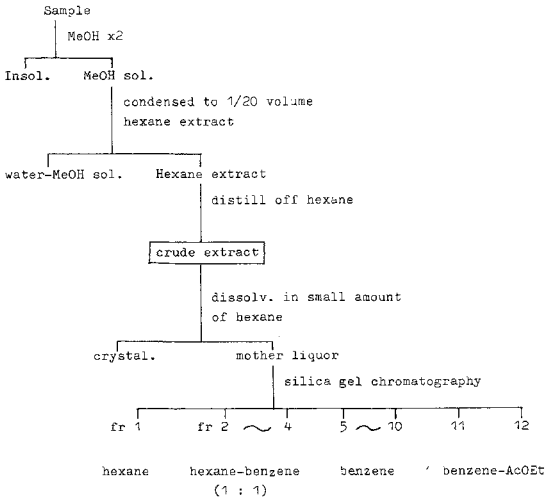

FIG. 2. Extraction and separation of growth inhibitor from plants.

a stainless column (2 m × 3 mm) packed with 10% DEGS on Celite 545 was used for quantitative analysis for *cis*- and *trans*-DME under 210° C isothermal condition with nitrogen carrier gas flow rate at 20 ml/min. Under the same conditions, 20% DEGS column was used for separation of three active components from *Erigeron* spp. Gas chromatographic trapping of each component was performed under the same conditions with a TC detector instead of the FI detector used above.

Spectrometers. IR spectra were recorded on a JASCO IRA-1 grating spectrometer as KBr disk for crystalline products and as film for liquid substances. PMR spectra were determined as a solution in CCl_4 on a JEOL MH-60 (60 MHz) spectrometer using TMS as an internal standard. UV spectra were recorded on a Hitachi 124 spectrophotometer as an ethanol solution. GC-MS were recorded using JMS-06 GC-MS spectrometer.

RESULTS

When the crude extract from *S. altissima* was dissolved in a small amount of hexane and stored in a cold place, needle crystals were separated and identified as *cis*-dehydromatricaria ester (methyl (*Z*)-2-decene-4,6,8-triynoate, *cis*-DME, I) from the melting point (116° C) and the spectrometric data as follows: IR; λ_{max} 2200, 2180, 1718, 1598 cm^{-1}, PMR; δ 2.08 (3H, s), 3.85 (3H, s), 6.30 (1H, d, J = 11.0 Hz), 6.32 (1H, d, J = 11.0 Hz). UV λ_{max} nm (ϵ) 245 (13200), 256 (18000), 286 (1100), 304 (3700), 324 (6600). The mother liquor was column chromatographed and *cis*-DME was eluted in fraction 2 (TLC Rf = 0.44 one spot) which showed very strong inhibition on the rice seedling growth. The inhibitory effect of *cis*-DME is depicted in Figure 3 from which 50% inhibition was supposed to appear at the concentration of 5.0–10.0 ppm. Table 1 shows that *cis*-DME could not suppress the germination of rice seeds, but at concentrations sufficiently strong to inhibit rice seedling growth, *cis*-DME was found to inhibit strongly germination of the competing plants, hog-weed and eluaria.

To prove *cis*-DME is an allelopathic substance, it would seem necessary to show that a reasonable amount of this compound exists in the plant body and is also present in the soil after being released from the plant body in quantities sufficient to affect the germination or growth of other plants. As summarized in Table 2, the content of *cis*-DME in the root was between 250 and 400 ppm, which is much higher than the concentration sufficient to inhibit other plants. From the soil extract, active fraction 2 was also obtained, but it contains *trans*-DME (II) along with *cis*-DME. The identification of *trans* isomer was accomplished by a direct comparison of the synthesized authentic sample from chromatographic and spectrometric methods.

