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Photograph courtesy of Dominik Skoneczny, PhD student at Charles Sturt University Wagga Wagga Australia. This is a photograph of Paterson's curse (*Echium plantagineum*) being visited by an insect pollinator. The photo was taken in Spain in May 2014 on a research trip on which Prof. Leslie Weston and Dominik Skoneczny collected a diverse group of native *Echium* species. for study of their genetics, using DNA barcoding and also their chemistry, in particular root-produced naphthoquinones and shoot- produced pyrrolizidine alkaloids."

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Metabolomics: an unexplored tool for allelopathy studies

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ABSTRACT

Allelopathy is a very complex phenomenon, influenced by many inherent and environmental factors. Due to this complexity, its elucidation is a challenging task, requiring expertises from several fields.

A valuable approach to study these interactions is systems biology, a holistic approach giving a broad view of biological systems and relying on omics technologies. Among omics sciences, metabolomics could be very powerful, but despite its potential it has been seldom applied to allelopathy studies.

This review aims to introduce metabolomics approach to researchers involved in the study of allelopathy: to this end, an overview of this approach is given along with examples of application of metabolomics to other ecological issues (effects of abiotic and biotic stresses). Furthermore, the scarce examples of application of metabolomics or metabolomics-related approaches to allelopathy studies are reported.

Keywords: Allelopathy, Ecometabolomics, Metabolomics.

Abbreviations

CE-MS: Capillary Electrophoresis – Mass Spectrometry
DFA: Detrended Fluctuation Analysis
DIMS: Direct Infusion Mass Spectrometry
ESI: ElectroSpray Ionization
FT-ICR-MS: Fourier Transform – Ionic Cyclotronic Resonance – Mass Spectrometry
FT-IR: Fourier Transform-Infra Red
GC-MS: Gas Chromatography – Mass Spectrometry
HCA: Hierarchical Cluster Analysis
HR-MS: High Resolution – Mass Spectrometry
LC-MS: Liquid Chromatography – Mass Spectrometry
MAS-NMR: magic-angle spinning – Nuclear Magnetic Resonance
MS: Mass Spectrometry

MS-MS (MSn): TANDEM Mass Spectrometry
NMR: Nuclear Magnetic Resonance
PCA: Principal Component Analysis
PCO: Principal Coordinates Analysis
PLS-DA: Partial Least Squares – Discriminant Analysis
QTOF: Quadrupole Mass Analyzer – Time Of Flight
ROS: Reactive Oxygen Species
SOM: Self Organizing Maps
TOF-MS: Time Of Flight – Mass Spectrometry
UPLC-DAD: Ultra-Performance Liquid Chromatography-Diode Array Detector
1D, 2D: one dimensional, two dimensional

Introduction

A small, but growing body of evidence suggests that plants may be capable of recognizing and responding to neighbouring plants in a species specific manner (Broz *et al.* 2010).

Among biotic plant interactions, allelopathy represents an important factor in determining species distribution and abundance as well as in regulating dynamic processes within plant communities (Reigosa *et al.* 2006). The study of this phenomenon is very challenging because of several reasons. Firstly, it is governed by different factors such as individual chemical structure, antagonistic, agonistic, additive, sequential, or synergistic effects. Secondly, the fate of allelochemicals in the soil, their interaction with

the microorganisms in the rhizosphere, biochemical, chemical and photochemical transformations, and many other factors need to be assessed. Moreover, the production of allelochemicals is influenced by abiotic (e.g. temperature, light, soil characteristics, rainfall) and biotic factors (e.g. life cycle, competition, pathogens, pests, parasites, herbivores), with a resulting unequal distribution of putative allelochemicals in a plant in different seasons and in different environmental conditions (Granéli & Salomo, 2010; Inderjit & Duke, 2003).

This complex scenario requires a number of different approaches to assess each distinct process (Scognamiglio *et al.* 2013). Thanks to its holistic view, systems biology could be a valuable tool in allelopathy. The combined use of all omics sciences

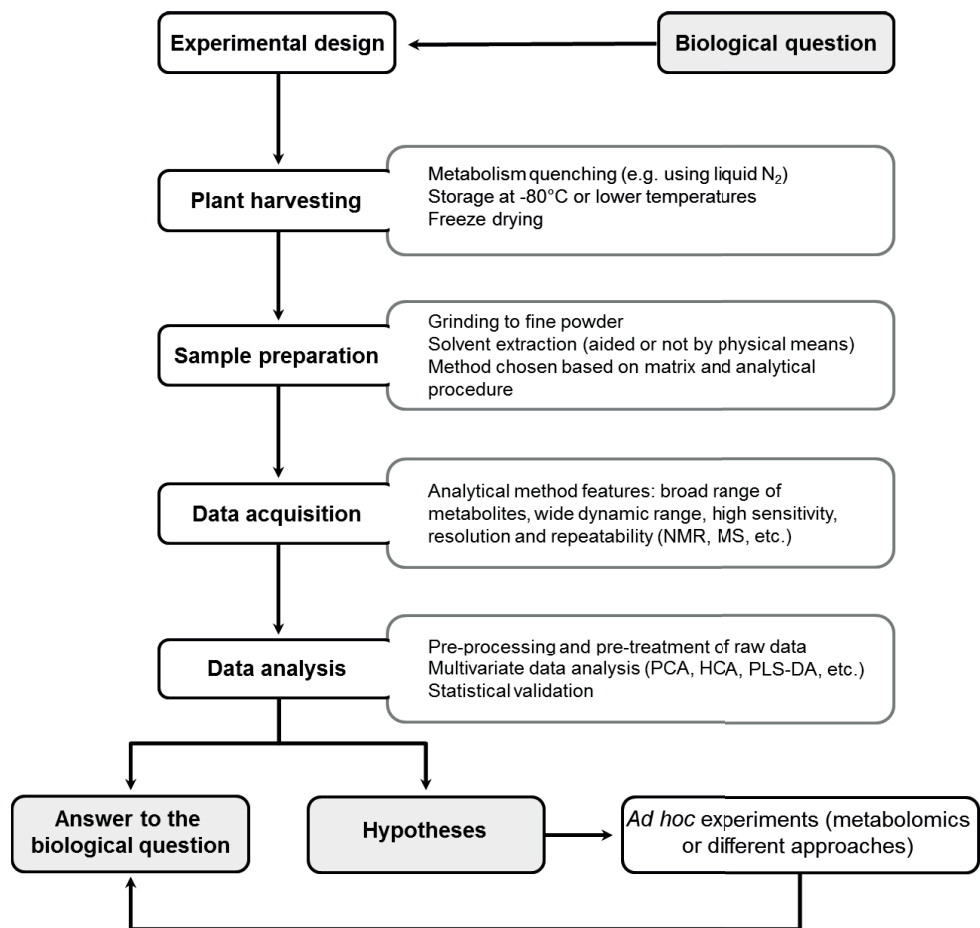


Figure 1. Metabolomics approach workflow.

can be very useful and helpful in this field, enabling scientists to have a full view of the organisms and of the complex processes (Moreno-Risueno *et al.* 2010; Urano *et al.* 2010). Among the omics sciences, metabolomics has several advantages in chemical ecology: because all metabolites are measured at once, also unstable compounds can be detected, additive and synergistic effects are also more likely to be evidenced, since metabolites are analysed together (Forseth and Schroeder 2011). Furthermore, it is a high throughput approach, thus allowing the analysis of many different samples in relatively short times. Finally, it gives a broad view of the biochemical status of the organism: the metabolome represents the end result of all processes in the cell from the transcriptional, translational and post-translational cellular events, defining cells' biochemical phenotype (Macel *et al.* 2010).

Based on these considerations metabolomics could be applied to allelopathy studies. Herewith the utility of metabolomics in the study of allelopathy is presented, and examples of application in several fields of chemical ecology are reported.

Metabolomics

Metabolomics is the non-targeted qualitative and quantitative analysis of all low molecular weight metabolites (<1500 Da) in a biological sample (Kim *et al.* 2011; Verpoorte *et al.* 2007). It is a component of systems biology, along with the other omics technologies. These analyses, based on the comprehensive monitoring of global metabolite networks and their fluctuation in response to various stimuli, give a broad view of the biochemical status of an organism, or more broadly, of a biological system. The metabolome, in particular, provides a snapshot of the end results of all processes in the cell from the transcriptional, translational and post-translational events, giving clues about what is going on in the system and defining its biochemical phenotype (Sumner *et al.* 2003). This one-time-point image might not be enough for studying dynamic processes, but this problem can be overcome by taking many subsequent pictures and integrating all the information, or by using fluxomics (Kim *et al.* 2011; Ratcliffe and Sachar-Hill 2006). It can be fairly stated that while genomic, transcriptomic and proteomic approaches predict what is expected to happen

in an organism, metabolomics portrays what is really happening (Aliferis and Jabaji 2011).

