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# Bioactivity of the Fungal Metabolite (8*R*,16*R*)-(−)-Pyrenophorin on Graminaceous Plants

MICHAEL A. KASTANIAS AND MARIA CHRYSAYI-TOKOUSBALIDES*

Pesticide Science Laboratory, Agricultural University of Athens, 75 Iera Odos, 118 55 Athens, Greece

A secondary metabolite was isolated from cultures of a *Drechslera avenae* pathotype with host specificity to *Avena sterilis* and identified as the macrodiolide (8*R*,16*R*)-(−)-pyrenophorin (8,16-dimethyl-1,9-dioxa-cyclohexadeca-3,11-diene-2,5,10,13-tetraone). A considerable yield of the substance was obtained after 8−12 days of incubation at temperatures of 15−20 °C. The compound at a concentration of 60 $\mu$M inhibited seed germination of wild oats (*Avena sterilis*, *A. fatua*), oat (*A. sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*). Root growth of pregerminated seeds of the graminaceous plants was stimulated, remained unaffected, or was inhibited by pyrenophorin at 10−30, 31−50, and >51 $\mu$M, respectively. The metabolite caused abnormal chlorophyll retention in leaf sections of all five graminaceous plants, but seedling cuttings partially immersed in 1000 $\mu$M solutions remained unaffected. The rate of chlorophyll dissipation was decreased by half in leaf sections treated with pyrenophorin at 320 $\mu$M compared with the control. These findings are discussed and compared with data on the production and bioactivity of the macrodiolide (5*S*,8*R*,-13*S*,16*R*)-(−)-pyrenophorol, which has a similar stereochemical configuration.

**KEYWORDS: macrodiolides; pyrenophorol; pyrenophorin; *Avena strerilis*; graminaceous plants**

## INTRODUCTION

Bioactive substances produced by fungi act as a supply of new structural types which can be models for development of novel pesticides. The majority of isolated fungal metabolites are produced via pathways of secondary metabolism and are characterized by chemical diversity. Several metabolites produced by phytopathogenic fungi have been found to be phytotoxic, such as de-*O*-methyldiaporthin, tryptofol, pyrenophorol, pyrenophorin, dihydropyrenophorin, members of the ophiobolins, curvularins, eremophilanes, triticones, and drechslerols, which were isolated from species of the genus *Drechslera* (*1*−*11*). Although a great amount of work has been done on the isolation, identification, and determination of bioactivity of fungal phytotoxins, their effect has not been associated with a particular stereochemistry with the exception of pyrenophorol with a (5*S*,8*R*,13*S*,16*R*)-(−) configuration. This stereoisomer has been isolated from a *Drechslera avenae* (Eidam) Scharif pathotype with host specificity for *Avena sterilis* L. and has shown selective phytotoxicity against wild oats (*11*). The objective of this work was to identify other stereoisomers of (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol or metabolites of similar structure in *D. avenae* cultures and study their bioactivity on graminaceous plants.

## MATERIALS AND METHODS

**Fungi and Plant Material.** An isolate of *D. avenae*, with host specificity for *A. sterilis*, was used (*11*). It was maintained and grown on a solid medium containing oatmeal (30 g), agar (20 g), and deionized water (1 L). Seeds of *A. sterilis* L. were collected from naturally grown plants. Seeds of *Avena fatua* L., *Avena* sativa L., *Triticum aestivum* L., and *Hordeum vulgare* L. were commercially purchased. All seedlings were grown in a growth chamber at 20 °C with a 12-h photoperiod.

**Chemicals and Reagents.** (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol was isolated from cultures of *D. avenae* (*11*). All solvents (pro-analysis grade) and reagents were from LabScan Analytical Reagents (Dublin, Ireland).