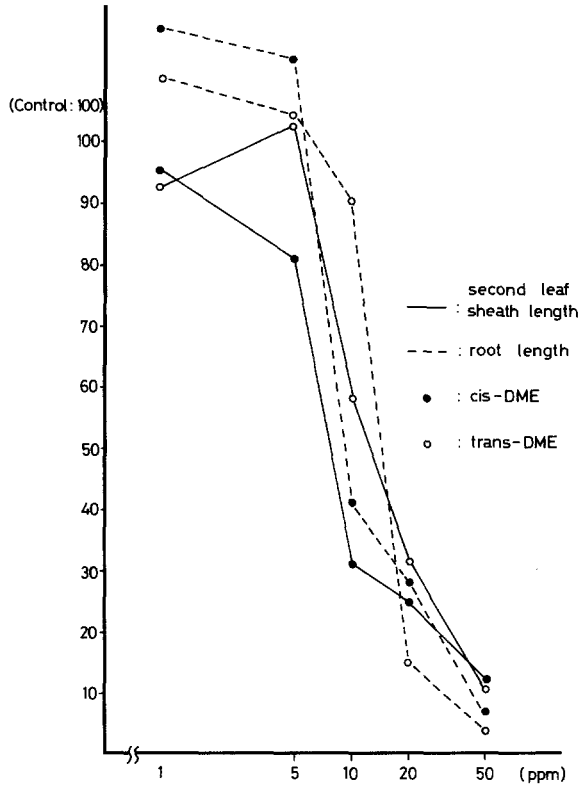

FIG. 3. Inhibitory effects of *cis*- and *trans*-DME on the growth of rice seedlings.

TABLE 1. INHIBITORY EFFECT OF *cis*-DME ON GERMINATION OF *Oryza sativa* L., *Ambrosia artemisiaefolia* VAR. *elatior* DESC. AND *Miscanthus sinensis* ANDERSS. (CONTROL: 100)

	cis-DME (ppm)			
	1.0	10	20	50
	% of Control			
O. sativa	100	90	88	88
A. artemisiaefolia var. *elatior*	72	55	50	25
M. sinensis	85	38	25	22

TABLE 2. YIELDS OF DMEs FROM ROOTS OF *S. altissima* AND SOIL

S. altissima	Crude extract	Crystal (mg)	Fraction 2 (mg)	Total (mg)
1. 3.9 kg	61.0 g (1.56%)	222	740	962 (247 ppm)
2. 1.2 kg	22.2 g (1.86%)	84	380	464 (387 ppm)
3. 0.77 kg	15.9 g (1.59%)		190	190 (247 ppm)
Soil 3.2 kg	770 mg		20	20 (6 ppm)

Although the same extracting and separating procedures were employed on both the plant and the soil samples, *trans* isomer was obtained only from the soil. Therefore we concluded that the *trans* isomer was not present in living cells and *cis*-DME was isomerized to the *trans* isomer in the soil after exudation from the root. It is well known that α, β-unsaturated ester is easily isomerized by light, pH, or other environmental factors to the more stable *trans* form. To monitor the *cis–trans* isomerization under experimental conditions for growth inhibition, 1% ethanol solution of *cis*-DME was placed under the illumination of 2000 lux at 29° C, and an aliquot of the sample was analyzed by GLC. Two days later, the *cis–trans* ratio had become 1 : 1; after an additional 5 days, the ratio had become 1 : 11. Therefore, it is probable that *cis*-DME can be isomerized to *trans*-DME under environmental conditions.

The *trans* isomer also showed an inhibitory effect on rice seedlings, and a 50% inhibition appeared at a concentration of 10–15 ppm (Figure 3). The ratio of *cis*- and *trans*-DME in fraction 2 of the soil extract was calculated from GLC (*cis* t_R = 11.6 min, *trans* t_R = 7.7 min) to be 3 : 2. The concentration of DMEs in the soil was approximately 6 ppm (Table 2), and this value was thought to be enough to influence the germination and early growth of plants growing on this soil.

The crude extracts from four *Erigeron* species also showed strong growth inhibitory effects,and their activities were concentrated at fraction 2 (Figure 2). In contrast to the fraction from the *S. altissima* extract, fraction 2 did not crystallize but remained a yellow viscous oil. On the gas chromatograms, there were three peaks—namely, peak A (t_R = 11.0 min), peak B (t_R = 13.5 min) and peak C (t_R = 16.2 min). To establish their carbon skeletons, 30 mg of fraction 2 were dissolved in 2 ml of methanol and hydrogenated with platinum oxide (30 mg) as a catalyst under hydrogen gas. The hydrogenated product showed one peak at 1.9 min under the same conditions described above and was identified as methyl decanoate by comparison with the authentic sample.

