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# Metabonomic Strategy for the Investigation of the Mode of Action of the Phytotoxin (5*S*,8*R*,13*S*,16*R*)-(−)-Pyrenophorol Using $^1$H Nuclear Magnetic Resonance Fingerprinting

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The biochemical mode of action of (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol isolated from a *Drechslera avenae* pathotype was investigated by using metabolic fingerprinting. $^1$H NMR spectra of crude leaf extracts from untreated *Avena sterilis* seedlings and *A. sterilis* seedlings treated with pyrenophorol were compared with those obtained from treatments with the herbicides diuron, glyphosate, mesotrione, norflurazon, oxadiazon, and paraquat. Multivariate analysis was carried out to group treatments according to the mode of action of the phytotoxic substances applied. Analysis results revealed that none of the herbicide treatments fitted the pyrenophorol model and indicate that the effect of the phytotoxin on *A. sterilis* differs than those caused by glyphosate, mesotrione, norflurazon, oxadiazon, paraquat, and diuron, which inhibit 5-enolpyruvylshikimate-3-phosphate synthase, 4-hydroxyphenyl-pyruvate-dioxygenase, phytoene desaturase, protoporphyrinogen oxidase, photosystem I, and photosystem II, respectively. The method applied, combined with appropriate data preprocessing and analysis, was found to be rapid for the screening of phytotoxic substances for metabolic effects.

KEYWORDS: *Avena sterilis*; $^1$H NMR fingerprinting; metabonomics; mode of action; phytotoxins; pyrenophorol

## INTRODUCTION

A natural product approach has been introduced in the discovery programs of new pesticides (*1*−*3*). The rationale behind it is to exploit structural diversity and to seek new modes of action. Regarding herbicides, natural phytotoxic compounds can be useful as chemical leads for new active ingredients. Furthermore, research on the mode of action of natural phytotoxins could reveal subcellular targets previously unknown as sites of herbicide action, and results from such studies would contribute to target-based discovery of novel herbicides (*3*).

Pyrenophorol is a fungal phytotoxin that was first isolated from cultures of *Byssochlamys nivea* and later from cultures of *Stemphylium radicinum* (*4*). Although various isomers have been synthesized in the laboratory (*5*−*7*), little attention has been given to the stereochemistry of natural pyrenophorol.

The isomer (5*S*,8*R*,13*S*,16*R*)-(−)-pyrenophorol has been isolated from cultures of a *Drechslera avenae* pathotype with host specificity for wild oat (*Avena sterilis*) and was found to be phytotoxic to *A. sterilis* but not to other related plant species (*8*). Such selectivity at the plant level combined with the observation that the structure of the metabolite is not shared by commercial herbicides is indicative of an interesting structure−activity relationship. Therefore, the elucidation of the mode of action of pyrenophorol was the objective of the present study.

Because the metabolic profile is a sensitive indicator of environmental influences, genotype, and developmental stage of an organism and can be used to detect changes of the total metabolic state due to pathophysiological or chemical stimuli, we investigated the mode of action of the phytotoxin by using metabolic fingerprinting. After exposure to a herbicide, biochemical interference with cell functions is recognizable in the plant's metabonome and can be detected in the NMR spectrum of the plant extract. Automated pattern recognition has established the biochemical mode of action of members of various herbicide classes (*9*, *10*).

Because $^1$H NMR spectroscopy facilitates the detection of low molecular weight metabolites, we applied a $^1$H NMR based metabonomic strategy to study the mode of action of pyrenophorol using $^1$H NMR fingerprints of *A. sterilis* crude leaf extracts. The task of the method is to establish differences between the spectra from sets of treatments and not to identify the metabolites. This approach to the analysis of NMR spectra has been developed in the biomedical field (*11*−*19*) and has also been extended to the analysis of extracts and materials of plant origin (*9*, *10*, *20*−*28*).