**Metabolite Isolation and Identification.** Agar cultures 10 days old were chopped, and the fragments were suspended in benzene (100 g/400 mL) for 24 h. After filtration, active charcoal (2.5 g/1 L) was added to the solvent extract. The slurry was paper-filtered (Whatman No 1). The solvent phase was dried over anhydrous sodium sulfate and reduced to dryness under vacuum at 36 °C. The residue dissolved in benzene (0.5 mL) and was subjected to sequential thin-layer chromatography (TLC, preparative TLC plates coated with a 0.75-mm layer of silica gel $60GF_{254}$, Merck, Darmstadt, Germany) using acetone:chloroform (4:6) and ethyl acetate:hexane (3:7). The quenching band viewed at 365 nm ($R_f$ = 0.36) was scraped off from the second chromatograph, suspended in benzene, and centrifuged, and the supernatant was brought to a volume of 0.1 mL. The addition of hexane gave white flakes that were obtained by centrifugation at 1000xg. The melting point of the purified metabolite (175 ± 1 °C) was obtained with a Buchi melting point apparatus and was uncorrected. The $[\alpha]_D^{20}$ was measured with a Perkin-Elmer 141 Polarimeter and was −64 (c1.0, acetone). IR spectra were taken with a Nicolet Magna 750 FT-IR spectrophotometer equipped with a DTGS detector and interfaced with a personal computer. A Spectra Tech Microcup DRIFTS accessory was used, and the background spectra were recorded by using pure dried potassium bromide. Spectra were acquired and evaluated with the use of Omnic ver. 3.1 FT-IR software at 4-$cm^{-1}$ resolution and 100 scans per sample. All spectra were smoothed by using the Savitsky-Golay algorithm with

* To whom correspondence should be addressed. Tel: +30-210-529-4545. Fax: +30-210-529-4514. E-mail address: mchrys@aua.gr.

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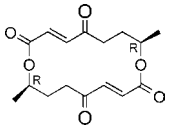

**Figure 1.** Structure of (8*R*,16*R*)-(–)-pyrenophorin.

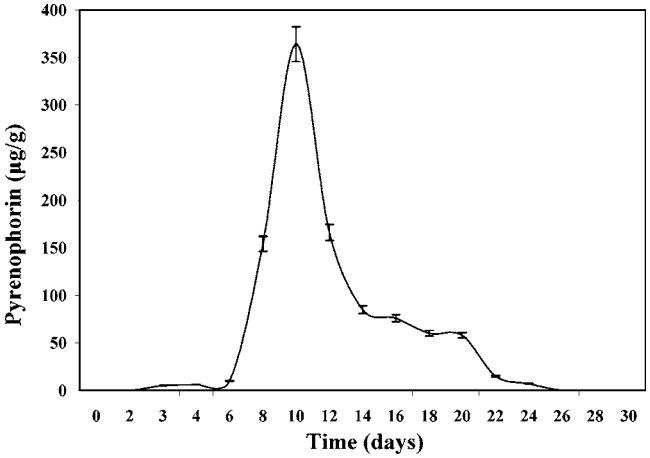


**Figure 2.** Production of (8*R*,16*R*)-(–)-pyrenophorin in oatmeal agar cultures of *Drechslera avenae* at 20 °C in the dark. The yield is expressed as $\mu$g/g of culture medium, and the values are means of five replications. Standard deviations are plotted as vertical bars.

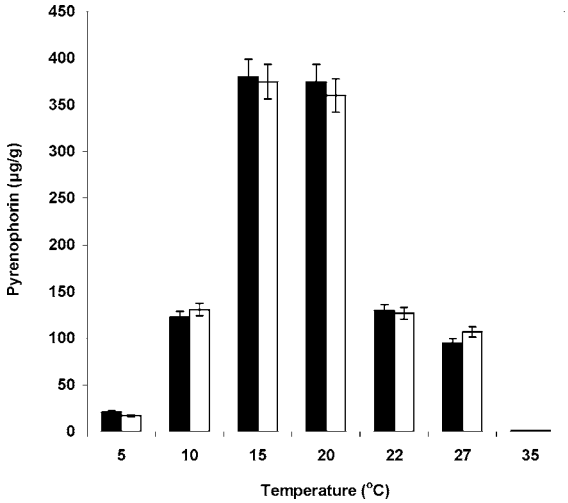


**Figure 3.** Production of (8*R*,16*R*)-(–)-pyrenophorin in oatmeal agar cultures of *Drechslera avenae*, following 10 days of incubation at various temperatures, in the dark. The yield is expressed as $\mu$g/g of culture medium, and the values are means of three replications. Standard deviations are plotted as vertical bars.