Metabolomics stands at the interface of biology and chemistry: its object is the entire complement of small molecules, but the subject is the study of biological phenomena. Noteworthy, due to its untargeted nature, it is a data-driven, hypotheses generating approach.

This field is relatively new: the first publication about metabolomics date back to 1998 (Oliver *et al.* 1998). It is slowly growing and not completely standardized, due to technical problems: as a consequence of its highly variable target, metabolomics does not have well defined methods, contrary to the other omics sciences.

Besides the application of metabolomics in different fields of research and analyses (Sumner *et al.* 2003, Verpoorte *et al.* 2007), it is usefully applied to the study of the responses to several stresses and some kind of interactions (Kim *et al.* 2011): this issue will be further examined in the present paper.

The use and contribution of metabolomics and related approaches in chemical ecology is thoroughly exemplified by Prince and Pohnert (2010), who reported two examples of how metabolomics could be useful, describing the available approaches and advantages (viz. detection of unstable compounds, detection of additive or synergistic effects, identification of chemical signals and definition of their effects at the phenotype level) and disadvantages (viz. problems mainly related to the inherent noisiness of biological and ecological systems). However, although it has been applied to several fields, its full potential in chemical ecology, and especially in plant-plant interactions is yet to be understood (Jones *et al.* 2013).

Besides metabolomics, other related technologies have been introduced, to date: metabonomics, metabolic profiling, target metabolite analysis, metabolite fingerprinting, and metabolite footprinting. The literature is somehow confusing when defining what the aim is of each of these, but the readers are invited to refer to the definition reported in the glossary (Box 1).

A true metabolomic study has the aim to investigate a problem and search for answers to one or more biological questions: it is not a simple report of the metabolite content of a biological system (Kim *et al.* 2011).

Metabolomics workflow: from experimental design to data analysis

Metabolomics requires the analysis of a wide range of different compounds, which differ in many aspects: chemical nature, solubility, and concentration (Schripsema, 2009).

An important premise: a single extraction technique and a single analytical platform do not provide the isolation and the detection of the complete metabolome (Fukusaki and Kobayashi 2005; Wolfender *et al.* 2013).

A broad literature base exists on the analytical and data analysis techniques used in metabolomics, and the readers are invited to refer to this literature for more in-depth understanding of the practical operations and for the analytical approaches (Table 1), as well as to the recent comprehensive review by Wolfender *et al.* (2013) and to the recommendations for reporting metabolite data (Fernie *et al.* 2011; Goodacre *et al.* 2007; Sumner *et al.* 2007). The entire metabolomic experimental scheme from the experimental design, to data mining and biological interpretation is accurately explained in the paper by Wolfender *et al.* (2013). However, in order to introduce metabolomics approach to non-expert readers, the typical workflow is illustrated in Figure 1, and the most relevant aspects are discussed below.

The first step is the experimental design, which will be based on the biological question underpinning the research. As metabolomics usually takes into account a large number of samples, and hence a great work load, the design of suitable experiments is a crucial point (Brown *et al.* 2005). Inherent biological variability has to be taken into account from an early stage of the research, for plants grown in both natural and in controlled conditions.

The second step is plant harvesting. The method used will depend on the study, but it is generally recommended to quench plant metabolism as soon as the plant is harvested in order to avoid unwanted enzymatic reactions (Fernie *et al.* 2011). Freeze-drying process is usually performed prior to extraction, in order to remove water for practical purpose (it may interfere with some extraction and analytical techniques) and to avoid reactivation of enzymes. An important issue is timing: if samples are to be compared, it is recommended to collect samples at the same moment of the day and at the same growth stage. It is also important to monitor all environmental conditions and/or annotate all metadata (Wolfender *et al.* 2013).

Sample extraction and preparation methods highly depend on the matrix to be analysed and on the analytical platform (Kim and Verpoorte 2010). In this perspective, NMR-based metabolomics has the advantage of minimum sample preparation: the extraction can be carried out directly in deuterated solvents, often with a mixture of phosphate buffer in D₂O and MeOD (1:1). This represents a great advantage in light of the need for standardization of procedure.

Once extracted, metabolites need to be analysed, and several techniques can be used. A list of these

Table 1. Reviews on analytical techniques used in metabolomics (gray box indicates the reviewed methodology).

Review paper	Analytical technique							
	NMR	MAS-NMR	LC-MS	GC-MS	CE-MS	DIMS	FT-ICR-MS	FT-IR
Allwood et al. 2008								
Dunn 2005								
Fukusaki and Kobayashi 2005								
Goodacre et al. 2004								
Hall 2006								
Kim et al. 2011								
McGhie et al. 2012								
Schripsema 2009								
Sumner et al. 2003								
Verpoorte et al. 2007								
Wishart 2008								
Wolfender et al. 2013								
Xiao et al. 2012								

techniques and of viewpoints on their advantages and disadvantages in metabolomics application go beyond the aim of the present review, as these topics have been extensively discussed in the literature (Table 1).

Among the techniques, NMR and MS seem to be the most powerful tools, for many reasons. NMR is a physical measurement of the resonances of magnetic nuclei: an external magnetic field is used to align the nuclear spins. Then, they are irradiated with specific frequencies in order to be promoted from a low to a high energy state and the radiation emitted during relaxation is detected. The frequency at which each nucleus resonates depends on its chemical environment. As a consequence, each compound has a highly specific spectrum, which is an information-rich graph. ¹H NMR is commonly used in metabolomics thanks to several advantages: first of all, easy sample preparation, ease of standardization and high reproducibility, being the solvent used and the magnetic field strength the only variables (Verpoorte *et al.* 2007). Furthermore, as the majority of metabolites contain hydrogen, ¹H NMR can be considered as unbiased compared to other platforms (Allwood *et al.* 2008), a desirable characteristic for metabolomics: it implies that in NMR based metabolomics the only bias are introduced by the solvent choice. Also, quantitative analysis is possible (Kim *et al.* 2011; Wishart 2008). The combined use of 1D and 2D NMR techniques allows the rapid identification of all of the compounds present in a mixture.

NMR metabolomics appears to be highly reproducible, it allows the contemporary identification and quantification of a large number of compounds, and needs short times of analysis (including the extraction procedures). The only limitation of NMR is its low sensitivity when compared to mass spectrometry, although sensitivity has been drastically increased with recent advances like the introduction of higher magnetic fields and microcryoprobes (Forseth and Schroeder 2011; Kim *et al.* 2011). A further drawback of NMR is that some classes of lipids can only be identified as total groups and not as individual compounds by means of 1D NMR. On the other hand, NMR, allows the identification of unknown compounds in the analysed mixtures, as it gives important structural information: recent advances in this framework have been registered lately (Forseth and Schroeder 2011).

In MS, ions are generated, separated by their mass-to-charge ratio and analysed by a detector. A number of ion sources and of analysers is available (Xiao *et al.* 2012). The main advantage of MS based techniques is sensitivity, but, on the other hand the identification of metabolites can be tricky, especially for the unknown compounds. The structural elucidation of compound structures is possible only by tandem MS (MS-MS), preferably HR(MS)ⁿ. However, for unknown metabolites, they should be isolated and characterized by NMR for definite structural elucidation (Sumner *et al.* 2007).

MS is usually combined with chromatography (GC or LC-MS): the hyphenated MS approach is useful for quantification and the retention time can be considered as a further hint of metabolite identity (pure standards are needed). However, the quantification requires suitable external calibration (calibration curves) with the use of chemical standards (Allwood *et al.* 2008). Furthermore, GC-MS of plant extracts (if not volatile) requires derivatization, which introduces bias, while LC-MS success mainly depends on the separation step efficiency. The newest LC-MS/MS approaches offer a useful tool for metabolite identification and quantification (Xiao *et al.* 2012), although it is recommended to follow published guidelines (Fernie *et al.* 2011; Goodacre *et al.* 2007; Sumner *et al.* 2007).

The principal drawback of MS-based approaches is their difficult standardization, as a consequence of a number of combinations of ion sources and analysers that strongly impact the analysis output.

It is worth to underline that the choice of the analytical platform and strategy largely depends on the aim of the study, and it is generally accepted that the best results can be achieved by a combination of extraction and analytical measurements (Allwood *et al.* 2008; Kim and Verpoorte 2010).

Raw data are processed and large data sets coming out from metabolomic studies are usually analysed by multivariate data analysis, with the aim to obtain as much information as possible from the samples with the aid of biostatistical softwares. Both unsupervised (PCA, HCA, SOM independent component analysis) and supervised algorithms (PLS-DA, hierarchical clustering, DFA) are used (Allwood *et al.* 2008; Fukusaki and Kobayashi 2005; Sumner *et al.* 2003). Once again, a detailed survey of the approaches is available in the literature (Brown *et al.* 2005; Goodacre *et al.* 2004; Wolfender *et al.* 2013), and dedicated papers deal with this particular topic (Boccard and Rudaz 2014; Broadhurst and Kell 2006; Jansen *et al.* 2010; Van den Berg *et al.* 2006).