GC-MS showed that peaks A and C have the same M^+ ion peak at 174 and their fragmentation patterns have many similarities (*m/e*: 174, 159, 143, 115, 103). Therefore their molecular formulae were $C_{11}H_{10}O_2$, and they are deduced to be isomers of matricaria ester. After GC trapping, peak C was crystallized with mp 33.5°C (Bruun et al., 1951) and was identified as *cis*-matricaria ester (methyl (2*Z*,8*Z*)-2,8-decadiene-4,5-diynoate, *cis*-ME II). On the other hand, peak A did not crystallize at room temperature, but its PMR spectrum showed α,β-unsaturated *trans* protons (δ: 6.1, 6.8 ppm, d, J = 15.8 Hz) and *cis* protons coupled with terminal methyl group (δ: 5.5, d.q, J = 11.0, 1.2 Hz, δ: 6.1, d. q, J = 11.0, 6.5 Hz). Therefore peak A was *trans*-matricaria ester [methyl (2*Z*,8*Z*)-2,8-decadiene-4,6-diynoate, *trans*-ME, III]. On GC-MS of peak B, M^+ peak appeared at 176 and its mp (31°C after GC trapping) or spectrometric data were identical to those of *cis*-lachnophyllum ester [methyl(*E*)-2-decene-4,6-diynoate, *cis*-LE, IV] (Tronvold et al., 1953). On PMR spectra of *cis*-ME and *cis*-LE, two protons attached to the α,β-unsaturated double bond have the same chemical shift; therefore, single peaks were observed at 6.2 and 5.9 ppm, respectively. In the presence of shift reagent (Shiever's reagent), the proton on the α-carbon shifted downfield. The coupling constant was determined to be 11.0 Hz.

The growth inhibitory effects of *cis*-LE, *cis*-ME, and *trans*-ME were tested using rice seedlings under the same conditions as the test of *cis*-DME. As shown in Figure 4, the activity of *cis*-LE is as strong as that of *cis*-DME, but the inhibitory effects of *cis*- and *trans*-ME are much lower than that of *trans*-DME. The difference in inhibition strength between *cis*-LE and *cis*-ME is obvious from the germinating test of *A. artemisiaefolia* var. *elatior* which is summarized in Table 3.

The growth inhibitory effect of main polyacetylenes, *cis*-DME, *cis*-LE, and *cis*-ME to *A. artemisiaefolia* var. *elatior* was tested by a single pot experiment to determine whether the growth inhibition was operative on the dominant plant at early stages of secondary succession under natural conditions. It was difficult to germinate the hog-weed seed at the same time in the pot, but the number of growing plants was calculated after 30 days and the results were as follows: control, 22, *cis*-DME, 19, *cis*-LE, 15, *cis*-ME, 20. Statistical analysis was impossible from these data, but the tendency of stronger inhibitory effect of *cis*-LE was apparent. The growth in each pot was compared by fresh weight and the length after 90-day cultivation (Figure 5). *cis*-DME was the most effective while *cis*-LE showed no influence on growth, although both compounds had shown the same inhibitory effect on rice seedlings grown on agar plates. In view of the differences which appeared under different experimental conditions, it seems *cis*-DME and *cis*-LE exercise their inhibiting effects in different ways in soil.

No polyacetylenes separated from the specimens showed any anti-

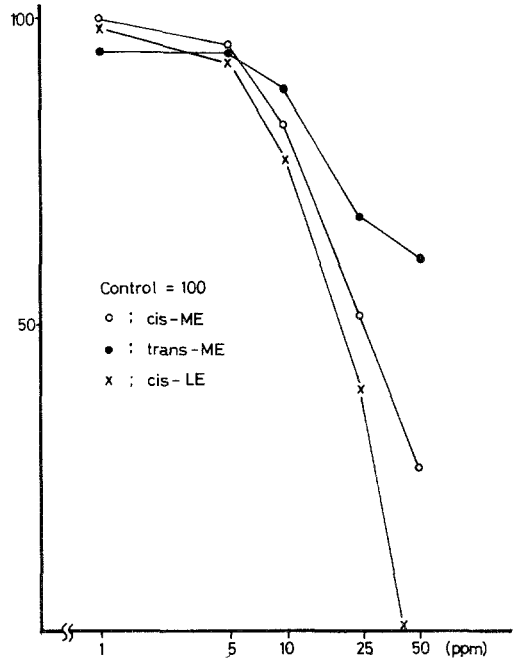

FIG. 4. Inhibitory effects of *cis*-ME, *trans*-ME, and *cis*-LE on the growth of second leaf sheath of rice seedling.

bacterial activity on the six typical soil bacteria at the concentration of 1000 ppm.