the automatic smooth function, and the baselines were corrected with the baseline correction function. IR absorption bands were at $V_{max}$ ($cm^{-1}$) 2984, 2941 (C—H), 2868, 1727 (—CO—), 1655 (—C═C—), and 1459. The lack of absorption at 1600 $cm^{-1}$ resulted from the presence of double-bonded carbon atoms, while the lack of absorption at 3302 and 3050 $cm^{-1}$ indicates that the molecule does not have hydroxy groups and aromatic structure, respectively. Mass spectra were taken with a Hewlett-Packard 5890 Series gas chromatograph equipped with an HP 5971A mass selective detector (MSD) and interfaced with a personal computer. The injection port and detector temperatures were 250 and 300 °C, respectively. The sample (1 $\mu$L) was injected in the splitless mode. A Restek RTX-5 capillary column (30 m × 0.25 mm, with 0.25 $\mu$m film thickness) was used. The initial column temperature was 100 °C for 3 min, increased to 280 °C at a rate of 10 °C/min, and held for 5 min. The carrier gas was helium at a flow rate of 1 mL/min. Spectra were acquired and analyzed with the use of HP G1034B software for MS Chemstation. The retention time was 17.7 min, and the mass spectrum main peaks were at *m*/*z* 154, 138, and 112. The peak at 155 corresponds to $M/2 + H^+$. $^1H$ NMR was recorded on a Bruker AC 200 (200 MHz, deuteriochloroform) and revealed the number and position of the hydrogen atoms of the molecule [$\delta$H: 1.29 (d, 6.3 Hz, 6H, $CH_3$), 1.95−2.00 (m, 4H, H-7, H-15), 2.18−2.22 (m, 4H, H-6, H-14), 5.11−5.17 (m, 2H, H-8, H-16), 6.50 (d, $J$ = 15.8, 2H, H-3, H-11), 6.94 (d, $J$ = 15, 8, H-4, H-12)]. Oxidation of (5*S*,8*R*,-13*S*,16*R*)-(−)-pyrenophorol was achieved with Jones reaction (*12*).

**Quantitative Estimation.** The production of the isolated metabolite was monitored in agar cultures grown at 20 (±1) °C for 30 days or at various temperatures for 10 days. The culture media (60−100 g) were extracted with benzene for 24 h. After filtration, the solvent extract was dried over anhydrous sodium sulfate and evaporated to dryness and the residue was taken up in benzene (1 mL). Mass spectra of the solutions were taken with GC-MSD operated in the selective ion mode and the characteristic for the compound ions (*m*/*z* 112, 138, 154, 155) were monitored. All calculations were made within the linearity limits under adjusted settings and conditions.

**Effect on Seed Germination.** Round filter papers were placed in Petri dishes (9 cm) and moistened with 5 mL of water or water solutions (10−640 $\mu$M) of the compound being tested. Seeds of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, and *H. vulgare* were placed on the moistened filter papers (20 seeds per dish), and then the Petri dishes (5 dishes for each treatment) were kept in a growth chamber in the dark at 20 °C. Seed germination was examined under an Olympus SZ40 stereoscope, and the germination percentage was calculated daily according to guidelines for seed testing of the International Seed Testing Association (*13*). Seeds with coleoptiles 2 mm long and active radicles were considered as germinated.

**Effect on Root Growth.** Seeds of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, and *H. vulgare*, pre-germinated in water, were put in glass test tubes (7.5 × 1 cm) containing 2 mL of 10−640 $\mu$M aqueous solutions of (8*R*,16*R*)-(−)-pyrenophorin or deionized water (5 replicates per treatment). The tubes were then placed in a growth chamber (20 °C and 12-h photoperiod), and the length of the main root was measured after 120 h.

**Phytotoxicity Bioassays.** Solutions of the metabolite in deionized water (1−1000 $\mu$M) were applied through the vascular system to seedling cuttings of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, and *H. vulgare*. Each cutting was put in a glass test tube (7.5 × 1 cm) with the lower part (1.5−2 cm) maintained in a 2-mL solution. The seedlings were kept in a growth chamber at 20 °C and a 12-h photoperiod. The development of phytotoxicity symptoms was assessed every 12 h.

**Effect on Leaf Chlorophyll Content.** The determination of the chlorophyll content was based on the procedures of Hiscox and Israelstam (*14*). The middle part (8 cm) of leaves from 12 to 15 day old seedlings of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, and *H. vulgare* was excised and cut into segments 1 cm long. The leaf segments were placed in 7.5 × 1 cm test tubes (four segments per tube) containing 3 mL of deionized water or solutions (320 $\mu$M) of (8*R*,16*R*)-(−)-pyrenophorin or (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol. Tubes were incubated in a growth chamber in the dark at 20 °C. In total, 16 tubes per treatment were prepared. Every 12 h, the segments from one tube per treatment were separated and transferred to four test tubes (10 × 1.6 cm), each containing 7 mL of dimethyl sulfoxide (DMSO). The tubes were placed in a GFL water bath at 65 °C for 30 min. The leaf pieces were then removed and dried at 105 °C prior to weighing. The leaf extracts were brought to a final volume of 10 mL with addition of DMSO. The absorbance of the obtained preparations was read at 665 nm with a Kontron Uvicon 922 spectrophotometer. Chlorophyll content was expressed as the ratio of the absorbance at 665 nm to the dry weight of the leaf tissue in grams. All calculations were made within the linearity limits of the instrument.