Micrometabolomics

The great improvements in analytical methods, especially with reference to sensitivity, prompted the birth of micrometabolomics and single cell metabolomics. Each tissue or cell in an organism has its own metabolome and each “cellular metabolome” can be differently affected by inherent or external stimuli: the analysis at this level can allow the detection of possible key metabolites, but also a better understanding of the response to biotic and abiotic interference (Moco *et al.* 2009).

This approach is possible thanks to laser-assisted

microdissection application, which is able to separate cells or tissue pieces. These samples are then analysed by MS and/or NMR. Clearly, MS (paired with chromatographic techniques) is the method of choice in this framework, due to its high sensitivity, but also NMR techniques are used thanks to the latest improvements in hardware, pulse sequences and spectral acquisition protocols (Moco *et al.* 2009; Schneider and Holscher 2007). Nonetheless, MS- and NMR-based imaging approaches have successfully been used in this field (Moco *et al.* 2009; Zenobi 2013).

Metabolomics in plant environmental responses - Ecometabolomics

The application of metabolomics in the study of interactions of organisms with their environment is known as ecometabolomics, or more generally, as environmental metabolomics (Sardans *et al.* 2011). This approach has many advantages for studying biotic and abiotic interactions between the organism and the environment, as well as for assessing organism function and fitness at molecular level (Bundy *et al.* 2009).

The emergence of environmental metabolomics represents one of the most important developments in plant physiology and ecology. Recently, the application of metabolomics and related techniques (Box 1) for profiling of plants in terms of small molecule content has been reviewed (Brunetti *et al.* 2013). Any change in growth conditions that modifies the metabolic homeostasis is defined as stress. Following a stress, plants adjust their metabolic pathways (acclimation) to face external solicitations. The plant responses to different abiotic and biotic stresses are also examined (Shulaev *et al.* 2008).

Metabolomics for abiotic plant stress responses

Plants regularly encounter adverse growth conditions. Climatic factors, such as extreme temperatures (heat, cold, freezing), drought (deficient precipitation, winds), irradiations, and contamination of soils (salt, heavy metals, pesticides) are the main abiotic plant stressors (Table 2). The metabolic network of plants is perturbed by these abiotic factors and must be reconfigured in order to allow both the maintenance of metabolic homeostasis and the production of compounds that ameliorate the stress resilience (Krasensky & Jonak 2012).

Based on metabolomics approaches, stress compounds such as proline, γ -amino butyrate and polyamines are reviewed (Obata & Fernie 2012). Specific reviews on plant responses to salinity or temperature stresses are available (Sanchez *et al.* 2008; Guy *et al.* 2008).

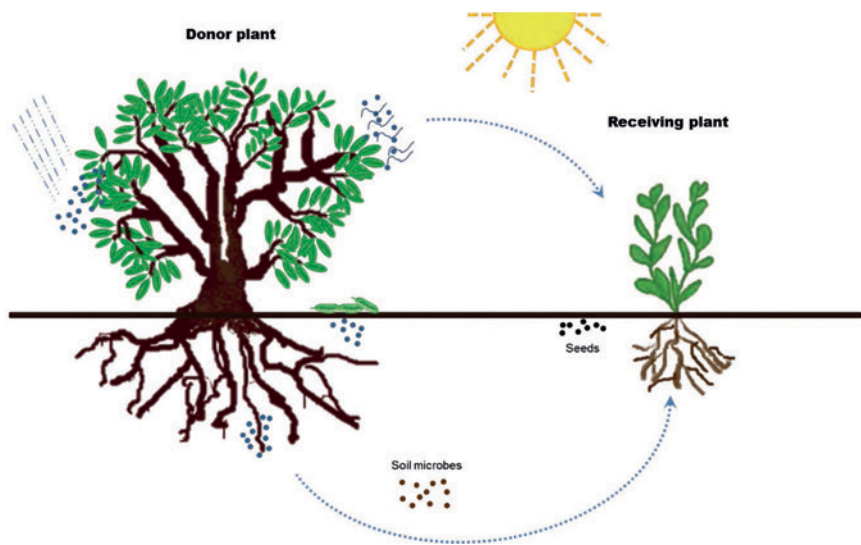


Figure 2. Schematic representation of the allelopathic phenomenon. The donor plant synthesizes and releases allelochemicals (blue circles). Virtually all plant organs can do it and the release pathways are variable. Once released, allelochemicals might be modified (or not) by biotic and/or abiotic factors and, finally, they can reach the receiving plant, affecting it in a direct or indirect manner.

Metabolomics for biotic plant stress responses

The high sensitivity and potentiality of metabolomic techniques in detecting molecular responses of plants to biotic interactions are reported in the literature (Sardans *et al.* 2011; Bundy *et al.* 2009; Jansen *et al.* 2009; Weingart *et al.* 2013). However, the studies available in this field are very scarce and carried out mainly on the basis of metabolic profiling analyses or on the modification of specific classes of secondary metabolites involved in specific processes. Some recent examples of studies on plant responses to biotic interactions are reported in Table 3. The most studied interactions are between plants and microorganisms, especially fungi and bacteria.

Potential for applying metabolomics approaches in allelopathy studies

Plant-plant interactions

Allelopathy is defined as the beneficial or harmful influence of chemical substances (belonging to diverse chemical groups) produced mainly from the secondary metabolism of plants, microorganism, and fungi that affect the growth and development of nearby plants or microorganisms, and interfere with the dynamical processes of agricultural and natural ecosystems (De Albuquerque *et al.* 2011). According to the definition of the International Allelopathy Society (IAS), it involves interactions between plants and other organisms, excluding animals (Torres *et al.* 1996), while according to other sources, allelo-

chemicals may be involved not only in plant-plant and plant-microorganisms interactions, but also in plant-insect or plant-herbivore communication (Weir *et al.* 2004).

Here plant-plant interactions will be considered. Two main actors are involved in this interaction: donor plant, which produces and releases the allelochemical, and the receiving plant that is somehow sensitive to it (Fig. 2).

Concerning the donor plant, allelochemicals are present in virtually all organs (leaves, stems, roots, rhizomes, seeds, flowers, pollen, fruits, etc.), which produce or just store them, and they can be found in different concentration in each compartment (Gatti *et al.* 2010). The quantity and emission pathway vary within species (Friedman 1995). Most allelochemicals are released into the environment by root exudation, vaporization, leaching (lixiviation) or decomposition of plant organs (Fernandez *et al.* 2009), depending on their physico-chemical properties and on the specific site of accumulation and/or production. To further complicate this picture, there is a plethora of abiotic (e.g. temperature, light, soil characteristics, rainfall) and biotic (e.g. life cycle, competition, pathogens, pests, parasites or herbivores) factors that may influence the production, release, and nature of allelochemicals (Rivoal *et al.* 2011).

Finally, allelochemicals have to be transported from the donor plant to the receiving plant (Fig. 2): besides the physical distance, the existing ecosystem (made up by microorganisms, fungi, soil organic matter, etc.) could contribute in modifying these molecules.

Table 2. Examples of metabolomics studies for abiotic plant stress responses.

Investigated species	Stress	An. technique	Reference
<i>Oryza sativa</i>	Cold	HPLC	Morsy et al. 2007
<i>Arabidopsis thaliana</i>	Cold	CG-TOF-MS	Cook et al. 2004
<i>Arabidopsis thaliana</i>	Cold	FT-ICR-MS	Gray & Heath 2005
<i>Arabidopsis thaliana</i>	Drought	GC-TOF-MS	Urano et al. 2009
		CE-MS	
<i>Arabidopsis thaliana</i>	Drought/Heat	GC-MS	Rizhsky et al. 2004
<i>Arabidopsis thaliana</i>	Heat/Cold	CG-MS	Kaplan et al. 2004
<i>Arabidopsis thaliana</i> *	Heavy metals	NMR	Le Lay et al. 2006
<i>Silene cucubalus</i> *	Heavy metals	NMR	Bailey et al. 2003
<i>Oryza sativa</i>	Oxidative stress	CE-MS	Ishikawa et al. 2010
<i>Arabidopsis thaliana</i> *	Oxidative stress	CG-MS	Baxter et al. 2007
<i>Oryza sativa</i>	Ozone	CE-MS	Cho et al. 2008
<i>Brassica napus</i>	Pesticides	UPLC-DAD	Petersen et al. 2011
<i>Phaseolus vulgaris</i>	Phosphorus deficiency	GC-TOF-MS	Hernandez et al. 2007
<i>Lycopersicon esculentum</i>	Salt	FT-IR	Johnson et al. 2003
<i>Arabidopsis thaliana</i> *	Salt	GC-MS	Kim et al. 2007
		LC-MS	
<i>Thellungiella halophila</i>	Salt	GC-MS	Gong et al. 2005
<i>Oryza sativa</i>	Salt	GC-MS	Zuther et al. 2007
<i>Arabidopsis thaliana</i>	Sulfur deficiency	GC-MS	Nikiforova et al. 2005
		LC-MS	
<i>Arabidopsis thaliana</i>	UV-B light	LC-QTOF-MS	Kusano et al. 2011
		GC-TOF-MS	
<i>Arabidopsis thaliana</i>	UV-B light	LC-QTOF-MS	Lake et al. 2009
<i>Vitis vinifera</i>	Water/Salt	GC-MS	Cramer et al. 2007
<i>Arabidopsis thaliana</i>	Water/Salt	LC-QTOF-MS	Arbona et al. 2010
<i>Thellungiella halophila</i>			