The response of *A. sterilis* to pyrenophorol was examined in comparison with that caused by the phytotoxic substances diuron [photosystem II (PSII) inhibitor], glyphosate [5-enolpyruvylshikimate-3-phosphate (EPSP) synthase inhibitor], mesotrione [4-hydroxyphenyl-pyruvate-dioxygenase (HPPD) inhibitor], norflurazon [phytoene desaturase (PDS) inhibitor], oxadiazon [protoporphyrinogen oxidase (PPO) inhibitor], and paraquat [photosystem I (PSI) electron diverter] (*29*, 30). For the classification and discrimination of the mode of action of

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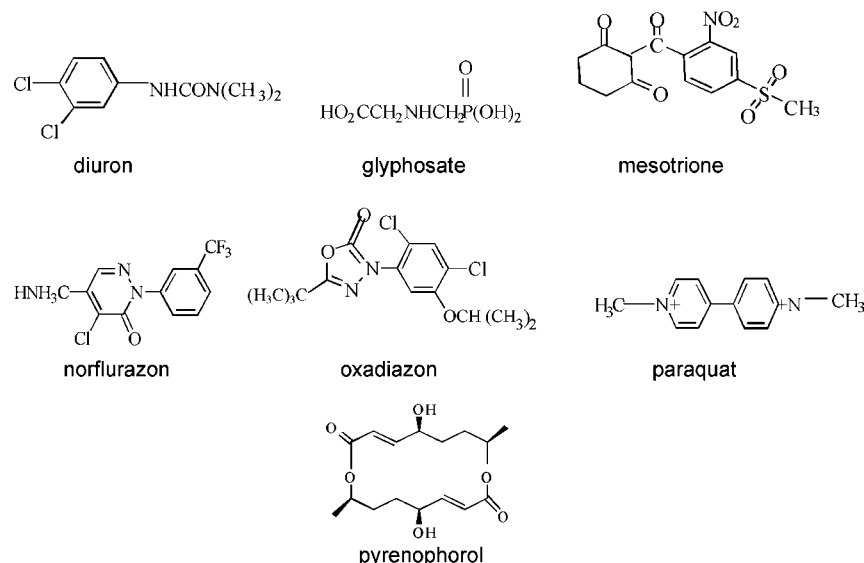
**Figure 1.** Chemical structures of the phytotoxic substances used in the study.

(5S,8R,13S,16R)-(−)-pyrenophorol from those of the herbicides included in tests, principal components analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and soft independent modeling of class analogy (SIMCA) were carried out. The objective of PLS-DA is to find a model that separates classes of observations on the basis of their *X*-variables, by accomplishing a rotation of the projection in a manner that focuses on class separation. SIMCA can be used as a complementary or alternative method to PLS-DA. It is a graphically oriented technique based on the observation that data in a class of similar observations can be well approximated by a few-components model. Observations in SIMCA are classified according to the tolerance intervals of the different classes and can be presented in Coomans plots (*31, 32*).

The results from this study on the investigation of the mode of action of pyrenophorol by applying ^{1}H NMR spectroscopy in combination with multivariate analysis are presented in this paper.

## MATERIALS AND METHODS

**Chemicals and Reagents.** (5S,8R,13S,16R)-(−)-Pyrenophorol (**Figure 1**) was isolated from cultures of a pathotype of *D. avenae* grown on oatmeal agar (*8*). Paraquat (1,1′-dimethyl-4,4′-bipyridinium) (33.5%), mesotrione [2-(4-mesyl-2-nitrobenzoyl)cyclohexane-1,3-dione] (99.7%), and norflurazon [4-chloro-5-methylamino-2-(α,α,α-trifluoro-*m*-tolyl)-pyridazin-3(2H)-one] (98.0%) (**Figure 1**) were kindly provided by Syngenta Hellas. Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (99.6%) and oxadiazon [5-*tert*-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2(3H)-one] (99.8%) (**Figure 1**) were kindly provided by Bayer CropScience Germany. Glyphosate [N-(phosphonomethyl)glycine] (99.5%) (**Figure 1**) was courtesy of Agan Chemical Manufacturers Ltd. Acetone was of analytical grade, purchased from Lab Scan Analytical Reagents (Dublin, Ireland); D₂O (99.9%) and trimethylsilyl-2,2,3,3-*d₄*-propionic acid, sodium salt (TSP), were obtained from Euriso-Top SA.

**Plant Material.** Seedlings of *A. sterilis* were obtained from seeds collected in Votanikos, Athens, in July 2002. The seeds were placed in a mixture of peat/perlite/soil (2:1:1 v/v/v) after glume removal. Seedlings were kept in a growth chamber at 22 ± 1 °C and a relative humidity of 85 ± 5% with a 16 h photoperiod. The light source used was cool white fluorescent lamps (Sylvania) providing illumination of ∼19.62 W m⁻².