## RESULTS AND DISCUSSION

**Identification of the Metabolite.** The results of the chemical analyses enabled the identification of the macrodiolide 8,16-dimethyl-1,9-dioxa-cyclohexadeca-3,11-diene-2,5,10,13-tetra-one with a molecular mass of 308 for which the name

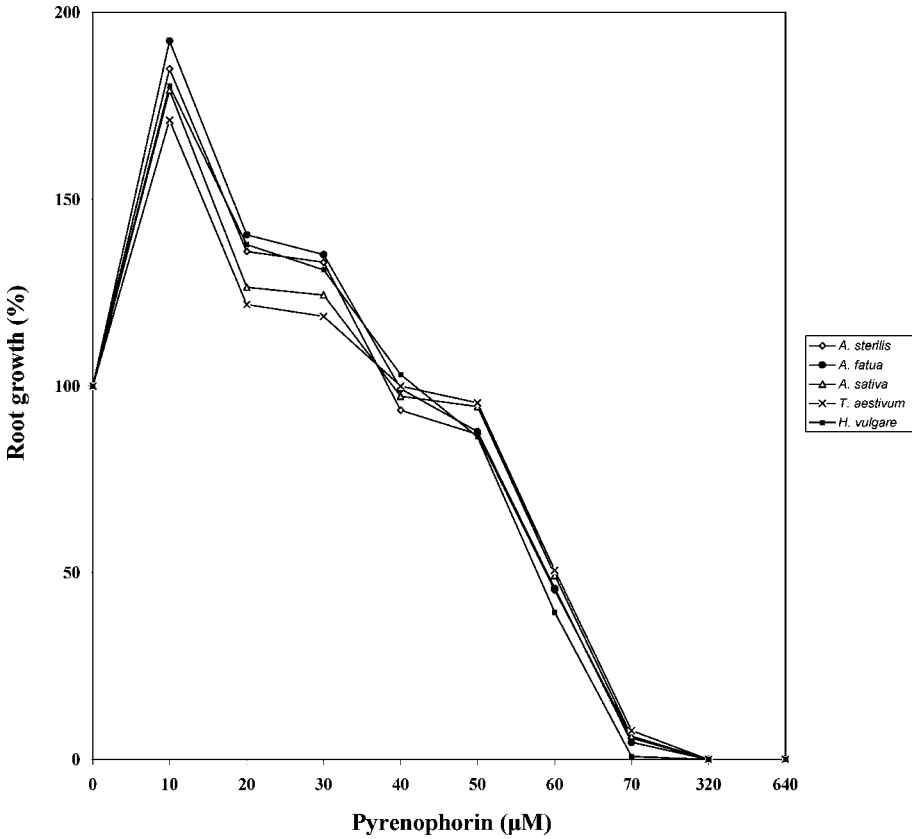


**Figure 4.** Effect of (8*R*,16*R*)-(–)-pyrenophorin on root growth of *Avena sterilis*, *A. fatua*, *A. sativa*, *Triticum aestivum*, and *Hordeum vulgare* 120 h after treatment.

**Table 1.** Seed Germination of Graminaceous Plants in the Presence of Various Concentrations of (8*R*,16*R*)-(–)-Pyrenophorin