*cells cultures

Once allelochemicals have reached the receiving plant, they can affect physiological functions like respiration, photosynthesis, ion uptake, enzyme activity, water status, transpiration, stomata opening, hormone levels, cell division and differentiation, signal transduction, gene expression and structure and permeability of cell membranes and walls, etc. (Chon and Nelson 2010; Zhuo and Yu 2006; Reigosa *et al.* 1999). Allelochemicals can also elicit the production of reactive oxygen species (Weir *et al.* 2004; Scognamiglio *et al.* submitted) or of secondary metabolites. The biochemical and metabolic alterations produce morphological changes in receiving plants: seeds appear darkened and swollen, the roots or radicles and shoots or coleoptiles can be reduced, root tips swollen or affected by necrosis, root axes can be curled, root hairs number reduced or increased, etc. (Badhoria 2011). Many allelochemicals seem to influence seed germination. Allelochemicals can also indirectly influence neighbouring species by modifying biotic and abiotic interactions, such as nutrient uptake and bioavailability (Tharayil *et al.* 2008; Tharayil 2009), or positively or negatively affect microorganism populations in the soil (Castaldi *et al.*, 2009).

This two-character picture is absolutely not exhaustive as the biological activities are governed by a number of different factors: individual chemi-

cal structure, antagonistic, additive, sequential, or synergistic effects (An *et al.* 2001). This is probably the situation in field conditions where the amount of allelochemicals is often too low to explain allelopathic activity, justified on the contrary, by interactions among allelochemicals. It has also been demonstrated that the specific mixture of allelochemicals and their specific ratio is more important than mere additive effects (An *et al.* 2001).

Allelopathy studies

Allelopathic interactions can be considered elucidated when many evidences, here discussed, are available (Box 2). Firstly, the existence of chemicals (or their mixtures) able to influence other plant performance should be proven. From the donor plant point of view, beyond the identification of active compounds, also the identification of production and storage sites are very important, along with the identification of emission pathways and release mechanisms. It should be demonstrated that metabolites are released into the soil (or, more generally, into the environment) and can reach the receiving plant. Then it should also be demonstrated that this receiving plant takes up or “senses” these compounds and responds to them. Finally, the modes of action and the possible molecular mechanisms involved should be established.

Table 3. Examples of metabolomics studies for abiotic plant stress responses.

Plant species	Organism species	An. technique	Reference
<u>Plant-fungus interaction:</u>			
<i>Brassica rapa</i>	<i>Leptosphaeria maculans</i>	¹ H NMR	Abdel-Farid et al. 2009
	<i>Aspergillus niger</i>		
	<i>Fusarium oxysporum</i>		
<i>Vitis vinifera</i>	<i>Uncinula nector</i>	¹ H NMR	Figueiredo et al. 2008
	<i>Plasmopara viticola</i>		
<i>Vitis vinifera</i>	<i>Phaeomoniella spp</i>	¹ H NMR	Lima et al. 2010
	<i>Fomitiporia spp.</i>		
<i>Triticum aestivum</i>	<i>Fusarium graminearum</i>	GC-MS	Paranidharan et al. 2008
<i>Horeum vulgare</i>	<i>Fusarium graminearum</i>	LC-ESI-LTQ-Orbitrap	Bollina et al. 2010
<i>Lupinus angustifolius</i>	<i>Colletotrichum lupini</i>	HPLC-DAD	Muth et al. 2009
		LC-MS	
<i>Solanum tuberosum</i>	<i>Rhizoctonia solani</i>	FT-ICR-MS	Aliferis and Jabaji 2012
		GC-MS	
<i>Oryza sativa</i>	<i>Magnaporthe grisea</i>	GC-MS	Jones et al. 2011
		LC-MS	
		¹ H-NMR	
<u>Plant-bacterium interaction:</u>			
<i>Arabidopsis thaliana</i>	<i>Pseudomonas syringae</i>	FT-IR	Allwood et al. 2010
<i>Medicago sativa</i>	<i>Sinorhizobium meliloti</i>	¹ H NMR	Barsch et al. 2006
<i>Citrus sinensis</i> ,	<i>Candidatus liberibacter</i>	CE-DAD	Cavillos et al. 2009
<i>Solanum lycopersicum</i>	<i>Pseudomonas syringae</i>	¹ H NMR	López-Gresa et al. 2010
		LC-MS	
<i>Catharanthus roseus</i>	<i>Phytoplasma</i>	¹ H NMR	Choi et al. 2004
<i>Chicorium intybus</i>	<i>Dickeya dadantii</i>	¹³ C NMR	Effantin et al. 2011
<i>tuberosum</i>			
<i>Brassica rapa</i>	<i>Bacillus subtilis</i>	¹ H NMR	Jahangir et al. 2008
	<i>Staphylococcus aureus</i>		
	<i>Escherichia coli</i>		
<i>Arabidopsis thaliana</i>	Soil microbiome	GC-MS	Badri et al. 2013
<u>Plant-virus interaction:</u>			
<i>Nicotiana tabacum</i>	tobacco mosaic virus	¹ H NMR	Choi et al. 2006
<i>Brassica oleracea</i>	<i>Pieris brassicae</i>	HPLC-DAD,	Ferreres et al. 2007
		HPLC-MS-MS	
<i>Brassica oleracea</i>	<i>Pieris rapae</i>	UPLC-MS	Jansen et al. 2009
<i>Prunus persica</i>	<i>Myzus persicae</i>	¹ H NMR	Poëssel et al. 2006
<i>Solanum lycopersicum</i>	tobacco mosaic virus	¹ H NMR	Lopez-Gresa et al. 2012
<u>Plant-insect interaction:</u>			
<i>Brassica oleracea</i>	<i>Pieris rapae</i>	UPLC-MS	Jansen et al. 2009
<i>Brassica rapa</i>	<i>Plutella xylostella</i>	¹ H-NMR	Widarto et al. 2006
	<i>Spodoptera exigua</i> Hubner		
<i>Vitis berlandier. × V. riparia</i>	<i>Daktulosphaira vitifoliae</i>	GC-MS	Lawo et al. 2011
<u>Plant-herbivore interaction:</u>			
<i>Brassica rapa</i>	<i>Plutella xylostella</i>	¹ H NMR	Schroeder et al. 2006
	<i>Spodoptera exigua</i>		

The demonstration of existence of active chemicals is not a problem as phytotoxicity is easy to check, and isolation and structural elucidation of natural products is well established (Bucar *et al.* 2013). Traditional approaches to discover and assess allelochemicals are based on bioassay-guided fractionation. This approach relies on analytical procedures to separate and characterize chemicals and on suitable bioassays: many successful examples have been reported and recently reviewed (De Albuquerque *et al.* 2011; Macías *et al.* 2007; Scognamiglio *et al.* 2013).

Far less literature is reported dealing with the definitive elucidation of the other phenomena, especially the mode of action. Up to now, the study of the mode of action of allelochemicals lacks reporting of established methods, with highly differing experimental designs being used (Macías *et al.* 2007). The mode of action of most allelochemicals is unknown (Duke 2013), and also the molecular mechanisms are rarely known, although the availability of new techniques and approaches offers useful tools to learn more (Golisz *et al.* 2008; Inderjit *et al.* 2009).

Box 1: Glossary*

Metabolome: the entire complement of small molecules (<1500 Da) in a biological system (organism, organ, tissue, cell).

Metabolomics: untargeted, comprehensive qualitative and quantitative analysis of the metabolome under a given set of conditions.

Metabonomics: measures of the changes (biochemical perturbations) induced by pathophysiological stimuli or genetic modification in biofluids.

Metabolic profiling: identification and quantification of a group of related compounds (frequently belonging to a specific metabolic pathway or sharing common chemical features).

Metabolic fingerprinting: screening tool to differentiate among samples of different origin or undergone different treatments. It is a high throughput qualitative screening of the metabolic composition of the system (no identification or quantitation).

Metabolic footprinting: measurements of metabolites secreted into extracellular growth medium.

Target metabolite analysis: analysis of few metabolites related to a specific metabolic reaction by using optimized extraction and separation/detection techniques.