**Plant Treatment and Sample Preparation.** Treatments of seedlings and sample preparation were performed according to the method of Aranibar et al. (*9*) with some modifications. For each experiment at least 40 plants were grown. Nine days after seed germination, 8 seedlings that were homogeneous in size and overall appearance were selected and carefully deplanted with caution to avoid root wounding, which might have an undesirable impact on ^{1}H NMR fingerprints. At this time, the first leaf of the seedlings had not been fully expanded and the tip of the second leaf had just emerged. The roots of the seedlings were washed with tap water, and the plants were placed in glass centrifuge tubes of 10.0 cm in height and 1.5 cm in diameter. Then, each herbicide solution (3 mL) in a mixture of acetone/water (5:95, v/v) was added to each tube. Namely, solutions of diuron (172 μM), glyphosate (296 μM), mesotrione (304 μM), norflurazon (165 μM), oxadiazon (72 μM), paraquat (4 μM), and pyrenophorol (1280 μM) were used. Appropriate controls with acetone/water (5:95 v/v) were also included. In each experiment one plant per treatment was used. The doses applied were the lowest effective concentrations that caused phytotoxicity symptoms on *A. sterilis* seedlings 24 h after treatment under the conditions set. Six experiments were conducted in total.

Twenty-four hours after treatment under continuous illumination, the seedlings were cut at the base and the lower part was discarded. The leaves of the seedlings (90−110 mg) were pulverized with a pestle in a mortar under liquid nitrogen and stored at −80 °C for a maximum of 4 days in 15 mL screw-cap glass bottles. Before further treatment, the samples were lyophilized for 24 h. Lyophilized plant preparations were suspended in D₂O (1.0 mL) containing trimethylsilyl-2,2,3,3-*d₄*-propionic acid (0.05 w/v) and centrifuged at 12000*g* for 60 min at 4 °C. The supernatants were then collected and further purified by centrifugation at 12000*g* for 30 min. The ^{1}H NMR samples were prepared from the supernatants (0.8 mL) and were frozen until ^{1}H NMR spectra were obtained.

**^{1}H NMR Spectroscopy, Data Preprocessing, and Analysis.** The ^{1}H NMR spectra of crude plant extracts were recorded using a Bruker DRX 500 NMR spectrometer equipped with a multinuclear inverse broad band 5 mm probe with 1 s solvent presaturation (60 dB) and 128 transients of 32K data points for each spectrum.

The data were Fourier transformed, and the baseline and the phase were manually corrected using MestReC software (version 461). The spectral regions δ 4.25−6.50 were removed to eliminate the effects of imperfect water suppression. Additionally, regions of the spectra were aligned prior to statistical analysis, as proposed by Defernez and Colquhoun (*23*). Each spectrum was then exported to Ms Excel and normalized prior to pattern recognition analysis by dividing individual values by the sum of all spectrum heights. Multivariate data analyses were carried out using SIMCA-P 10 (Umetrics, Umeå, Sweden) software. Data were Pareto scaled (1/√SD), which has been proposed as a compromise between unit variance scaling (UV) and no scaling and used in metabonomics studies (*32*). Finally, data were visualized