| (8*R*,16*R*)-(-)-pyrenophorin (μM) | inhibition of seed germination (%)[a] | | | | |
|---|---|---|---|---|---|
| | *A. sterilis* | *A. fatua* | *A. sativa* | *T. aestivum* | *H. vulgare* |
| 0 | 0 | 0 | 0 | 0 | 0 |
| 10–50 | 0 | 0 | 0 | 0 | 0 |
| 60 | 15.0 ± 2.1 | 12.5 ± 2.3 | 14.5 ± 3.6 | 12.4 ± 2.8 | 10.1 ± 1.0 |
| 70 | 68.5 ± 4.3 | 69.9 ± 4.6 | 71.5 ± 3.4 | 67.8 ± 2.4 | 67.5 ± 3.3 |
| 80 | 84.4 ± 1.2 | 82.0 ± 3.4 | 82.6 ± 4.1 | 81.4 ± 3.2 | 87.7 ± 3.1 |
| 90 | 89.6 ± 2.6 | 87.8 ± 3.2 | 89.0 ± 2.3 | 86.2 ± 4.1 | 88.2 ± 0.0 |
| 100 | 96.8 ± 1.9 | 95.3 ± 2.1 | 93.5 ± 2.4 | 90.8 ± 3.0 | 95.4 ± 4.1 |
| 200 | 100 ± 0.0 | 97.9 ± 3.2 | 95.6 ± 3.8 | 90.6 ± 4.3 | 99.0 ± 1.2 |
| 320 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 98 ± 1.0 | 100 ± 0.0 |
| 640 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |

[a] The values are means of five replicates, 20 seeds each.

pyrenophorin was introduced in 1961 (*15*), and its molecular structure was elucidated 4 years later (*16*). Pyrenophorin was also isolated from cultures of *Stemphylium radicinum* (Meier, Drechs and ED Eddy), Neergard (*17*), and *Pyrenophora avenae*, Ito and Kuribayashi (*1*). Although optical stereoisomers of pyrenophorin have been synthesized by various methods (*18*−*23*), the stereochemical structure of the natural product had not been elucidated. The chemical profile (melting point, $[\alpha]_D^{20}$, IR, MS, $^1$H NMR) of pyrenophorin, obtained from (5*S*,8*R*,13*S*,-16*R*)-(−)-pyrenophorol oxidation through the Jones reaction, was identical to that of the natural pyrenophorin isolated from *D. avenae* cultures. Our data support a relative configuration (8*R*,16*R*)-(−) of the isolated metabolite (**Figure 1**).

**Production in Culture.** The metabolite was found at detectable levels (≥5 μg/g of culture medium) after 3 days of incubation at 20 °C, with a maximum yield (364 μg/g of culture medium) at 10 days after inoculation and below detectable levels in 28 day old cultures (**Figure 2**). The production was favored, as in the case of (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol (*11*), by relatively low temperatures (15−20 °C) reaching a maximum of 385 μg/g of culture medium (**Figure 3**). The decline in (8*R*,-16*R*)-(−)-pyrenophorin concentration was adversely related to the production of (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol, which started to accumulate in culture 10 days after inoculation, reaching a maximum 20 days later (*11*), indicating that (8*R*,-16*R*)-(−)-pyrenophorin might be a metabolic precursor of (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol.

**Effect on Seed Germination and Root Growth.** (8*R*,16*R*)-(−)-pyrenophorin at concentrations of 60 μM or more affected seed germination of all five plant species tested (**Table 1**). Radicle development was severely inhibited, while coleoptiles remained unaffected in the presence of the phytotoxin. Further seed exposure to (8*R*,16*R*)-(−)-pyrenophorin resulted in seedling death. The effect of (8*R*,16*R*)-(−)-pyrenophorin on root growth of pregerminated seeds in water seems to be dependent upon concentration, since enhancement of, no effect on, or inhibition of root growth were observed at concentrations of 10−30, 31−50, and >51 μM, respectively (**Figure 4**). More specifically, root growth of seedlings exposed to 10 μM of (8*R*,16*R*)-(−)-pyrenophorin was twice as much as in the control. The dose−response relationship indicates a hormone type of action of (8*R*,16*R*)-(−)-pyrenophorin, which resembles with that of cytokinins (*24*−*25*). On the other hand, according to data reported elsewhere (*11*), (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol has no effect on seed germination, seedling growth, or root development of graminaceous or other plant species.

**Phytotoxicity on Seedling Cuttings.** (8*R*,16*R*)-(−)-pyrenophorin, even at a concentration of 1000 μM, did not cause any phytotoxicity effects on stems or leaves of seedling cuttings of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, or *H. vulgare*, while