*Sources: Allwood *et al.* 2008; Dunn *et al.* 2005; Fiehn 2001; Goodacre 2004; Hall 2006; Kim *et al.* 2011

Although the phenomena hitherto analysed are very complex, there is something even more difficult to study: the cosmos existing between the donor and the receiving plant. Secondary metabolites undergo degradation and/or transformation processes activated by biotic (e.g. soil microbes) and/or physicochemical processes (e.g. binding to organic matter, leaching, etc.), phenomena which affect their bioavailability, or more drastically, their nature and activity (Macías *et al.* 2007; De Albuquerque *et al.* 2011).

Based on these considerations, it is clear that greater understanding of allelopathy calls for new methods and new approaches, mainly based on systems biology. Up to now, notwithstanding the need of a global overview in order to elucidate the complicated allelopathic interactions, few papers deal with a system biology approach in this field.

The combined use of all “omics” sciences can be very useful and helpful in chemical ecology, enabling scientists to have a full view of the organisms and of the complex processes related to allelopathy.

Metabolomics and related approaches in allelopathy

Few studies reported a metabolomics approach in allelopathy investigation. A method for the study of plant-plant interference by metabolic fingerprinting has

been reported (Gidman *et al.* 2003). Two species were chosen in order to study their chemical interactions: *Brachypodium distachyon* and *Arabidopsis thaliana*. Plants of the two species were grown solitary and mixed. Samples were harvested at three time points and analysed by FT-IR and PCA. The study demonstrated the power of this approach in detecting changes in the total metabolic profiles of plants in response to biotic interactions, with FT-IR as an appropriate rapid screening prior to targeted metabolite analyses.

Recently, a co-culturing method/metabolomics approach was introduced to study chemically mediated interactions of planktonic organisms (Paul *et al.* 2013). The authors set up a device where different organisms could be grown, with no direct contact, but allowing metabolite exchanges. Diatoms *Thalassiosira pseudonana* and the bacterium *Dinoroseobacter shibae* were grown in this system and metabolic profiling of the diatoms was carried out. Diatoms were extracted, the extracts were derivatized using methoxamine hydrochloride followed by silylation and analysis by GC-MS. Data were analysed by principal coordinate analysis (PCO) and canonical analysis of principal coordinates. MS data and retention times were used to tentatively identify metabolites. The authors found that many metabolites were increased in diatoms co-cultured with bacteria.

Box 2: Evidences needed for allelopathic phenomenon elucidation

Donor plant:

- Existence of chemicals (or their mixtures) able to influence other plant performance.
- Identification of production and storage sites.
- Identification of emission pathways and release mechanisms.

Fate of emitted allelochemicals.

Receiving plant:

- Uptake of allelochemicals or their interaction with receptors (or different indirect effects).
- Mechanisms of action.
- Modes of action.

Very recently, a new approach has been developed for allelochemical studies (D'Ambrosia *et al.* 2013; Scognamiglio *et al.* 2014). In this approach, NMR-based metabolomics is applied to analyse donor plants, the extracts of the donor plants are screened for their activity on a test species, and the receiving plant's morphological and metabolic changes are measured.

From the donor plant point of view, by using multivariate data analysis, plant extract components that correlate with growth inhibition activity can be identified and also synergistic and additive effects can be found.

On the other hand, from the analysis of the receiving plant, the molecular changes induced by the allelochemicals can be observed and their fate in the receiving plant can be studied. This information from a single experimental design that also gives clues about the mode of action to be further explored.

The method has been standardized on a test species: *Aegilops geniculata*. A first experiment was carried out with a known allelochemical (coumarin) in order to understand the best stage of the plant life cycle at which to perform treatment and harvesting. This was achieved by daily sampling of plants and multivariate data analysis of non-treated plants and of plants treated with coumarin. From this first study, it was able to observe how dynamic the response was: harvesting at different time points gave important clues. However,

for practical purposes, a one-time point sampling was chosen for further studies.

At this point, the method was standardized with known allelochemicals (besides coumarin, catechol, p-hydroxybenzoic acid, p-coumaric acid and juglone). From metabolomics analysis of treated plants, the molecular changes upon treatment were detected and were found to be mainly in accordance with the allelochemicals' known mode of action.

The method has been applied to study the effects of *Phillyrea angustifolia* plant extracts prepared at different phenological stages (Scognamiglio *et al.* 2013). Different plant extracts produced a very different response in the target plant metabolome. The most active were those of the cold season (from November to March), characterized by the highest amount of oleuropein and inducing a decrease of *cis*-aconitate and an increase of citrate and malate. This indicated a perturbation in the tricarboxylic acids cycle, suggesting the inhibition of *cis*-aconitase. Based on this and on further observations, as this enzyme is considered an indicator of oxidative stress, the induction of ROS formation was proposed (Baxter *et al.* 2007; Zhao *et al.* 2005).

Hence, the method allowed the observation of responses specific for each extract composition, which depended on the collection time and made it possible to study how phenology could impact plant allelopathic potential.

Recently, the same approach has been applied to a set of plants from the Mediterranean region (Scognamiglio *et al.* submitted).

Metabolomics and allelopathy: a look forward

Metabolomics and metabolic profiling of donor plant can offer useful insights in the identification of active compounds. Plant extracts assayed for their biological activity can be characterized by metabolomics analysis, in order to identify putatively active compounds. Besides the methods discussed in the previous sections, it has never been applied in allelopathy, although many examples exist using metabolomics based techniques to uncover compounds involved in bioactivity from complex natural sources (e.g. plant extracts) (Yuliana *et al.* 2013).

Once the allelochemical has been identified, its production and/or storage tissues could be studied by subsequent restriction of the scale of analysis: from whole plant, to plant organ, to plant tissue, to plant cell. Hence, micrometabolomics and single cell metabolomics could be very helpful. Once these sites are defined, it follows that the way by which these metabolites are released into the environment can be elucidated, as this will depend both on the physicochemical features of the metabolites and on the sites where they are produced or accumulated. While metabolites present in roots are likely to be released by exudation, leaf allelochemicals can follow different pathways for their release. Obviously, volatilization will depend on the volatility of allelochemicals. Furthermore, the study of this emission pathway is easy as the analysis of plant volatiles is well established and maybe the oldest example of a metabolomics-like approach (Beck *et al.* 2013). Leaching will be possible, on the other hand, only if metabolites are deposited on the leaf surface. Otherwise, compounds are likely to be released into the environment by decomposition (Fig. 2). Ad hoc experiments could be useful to validate this hypothesis.

From metabolomic analysis of receiving plant and its organs, it is possible to study the fate of allelochemicals and the metabolic changes induced in the plant.

Concerning the mode of action, metabolomics has been proposed as a tool to explore the mechanism (molecular target site) and mode of action (complete sequence of processes: viz. how a compound is taken up, distributed, metabolized, binds to the target site, and results in changes in the regulation of pathways and in a specific phenotype) of phytotoxic compounds and pesticides (Aliferis and Jabaji 2011; Tresch 2013). Metabolomics can, indeed provide invaluable information on the biochemical status of the organisms

after exposure to bioactive compounds. In this context, plant metabolomics and plant fingerprinting technologies could also be used in the discovery of mode of actions of newly discovered candidate herbicides of natural origin (Aliferis *et al.* 2009).

The potential of metabolomics in the field of allelopathy is also well-supported by many applications in plant stress-response to biotic and abiotic stressors (Tables 2 and 3).

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Phenolic acids in plant-soil interactions: a microcosm experiment

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ABSTRACT

In soil naturally occurring phenolic acids act as allelochemicals and are involved in several plant-soil interactions. In the present study, a microcosm-style experiment was used to investigate the allelopathic responses induced by individually applied *trans*-cinnamic and ferulic acids and coumarin on the root system of maize seedlings grown for 288 h (12 days) under controlled conditions on a sterile 70/30 (v/v) soil/perlite mixture. Preliminary soil incubation assays were carried out to assess the potential bioavailability of these compounds as affected by abiotic sorption/desorption processes. The concentration-dependent sorption coefficients (Freundlich) showed that phenolic acids were rapidly adsorbed onto the soil surfaces following two distinctive sorption mechanisms. Further, recoveries from a 96-h depletion experiment indicated that the bioavailable amount of *trans*-cinnamic and ferulic acids markedly (-97 and -94%, respectively) and continuously decreased over time; whereas available coumarin strongly decreased (-92%) soon after addition remaining unchanged over time. According to these findings, single (coumarin) and pulsed (*trans*-cinnamic and ferulic acids) applications were established during the microcosm experiment to provide resident soil concentration at 0.02-0.15 $\mu\text{mol g}^{-1}$ still able to induce plant responses. Total root length, biomass allocation, root fineness and tissue density of the whole root system and different root types (radicle, seminal, nodal, lateral of the radicle and seminal roots) of maize seedlings were evaluated. Coumarin appeared to be the strongest phytotoxic compound reducing total root length and completely inhibiting the elongation of radicle root and the formation of the other root types. *Trans*-cinnamic acid exerted a marked and rapid action in reducing the total root length showing also a selective action towards the different root types more than ferulic acid. Further, *trans*-cinnamic acid affected both biomass allocation and structural parameters; whereas ferulic acid mainly influenced the root fineness. Results suggested: i) a specific response of maize root system in relation to the allelochemical and time of exposure; ii) maize root types showed a different response to the phenolic acids, and iii) their morphological parameters were selectively affected.