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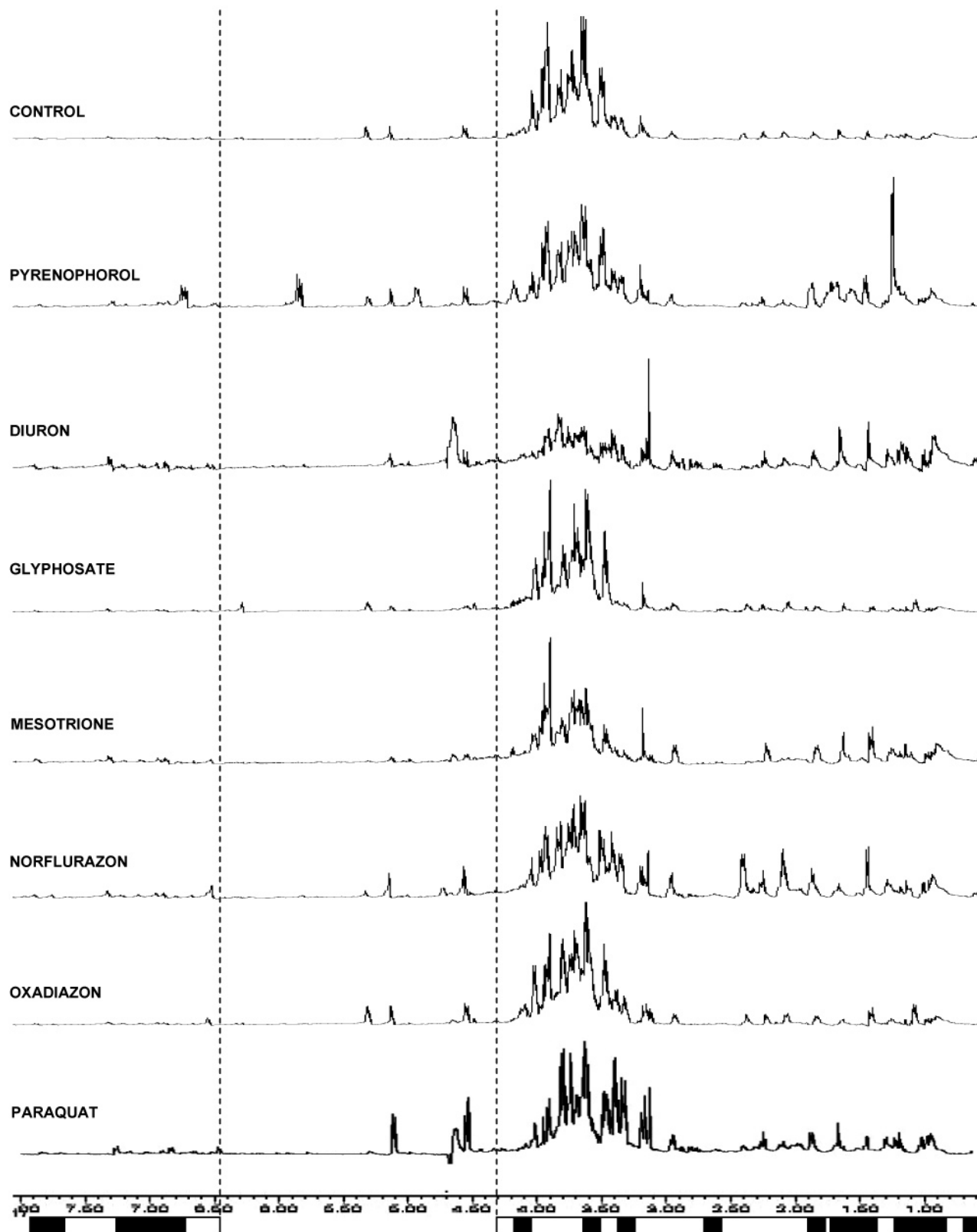*J. Agric. Food Chem.,* Vol. 54, No. 5, 2006 **1689**



**Figure 2.** ¹H NMR spectra of *A. sterilis* crude leaf tissue extracts of untreated seedlings and seedlings treated with phytotoxic substances prior to normalization. Spectra regions δ 4.25–6.50 (between dashed lines) were excluded from analyses. Areas that influenced the separation of pyrenophorol treatments are indicated with solid black rectangles.

by plotting the principal components scores where each coordinate represents an individual treatment.

## RESULTS AND DISCUSSION

*A. sterilis* seedlings treated with pyrenophorol or the herbicides showed slight or no observable effects at the time of plant

harvest, expressed as slight twisting and/or discoloration of leaf blades. Such symptoms were consistent in all experiments. In **Figure 2**, representative ¹H NMR spectra of crude leaf extract of *A. sterilis* seedlings treated with pyrenophorol, diuron, glyphosate, mesotrione, norflurazon, oxadiazon, and paraquat
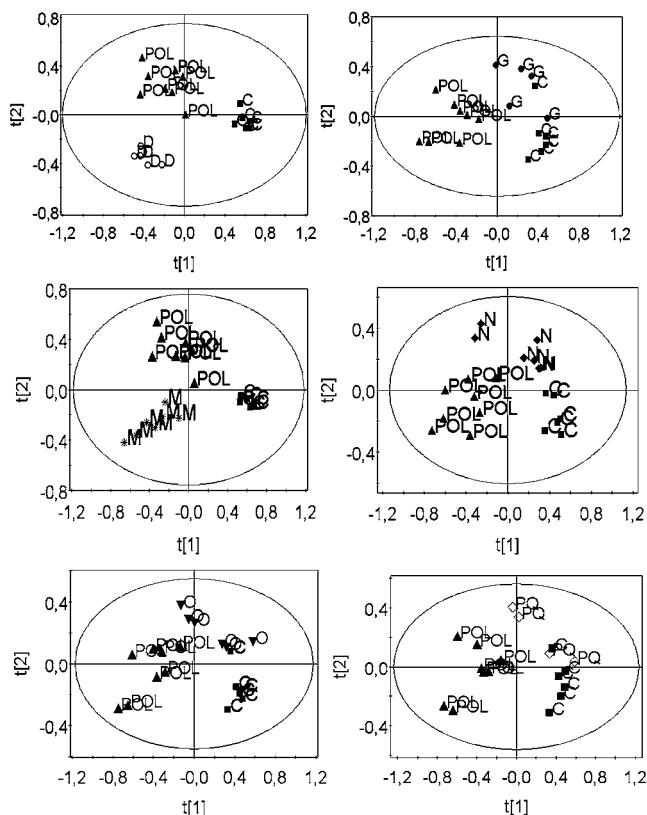
**Figure 3.** PLS-DA $t_1/t_2$ score plots based on the observations of control (C) and pyrenophorol (POL) groups plotted versus observations of diuron (D), glyphosate (G), mesotrione (M), norflurazon (N), oxadiazon (O), or paraquat (PQ) groups. The ellipse represents the Hotelling $T^2$ with 95% confidence.