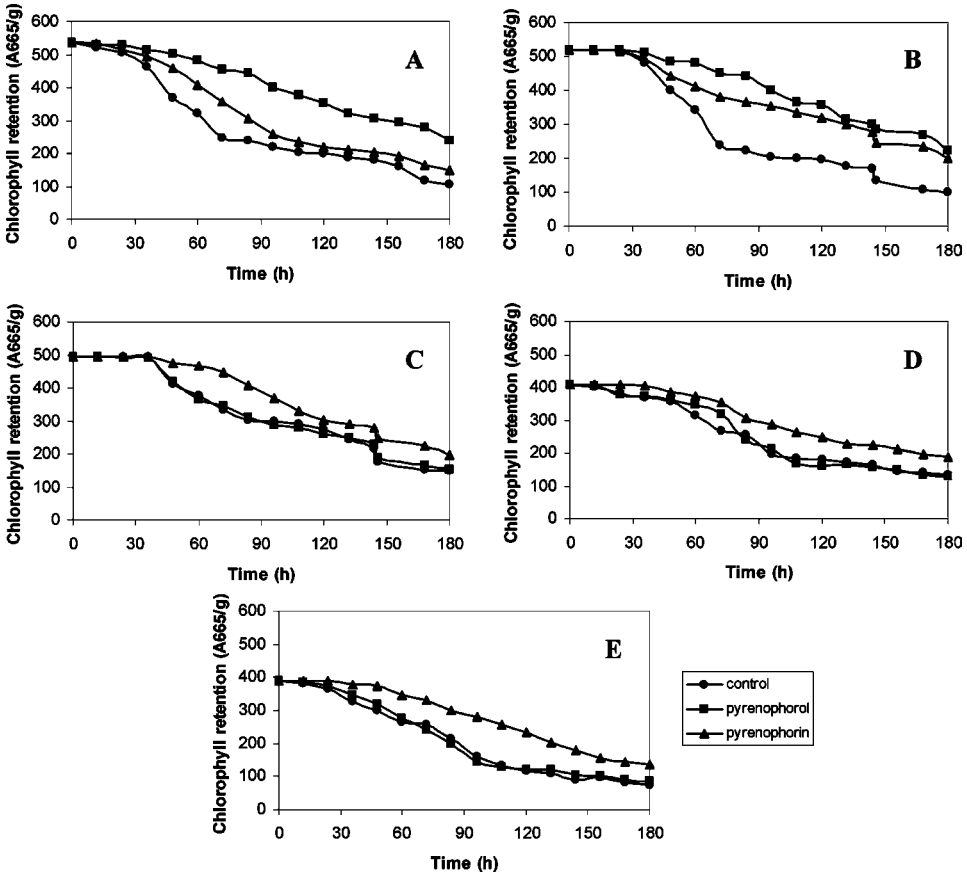


**Figure 5.** Effect of (8*R*,16*R*)-(–)-pyrenophorin and (5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol on leaf chlorophyll content of *Avena sterilis* (**A**), *A. fatua* (**B**), *A. sativa* (**C**), *Triticum aestivum* (**D**), and *Hordeum vulgare* (**E**) expressed as absorbance at 665 nm per gram of leaf dry weight. The values are means of four replications.

(5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol was phytotoxic to wild-oat cuttings (*A. sterilis* and *A. fatua*) (*11*).

**Effect on Leaf Chlorophyll Content.** Although graminaceous seedlings were not affected when partially immersed in (8*R*,16*R*)-(−)-pyrenophorin solution, leaf sections of the same plants were more sensitive to the phytotoxin showing abnormal chlorophyll retention, confined to cut leaf edges. This effect is also indicative of lack of translocation of the metabolite to the inner leaf cells. The rate of chlorophyll dissipation in the treated tissues of *A. sterilis*, *A. fatua*, *A. sativa*, *T. aestivum*, and *H. vulgare* (**parts A−E of Figure 5**), in the presence of (*8R*,16*R*)-(−)-pyrenophorin (320 $\mu$M) was 1.4−2 times slower than in the control, indicating a nonselective action of the compound. (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol at 320 $\mu$M, on the other hand, caused chlorophyll retention observed throughout the entire leaf segments of *A. sterilis* and *A. fatua*, and the rate of chlorophyll dissipation was the same in treated tissues of the sensitive plants (**parts A and B of Figure 5**). Chlorophyll dissipation rates in leaf tissues of *A. sativa*, *T. aestivum*, and *H. vulgare* (**parts C−E of Figure 5**) treated with (5*S*,8*R*,13*S*,-16*R*)-(−)-pyrenophorol were not significantly different from those of the untreated. The selectivity of (8*R*,16*R*)-(−)-pyrenophorin and (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol observed at the plant level (seed germination, root growth, seedling response, chlorophyll dissipation) might be attributed to differences in either the uptake or biochemical mechanisms at the subcellular level. Work is in progress to study the basis of specificity of those two phytotoxins and their role in pathogenesis.

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