Keywords: Allelopathy, phenolic compounds, root morphology, soil sorption, *Zea mays* L.

Introduction

Allelopathy is known to play an important role in the agro-ecosystems as it has a great potential in improving crop productivity, maintaining genetic diversity and providing biological control of weeds and pests. The increased public concern about environmental health combined with the emergence of herbicide-resistant weed species have made allelopathy an attractive non-chemical strategy for weed management in sustainable agriculture (Weston and Duke, 2003; Macias *et al.*, 2007). Indeed, due to their low environmental persistence, high bioactivity and multiple mode of action, allelochemicals can be used directly or as template molecules for the development of new environmentally-friendly herbicides (Duke *et al.*, 2000). In spite of considerable literature appeared on the phytotoxic effects of allelochemicals and their

mechanisms of action on plant metabolism, a little attention has been given to evaluation of allelopathic interactions at the plant-soil interface. In soil the fate of an allelochemical is primarily governed by several biotic (microbial activity) and abiotic (chemistry of colloidal soil particles) processes, which both influence their activity, mobility and persistence (Rice, 1984; Cheng, 1992; Cheng, 1995; Blum, 1996). In fact, to be effective in influencing the plant growth under field conditions, allelochemicals have to enter the soil, escape sorption, survive chemical and biochemical breakdown and eventually come in contact with target plants (Choesin and Boerner, 1991). Beside microbial activity, organic matter and clay minerals provide soil particles with highly chemically-reactive surface areas, which are able to control the availability and the phytotoxic potential of allelochemicals towards the target plants (Leham and Blum, 1999; Blum, 2006; Tharayil *et al.*,

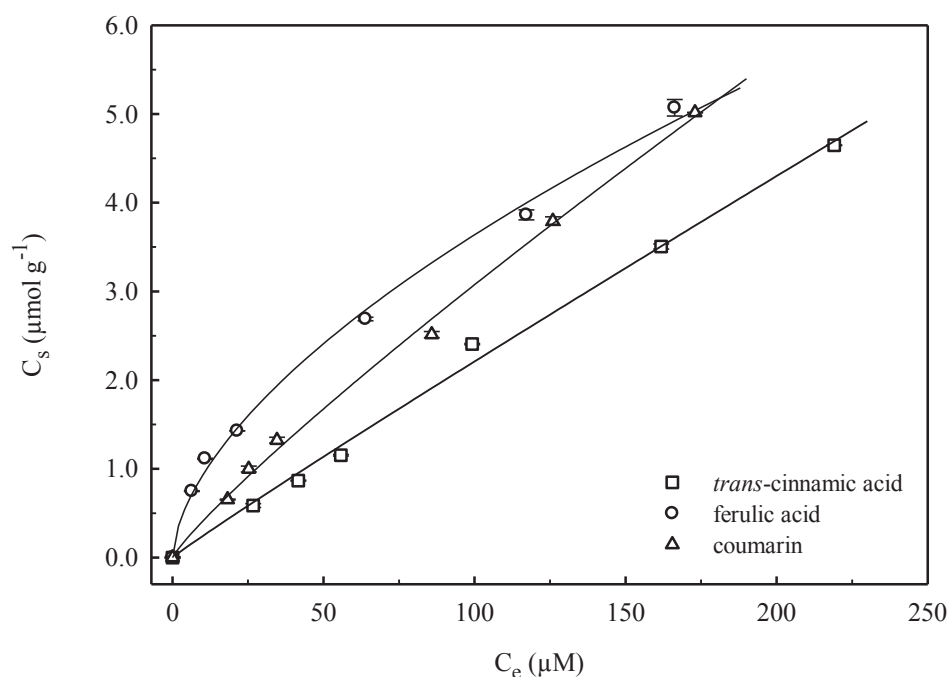


Figure 1. Freundlich adsorption isotherms of *trans*-cinnamic acid, ferulic acid and coumarin on sterile growing substrate. Values are means $\pm$ standard error bars ($n = 3$). Sorption parameters are shown in Table 1.

2006). Allelopathic substances include a range of secondary metabolites such as alkaloids, terpenoids, and phenolics. Among them, phenolic compounds are common and ubiquitous molecules entering the soil through leaching, root exudation and rhizodeposition, litter degradation and microbial synthesis (Bates-Smith, 1956; Harborne, 1998; Blum, 2004 and 2006). Phenols drive complex soil-plant-microbes interactions and they are considered to play a pivotal role in both natural and agricultural ecosystems (Rice, 1984). They inhibit the root growth of many different species (Fujita and Kubo, 2003; Abenavoli *et al.*, 2004), interfere with water and nutrient uptake (Pospíšil *et al.*, 1987; Booker *et al.*, 1992; Yu and Matsui, 1997), and control plant succession in natural ecosystems (Inderjit and Mallik, 2002; Vermerris and Nicholson, 2006; Zeng *et al.*, 2008).

For instance, using different mixtures of plant phenolic acids representative of plant exudates composition, Tharayil *et al.* (2006) confirmed the important role of site-specific soil sorption in influencing the availability and the phytotoxic action of various plant exudates. Within this context it becomes of great interest to investigate how the potential bioavailability of allelopathic compounds is governed by sorption/desorption processes occurring at the soil surface level.

In the present study the soil-plant responses to some phenolic compounds in microcosm-

style experiment were studied. In particular, the present work was addressed to investigate the morphological responses (length, fineness, density) of the root system in maize seedlings grown for 288 h (12 days) under controlled conditions into a sterile soil-based substrate amended with *trans*-cinnamic acid, ferulic acid and coumarin. In order to realistically describe these processes and provide a reliable estimate of the bioavailable amount of these phenolic compounds as derived by abiotic interactions occurring in the soil matrix, equilibrium concentration and persistence of single compounds was previously investigated by using sorption and depletion experiments.

Material and Methods

Chemicals

Three phenolic acids, namely *trans*-cinnamic acid (*trans*-3-phenyl-2-propenoic acid), ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid] and coumarin (1,2-benzopyrone) of analytical grade (> 99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Aqueous phenolic solutions were purified by passing them through a sterile 0.22 μm-pore cellulose acetate filter (Millex®-GV, Millipore Corporation). All solvents were of HPLC grade.

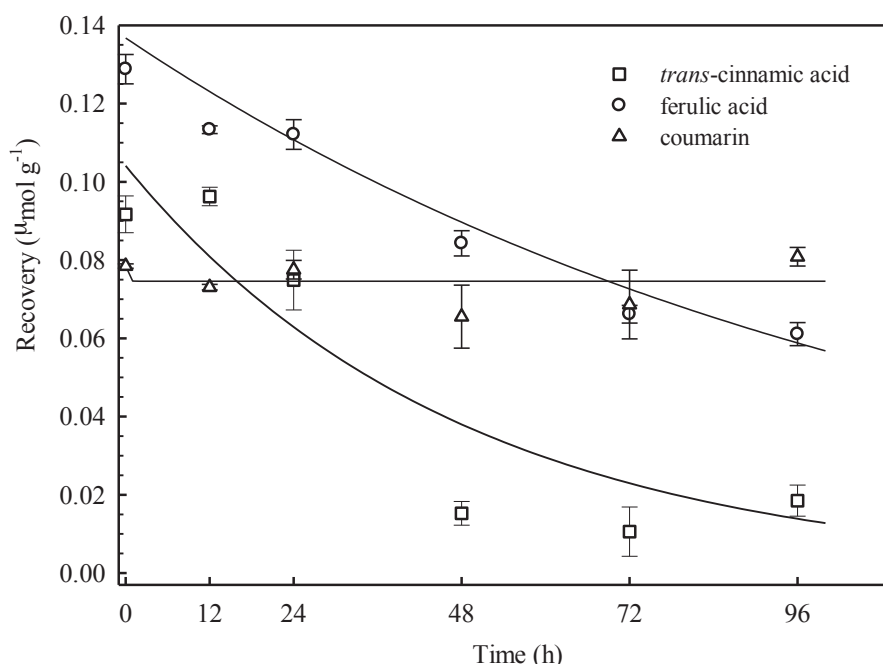


Figure 2. Kinetics of depletion of *trans*-cinnamic acid, ferulic acid and coumarin (added amount was 1 $\mu\text{mol g}^{-1}$ growing substrate) from sterile growing substrate under short-term incubation assays. Recoveries following 0.5 M K_2SO_4 extraction are shown as means $\pm$ standard error bars ($n = 3$) and were fitted to the Eq. (3). Depletion parameters are shown in Table 2.