The combination of a simple separating procedure of the active substances and a gas chromatographic analysis of fraction 2 made it easy to determine the composition of C_{10}-polyacetylenes in plants. An extraordinarily high content of *cis*-LE was observed in both aerial shoots and roots of

TABLE 3. EFFECTS OF *cis*-LE AND MEs ON GERMINATION OF *A. artemisiaefolia* var. *elatior* (CONTROL: 100)

	cis-LE and MEs (ppm)				
	1	5	10	25	50
Compounds	% of Control				
cis-ME	83	75	75	83	75
trans-ME	83	75	83	75	67
cis-LE	50	58	33	13	7

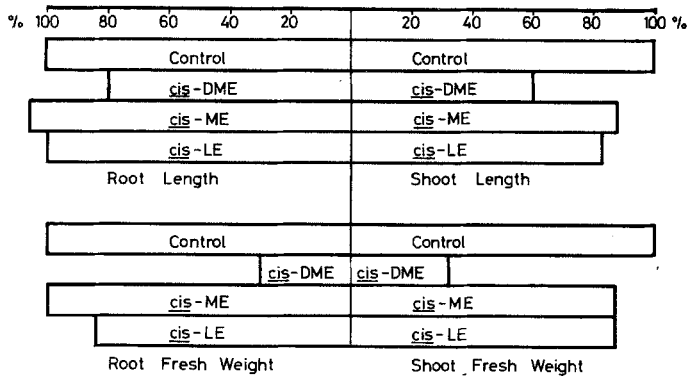

FIG. 5. Effect of DME, ME, and LE to pot cultured hog-weed.

E. floribundus (Figure 6) and the amount is higher than that of *cis*-DME in *S. altissima*. *E. philadelphicus* is observed to overwhelm *E. annuus* if the two plants grow in the same site and, in view of the results indicated in Figure 6, the higher content of C_{10}-polyacetylenes in the former is one of the factors in its dominancy.

DISCUSSION

The chemical constituents of the essential oil from Compositae have been widely studied by many researchers, and it is apparent that various polyacetylenes, especially C_{10}-polyacetylenes related to dehydromatricaria

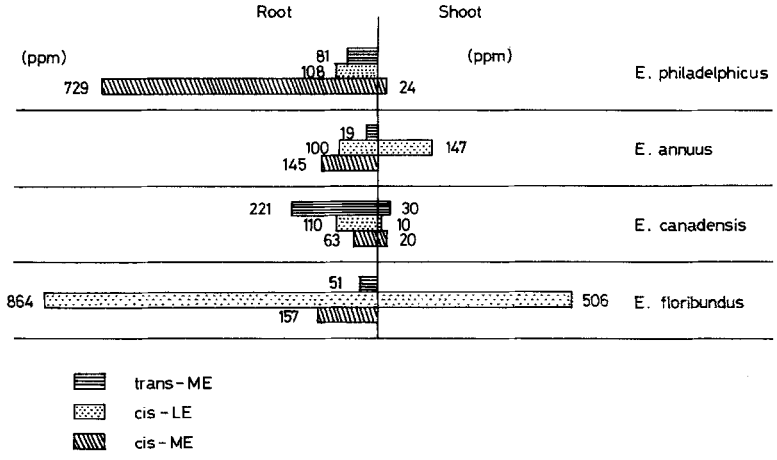

FIG. 6. C_{10}-Polyacetylene contents in *Erigeron* species.

ester, are generally distributed in this family (Bohlmann et al., 1973). However, their physiological or ecological roles have not been recognized. The composites are famous for their aggressive character and are spreading over the world as ruderal plants. Some of them, such as *S. altissima* and *E. floribundus* are well known as dominants in early stages in urban wastelands or abandoned fields in Japan.