of such signal to the overall complex metabolic fingerprint might be minimal. The spectra were further analyzed by multivariate analysis to examine variation between treatments.

Because PLS-DA requires the absence of strong outliers, PCA was initially carried out on the whole dataset. Such analysis revealed two strong outliers outside the Hotelling $T^2$ 95% confidence ellipse, which were excluded to avoid possible high leverage on the model. The presence of outliers might be attributed to unidentified factors of experimental nature. PCA, which was carried out on the remaining dataset, revealed the absence of strong outliers. The corresponding values of $R^2X = 0.78$ and $Q^2$(cum) = 0.64 (6 PCs) can be considered to be satisfactory for metabonomics experiments (*32*). To classify and separate treatments with various phytotoxic substances, PLS-DA and SIMCA were applied. By performing PLS-DA, models were developed from training sets of observations of known membership classes. Thus, the development of the above models was based on observations of the control and pyrenophorol groups, which were plotted versus observations related to each of the rest of the groups. Because the use of more than four groups could make interpretation tricky (*32*), the number of groups used in this study for simultaneously performing PLS-DA was confined to three. PLS-DA $t_1/t_2$ score plots for all combinations are given in **Figure 3**. Cross-validation was carried out by using the default software options. It is evident that control and pyrenophorol groups could be separated from those of diuron [3 PCs, $R^2X = 0.62$, $R^2Y = 0.95$, and $Q^2$(cum) = 0.82], glyphosate [3 PCs, $R^2X = 0.61$, $R^2Y = 0.79$, and $Q^2$-(cum) = 0.47], mesotrione [3 PCs, $R^2X = 0.64$, $R^2Y = 0.91$, and $Q^2$(cum) = 0.81], norflurazon [3 PCs, $R^2X = 0.59$, $R^2Y = 0.82$, and $Q^2$(cum) = 0.64], oxadiazon [3 PCs, $R^2X = 0.59$, $R^2Y = 0.78$. and $Q^2$(cum) = 0.36], or paraquat [3 PCs, $R^2X = 0.65$, $R^2Y = 0.73$, and $Q^2$(cum) = 0.37]. PLS-DA gave satisfactory models with high values of $Q^2$(cum) for diuron, glyphosate, mesotrione, and norflurazon, whereas the corresponding values for oxadiazon and paraquat were lower. Furthermore, profiles of the untreated seedlings were rather consistent (**Figure 3**), indicating that the *A. sterilis* metabonome was not affected by experimental conditions. Overall, variation of control batches of *A. sterilis* seedlings has been checked over a period of 1 year.

Additionally, SIMCA was carried out for the control and pyrenophorol groups. For each group, a separate PCA model
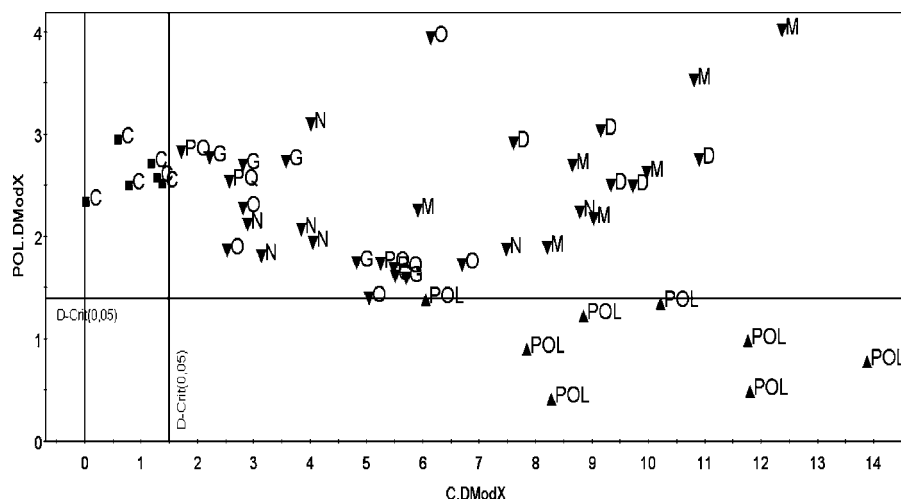
are compared with a spectrum of the untreated seedling leaf extract. The herbicides and their metabolites are usually not visible in the NMR spectra because the herbicides are applied at low concentrations to the roots of the plant and only a negligible amount is absorbed and translocated (*9, 10*). The amount of preparation left in each tube after a 24 h plant exposure was ∼2.7 mL. Therefore, the short plant exposure to the preparations of phytotoxic substances would also be a factor for limited uptake. Although the presence of the phytotoxic substance in the leaf extract is not totally excluded, the impact