Soil and growing substrate

The soil used for the microcosm experiment was an arable clay loam soil (sand 34.1%, silt 32.2%, clay 33.7%, $\text{pH}_{\text{H}_2\text{O}}$ 7.2 ± 0.1 , total organic C 19.3 ± 0.4 g kg^{-1} dw soil, total N 1.8 ± 0.2 g kg^{-1} dw soil, C/N ratio 10.7, cation exchange capacity (CEC) 20.9 ± 1.3 $\text{cmol}_{(+)}$ kg^{-1} dw soil, total CaCO_3 8.4 ± 0.5 g kg^{-1} dw soil, electrical conductivity ($\text{EC}_{1:1}$) at 25°C 0.271 ± 0.021 dS m^{-1} , bulk density 1.23 ± 0.04 kg dm^{-3}) freshly collected from the Ap layer (0–15 cm) of an agricultural field located at the Agricultural Experimental Station of the University of Reggio Calabria (South Italy). Soil properties were determined according to the standard methods recommended by the Soil Science Society of America (Sparks, 1996). After collection, the arable soil was air-dried (1 day), coarse sieved at <4 mm particle size, and then thoroughly mixed with commercial perlite (Agrilit[®]3, purchased from Perlite Italiana s.r.l., Milano, I) to prepare a 70/30 (v/v) soil/perlite mixture (growing substrate). Perlite properties were: particle size 2–5 mm, bulk density 100 ± 20 kg m^{-3} , pH 6.5–7.5, CEC 0.79 $\text{cmol}_{(+)}$ kg^{-1} , EC at 25°C 0.02 dS m^{-1} (1 $\text{dS m}^{-1} = 1000$ $\mu\text{S cm}^{-1}$), water content at -1 kPa 26.30%, water availability 12.97%. The growing substrate was autoclaved at 120°C and 1 bar for 20 min, incubated at 25°C for 24 h and autoclaved an additional 20 min, according to Wolf and Skipper (1994). Absence of culturable

microorganisms was confirmed by plating soil dilutions on half-strength nutrient agar.

Sorption experiment

Batch adsorption isotherms on sterile growing substrate were determined at $25 \pm 2^\circ\text{C}$. Samples of 1 g of sterile growing substrate were equilibrated in sterile polyallomer centrifuge tubes with 5 ml of the aqueous phenolic acid solution ranging from 0 to 3.2 mM, consistently with the water solubility of each allelochemical. Tubes were stirred using Teflon-coated magnetic stir bars for 4 h in the dark. Preliminary studies indicated that 95% adsorption occurred within the first 30 min. After the equilibrium was reached, the suspension was centrifuged (5000 g, 5°C , 15 min) and the clear supernatant recovered for HPLC analysis. The pH of aqueous suspensions was also checked. All studies were done in triplicate and included a blank tube (reference) containing the solution without the sterile growing substrate.

Depletion experiment

Depletion of available phenolic acids was determined by incubating the sterile growing substrate with *trans*-cinnamic acid, ferulic acid or coumarin at a fixed concentration (1 $\mu\text{mol g}^{-1}$ growing substrate) under controlled conditions (temperature

25±1°C, relative humidity 75%, day/night 16/8 h, photosynthetically active radiation, "PAR" 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 0, 12, 24, 48, 72 and 96 h. At each sampling time, the growing substrate was extracted with 0.5 M K_2SO_4 (1:4, w/v, soil:solution) under agitation (120 rpm, 60 min) at room temperature and then the soil slurry was filtered through a Whatman® No. 42 filter paper. Clear extracts were stored at -20°C prior to analytical determination. All studies were done in triplicate and included a blank tube (reference) containing the solution without the sterile growing substrate.

Microcosm experiment

The microcosm-scale experiment was carried out in a growth chamber located at the University of Reggio Calabria for 288 h (12 days). The microcosm units consisted of 1.5 L polyvinyl chloride (PVC) pipes (30 cm height x 8 cm i.d.) closed at the bottom by a layer of 35 μm -thick nylon stocking material (Normesh Limited, Lancashire, UK; code NN35/16115R). Before using, the PVC pipes were cleaned carefully and surface sterilized with GNeoSterixidina® (GOLMAR® SpA, I) and then immediately filled with the sterile 70/30 (v/v) soil/perlite mixture (growing substrate). A sterile soil-based substrate was used to give prominence to abiotic sorption processes. The microcosms were randomly set up in a 2 x 2 x 3 experimental block (3-time replicated in the experimental design) with 2 plant treatments (with and without maize plant), 2 soil treatments (with and without phenolic acids) and 3 phenolic acids. At the beginning of the trial (time zero), phenolic acid-amended microcosms were supplied with sterile aqueous solutions of *trans*-cinnamic acid, ferulic acid or coumarin (each at an equivalent concentration of 1 $\mu\text{mol g}^{-1}$ growing substrate). Additional amounts of *trans*-cinnamic acid and ferulic acid (at 1 $\mu\text{mol g}^{-1}$ growing medium) were added at 48, 96 and 192 h. A same amount of sterile distilled water was added to control (no phenolic acid amendment) soil microcosms. Phenolic acids in soil materials were extracted with 0.5 M K_2SO_4 (as seen for the desorption study) at time zero, immediately (30 min) before and after supplementary additions at 48, 96, 192 and then at 288 h to quantify short-term depletion of phenolic acids. Unamended microcosms were monitored accordingly. Pre-germinated maize seeds (*Zea mays* L., var. Cecilia, PR34N84-250, kindly provided by Pioneer HI-Bred Seeds Italy s.r.l., PR, I) (~1 cm root length) were transplanted onto each soil microcosm (1 plant for microcosm) (start of the trial) and let grow for 288 h. Growth chamber conditions were as previously reported in the decay experiment. No nutrients were provided during the growing period.

After 48, 96, 192 and 288 h from transplanting, soil microcosms were destructively sampled and maize plants were gently removed from soil, separated into shoot and root, rinsed with de-ionized water and their fresh weight determined. The root system was stained with 0.1% (w/v) toluidine blue O for 5 min and then scanned (WinRhizo STD 1600, Instruments Régent Inc., Canada) at a resolution of 600 dpi for morphological analyses (root length Lr, root volume Vr). Afterwards, root dry weight (DWr) was determined by oven-drying at 70°C until a constant weight was reached. Root fineness ($\text{Fr} = \text{Lr}/\text{Vr}$) and tissue mass density ($\text{TMDr} = \text{DWr}/\text{Vr}$) were then calculated.

HPLC analysis

Before the chromatographic analysis, a 2-ml aliquot of supernatant was filtered through a sterile 0.22- μm pore cellulose-acetate filter (Millex®-GV, Millipore Corporation, MA) and then a 1-ml aliquot of the filtrate was further purified by passing it through a Waters Sep-Pak® classic C_{18} cartridge (Millipore Corporation). The high-performance liquid chromatography (HPLC) analysis was carried out using a PerkinElmer LC 250 binary pump (Norwalk, CT) equipped with a Rheodyne 7125 injector (20- μl loop), a 4.0 x 3.0 mm SecurityGuard™ Max-RP (C_{12}) cartridge (Phenomenex®, CA) and a 250 x 4.6 mm i.d. Synergi™ Hydro-RP C_{12} (4 μm) analytical column (Phenomenex®). The mobile phase (0.7 ml min^{-1} flow rate) was acetonitrile/acetic acid/water (20:1:79 by vol) for the ferulic acid elution and acetonitrile/acetic acid/water (15:1:84 by vol) for the *trans*-cinnamic acid and coumarin elution. A PerkinElmer LC 135 diode array detector operating at 275 nm (*trans*-cinnamic acid and coumarin) or at 325 nm (ferulic acid) was used for analytical detection. Identification and processing of the chromatographic data was done by a PerkinElmer Omega-2 Analytical Workstation.

Data analysis

Data were firstly tested for deviation from normality (Kolmogorov-Smirnov test) and homogeneity of within-group variances (Levene's test) (Brown and Forsythe, 1974). After running a one-way ANOVA to check any significant effect of the treatment on the variability of the data (the block effect in the experimental design was found to be not significant at $P < 0.05$), multiple pairwise comparison of means was done by Tukey's HSD (Honestly Significant Difference) test at $P < 0.05$ level of significance.