The growth inhibitory effect of *cis*-DME in *S. altissima* was first pointed out by Kawazu et al. (1969). To prove that this inhibitory substance is participating in competition or succession as an allelopathic substance, it is necessary to show the method by which this substance is released into the environment and inhibits other species. The facts that *cis*- and *trans*-DME affect plants profoundly in minute amounts and that these substances are present in the soil at the border of *S. altissima* communities with effective concentrations seem to support the hypothesis that *cis*-DME in plants affects germination and early growth of nearby plants of the community. In addition, the isomerization of *trans* isomer does not indicate a stage of degradation but the maintenance of the inhibitory effect.

Another substance obtained from *S. altissima* showing a weak inhibition to rice seedlings (50% inhibition at 50–100 ppm) was obtained from fractions 9 and 10 (Figure 1). This compound was also deduced to be an acetylenic compound from its IR spectrum. Later, Ichihara et al. (1976) found a new acetylenic compound from *S. altissima* and determined the structure as methyl 10-[(Z)-2-methyl-2-butenoyloxy]-(2Z,8Z)-2,8-decadiene-4,6-diynoate (V), which was identical to the fraction 9 and 10 components. The same authors demonstrated that V inhibits growth of barnyard millet (*Echinochloa crus-galli* Beauv. var *frumentacea* Trin.) as effectively as *cis*-DME does. Moreover, they showed that more than twice as much V as *cis*-DME accumulates in the root of *S. altissima* (Ichihara et al., 1978). The authors synthesized V and showed that its inhibitory effect is not so strong as *cis*-DME on the rice seedlings (Kobayashi et al, 1976). Since both compounds have species-specific inhibitions and V is stable enough to accumulate in soil as *cis*-DME, we may conclude that *S. altissima* produces two acetylenic compounds probably as allelopathic substances, which strongly inhibit growth of many other plant species, and consequently the plant becomes a dominant in an early stage of secondary succession. Moreoever, the relatively short occupation by *S. altissima* in secondary succession may be a consequence of the accumulation of such polyacetylenes in soil which act as an autotoxic substance.

The general response of germinating seeds to the polyacetylenes is the inability of the young root to spread into the agar gel or the soil containing inhibiting amounts of these compounds. Consequently the young plant is pushed up to the surface and cannot grow normally. This tendency has also been observed by Nishida and Kasahara (1975) in the pot experiment of *E.*

floribundus. The young plant could not spread its root into soil which was mixed with fragments of the same plant body and finally withered. From Figure 6 it is obvious that the inhibitory effect of *E. floribundus* litter on its own species is due to the high concentration of *cis*-LE. The short occupation by *E. floribundus* in early stages of secondary succession may also be attributed to the accumulation of *cis*-LE in soil through litter.

Allelopathy occurs not only by the direct interaction between two plant species but also through changes in the rhizosphere of a plant. Rice (1964, 1974) pointed out that some weeds produce an allelopathic substance that inhibits soil bacteria and changes the soil environment beneficial to the weeds' prosperity. Therefore it is necessary to test the allelopathic substances for antibacterial activity. The absence of any antibacterial activity by the C_{10}-polyacetylenes suggested that these compounds directly affect the growth or germination of other plants. Lack of the antibiotic activity by polyacetylenes originating in plants was also reported by Tsuji et al. (1975).

Recently other biologically active polyacetylenes in plants have been reported. Kawazu et al. (1973) separated a strong antifungal substance from *Dendropanax trifidus* Makino and determined the structure of C_{18}-polyacetylenic alcohol, which has since been synthesized, and its antifungal activity has also been discussed (Kobayashi et al, 1977). Nematocidal substances were isolated from roots of *Carthamus tinctorius* L. (Kogiso et al., 1976), and their structures were confirmed as highly unsaturated polyacetylenic hydrocarbon and alcohol by the same authors (1976). These biologically active polyacetylenes seem to contribute to the prosperity of the producing plant. Similarly, the production of the C_{10}-polyacetylenes is beneficial to the producing plant because of strong inhibitory action on growth or germination of neighboring plants. We propose that these polyacetylenes are allelopathic substances but the question of the extent of their impact on the formation of plant communities or on the sequence of succession is to be answered in the future.

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