**Figure 4.** Coomans plot with distance (moderate outliers, DModX) to the control (C) model (*X*-axis) plotted versus distance to the pyrenophorol (POL) model (*Y*-axis). The critical distances (DCrit) are indicated by solid lines and correspond to 95% tolerance intervals [diuron (D), glyphosate (G), mesotrione (M), norflurazon (N), oxadiazon (O), and paraquat (PQ)].

was computed. Those two models were four-dimensional and were used to infer membership class of all treatments. The results are summarized in Coomans plots (**Figure 4**). The essence of a Coomans plot is that distances between two classes are plotted against each other in a scatter plot. By plotting also the critical distance for each model, four areas of diagnostic interest are created, contributing to visualization of differences between classes. As **Figure 4** shows, there are no observations in the lower left-hand part of the plot, which means that there are no prediction set treatments that fit both models. On the other hand, the observations in the lower right-hand and the upper left-hand part fit the pyrenophorol and control model, respectively. Also, observations related to diuron, glyphosate, mesotrione, norflurazon, oxadiazon, and paraquat treatments are clustered into the upper right-hand area in which treatments that do not fit either of the above models are supposed to be found. These results are in accordance with those obtained through PLS-DA presented above.

Spectral analysis also revealed that the differentiation of pyrenophorol treatments compared to treatments with other phytotoxic substances and to control was mainly due to differences at several spectra areas such as $\delta$ 0.55−0.60, 0.87−1.21, 1.37−1.74, 1.80−1.86, 2.29, 2.58−2.66, 3.20−3.35, 3.47−3.51, 4.17−4.21, 6.77−7.34, and 7.73−7.94 (**Figure 2**).

The fact that none of the treatments with the herbicides applied could fit the pyrenophorol model indicates that the metabolic effects of the phytotoxin on *A. sterilis* differ from those caused by glyphosate, mesotrione, norflurazon, oxadiazon, paraquat, and diuron, which inhibit EPSP synthase, HPPD, PDS, PPO, PSI, and PSII, respectively. The inclusion of more herbicides with modes of action the same as or different from those of the above herbicides and the identification of the metabolites with the largest contribution to the $^1$H NMR spectra would support more solid conclusions.

The results of this study show that a metabonomics approach using $^1$H NMR spectroscopy of crude plant extracts combined with appropriate data preprocessing and analysis is a rapid method of high potential for the initial screening, which is useful for the investigation of the mode of action of natural phytotoxic substances. PLS-DA and SIMCA seem to be powerful methods of multivariate analysis for obtaining discrimination among various treatments. Also, *A. sterilis* seedlings seem to be an appropriate plant material for such application.

The above findings encourage further investigation of the mechanism of action of pyrenophorol by performing $^1$H NMR fingerprinting in combination with biochemical tests.

## ABBREVIATIONS USED

EPSP, 5-enolpyruvylshikimate-3-phosphate; HPPD, 4-hydroxyphenyl-pyruvate-dioxygenase; PCA, principal components analysis; PDS, phytoene desaturase; PCs, principal components; PLS-DA, partial least-squares discriminant analysis; PPO, protoporphyrinogen oxidase; PSI, photosystem I; PSII, photosystem II; $Q^2$(cum), the cumulative fraction of the total variation of $X$'s that can be predicted by the extracted components; $R^2X$ and $R^2Y$, fractions of the sum of squares of all $X$'s and $Y$'s, respectively; SIMCA, soft independent modeling of class analogy; UV, unit variance scaling.

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