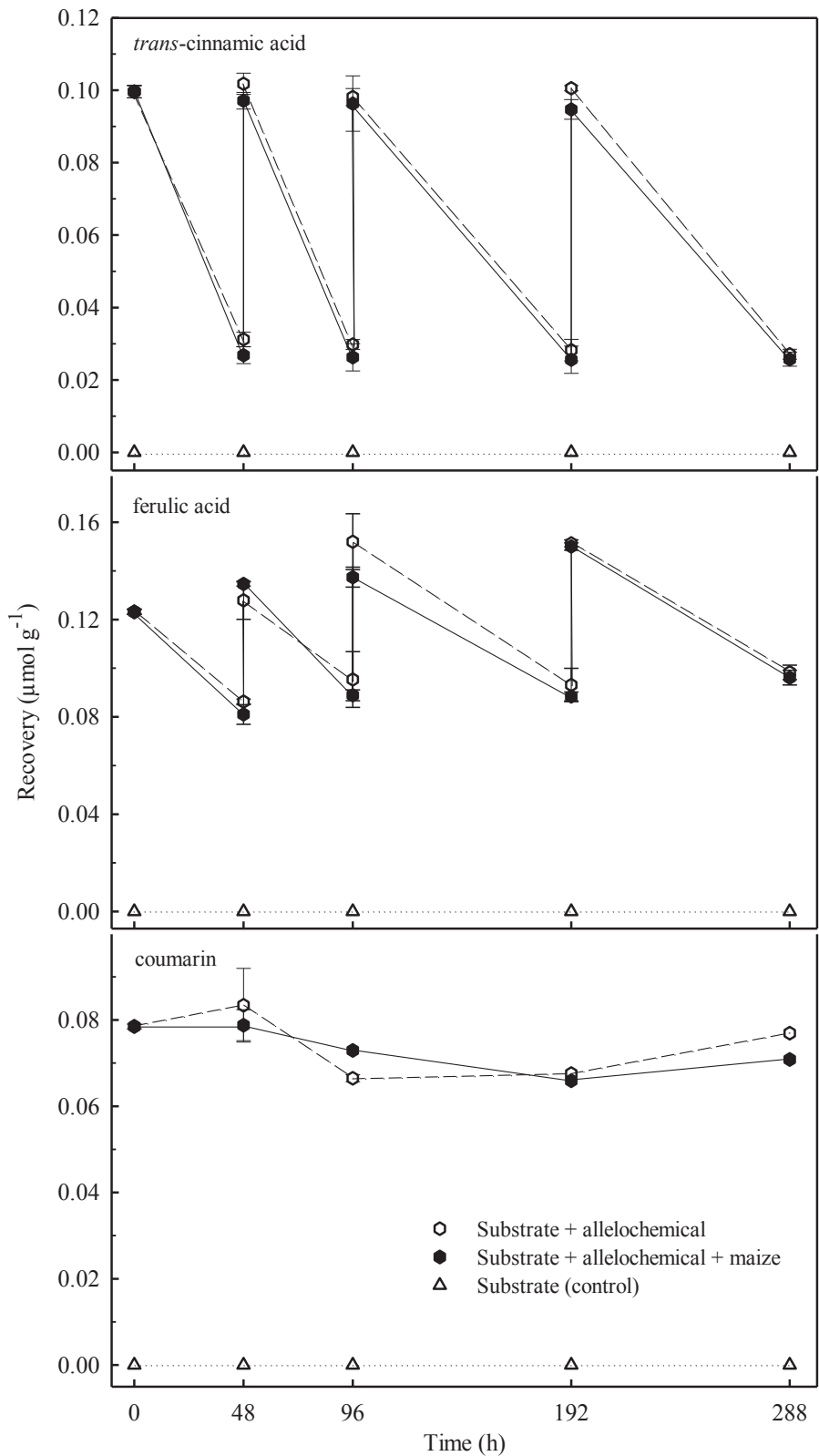


Figure 3. Changes in available amount of *trans*-cinnamic acid, ferulic acid and coumarin in soil microcosms following initial (time zero) addition ($1 \mu\text{mol g}^{-1}$ growing substrate). Supplementary additions ($1 \mu\text{mol g}^{-1}$ growing substrate) of *trans*-cinnamic and ferulic acids were done after 48, 96 and 192 h. Recoveries were determined by $0.5 \text{ M K}_2\text{SO}_4$ extraction 30 min before and after 48, 96 and 192 h, followed by a final sampling after 288 h. Values are means $\pm$ standard error bars ($n = 3$).

The extent of allelochemical sorption on sterile growing substrate was estimated as the difference between initial and final concentration in the solution. Sorption data were fitted to the Freundlich equation $C_s = K_f C_e^{1/n}$ (Eq. 1), where the amount adsorbed per mass of sorbent (C_s , in $\mu\text{mol g}^{-1}$) is plotted *vs* the equilibrium concentration (C_e , in mM). K_f and $1/n$ are empirical constants describing the sorption capacity and sorption intensity, respectively. The distribution coefficient $K_d = C_s/C_e$ was also calculated at $C_e=10 \mu\text{M}$ level.

Recoveries of allelochemical from the sterile growing substrate were used to draw a time-dependent curve showing depletion of phenolic acid from the autoclaved substrate. Depletion kinetics was described according to a mathematical model representing a non-linear, first order equilibrium equation:

$$-\frac{dC}{dt} = k_{ad} \cdot C_t - k_{des} \cdot (A_0 - C_t) \quad (\text{Eq. 2})$$

where A_0 is the initial concentration of each compound; C_t is the recovered amount at time t ; k_{ad} and k_{des} are, respectively, the forward (adsorption process) and reverse (desorption process) rate constants. The Eq. 2 may be written in integrated form as the exponential function:

$$f(t) = \frac{A_0 \cdot (k_{des} + k_{ad} \cdot \exp(-t \cdot (k_{ad} + k_{des})))}{(k_{ad} + k_{des})} \quad (\text{Eq. 3})$$

where $f(t)$ represents the concentration of the allelochemical, in $\mu\text{mol g}^{-1}$ of substrate, in response to the incubation time t , in hours; A_0 , k_{ad} and k_{des} are same as above. Eq. 3 could also be used to describe the phenolic acid depletion as a consequence of adsorption/desorption equilibrium processes occurring at the solid-liquid interface. After a certain period of time, this decline reaches an equilibrium concentration (C_{eq}) that can be estimated according to the following equation $C_{eq}=A_0 \cdot (k_{des}/(k_{ad}+k_{des}))$ (Eq. 4), which is derived from Eq. (3) when longer times are being considered. The best fit of Eq. 1, 2 and 3 to analytical data, and estimates of empirical parameters (K_f , $1/n$, A_0 , k_{ad} , k_{des} and C_{eq}) were carried out through non-linear regression analysis using the algorithm of Levenburg-Marquardt (TableCurve 2D v.5.01 software, SYSTAT Software Inc.). Non-linear regressions were repeated several times in order to minimize the sum of the square of deviation between the predicted and experimental values to less than 0.01% between two consecutive fits. The quality of curve fitting was assessed by F test for non-fit based on analysis of variance at $P < 0.05$. Graphs were drawn by using the SigmaPlot v.10.0 (SYSTAT Software Inc.).

Results and Discussion

Adsorption dynamics of phenolic acids

Sorption isotherms of *trans*-cinnamic and ferulic acids and coumarin showed a different binding mechanism on the sterile growing substrate (Fig. 1). According to the classification proposed by Giles *et al.* (1974), *trans*-cinnamic acid and coumarin isotherms were of C-type, which describes a constant partition of the binding molecule between the aqueous solution and the adsorption sites of the growing substrate. Linearity shows that the number of sites for adsorption remains constant throughout the whole range of solute concentration up to saturation of the substrate. This finding suggests that the more the substrate is being added, the more the sorption can take place. On the other hand, the ferulic acid sorption isotherm was of L-type (Fig. 1). This indicates that sorption becomes increasingly difficult for a bombarding molecule substrate and the affinity between the molecule and the adsorbent surface decreases as more solute is being adsorbed. The occurrence of these two distinctive mechanisms of allelochemical adsorption on the sterile growing substrate was further confirmed by the empirical sorption parameters K_f (capacity) and $1/n$ (intensity), which have been estimated by fitting the empirical Freundlich relationship (Eq. 1) to sorption data. In fact, $1/n$ was close to 1 for *trans*-cinnamic acid and coumarin, and this indicates a sorption curve with steady slope close to linearity (Table 1). Whereas in the experiments with ferulic acid $1/n$ deviated considerably from linearity, thus confirming a concentration-dependent sorption dynamics (Table 1). The sorption affinity coefficient K_f was rather low in all cases and the general order of sorption magnitude was ferulic acid > coumarin > *trans*-cinnamic acid. The distribution coefficient K_d ranked the allelochemicals with the same sorption affinity (Table 1). The occurrence of dynamic sorption/desorption processes represents a key soil factor affecting the environmental fate (i.e. mobility, persistence, etc) and the phytotoxic activity of allelochemicals in soil (Kobayashi, 2004). In fact, soil surfaces are able to adsorb phenolic acids through several different sorption mechanisms involving either the mineral or the organic fractions (Dalton *et al.*, 1989; Blum *et al.*, 1999; Ohno, 2001). In general, such an order of sorption affinity on soil surfaces as observed here (ferulic acid > coumarin > *trans*-cinnamic acid) has previously been reported (Tharayil *et al.*, 2006); and the greater adsorption of ferulic acid was related to the methoxy substitution on the aromatic ring (Dalton *et al.*, 1989). Moreover, the adsorption of *trans*-cinnamic acid and,

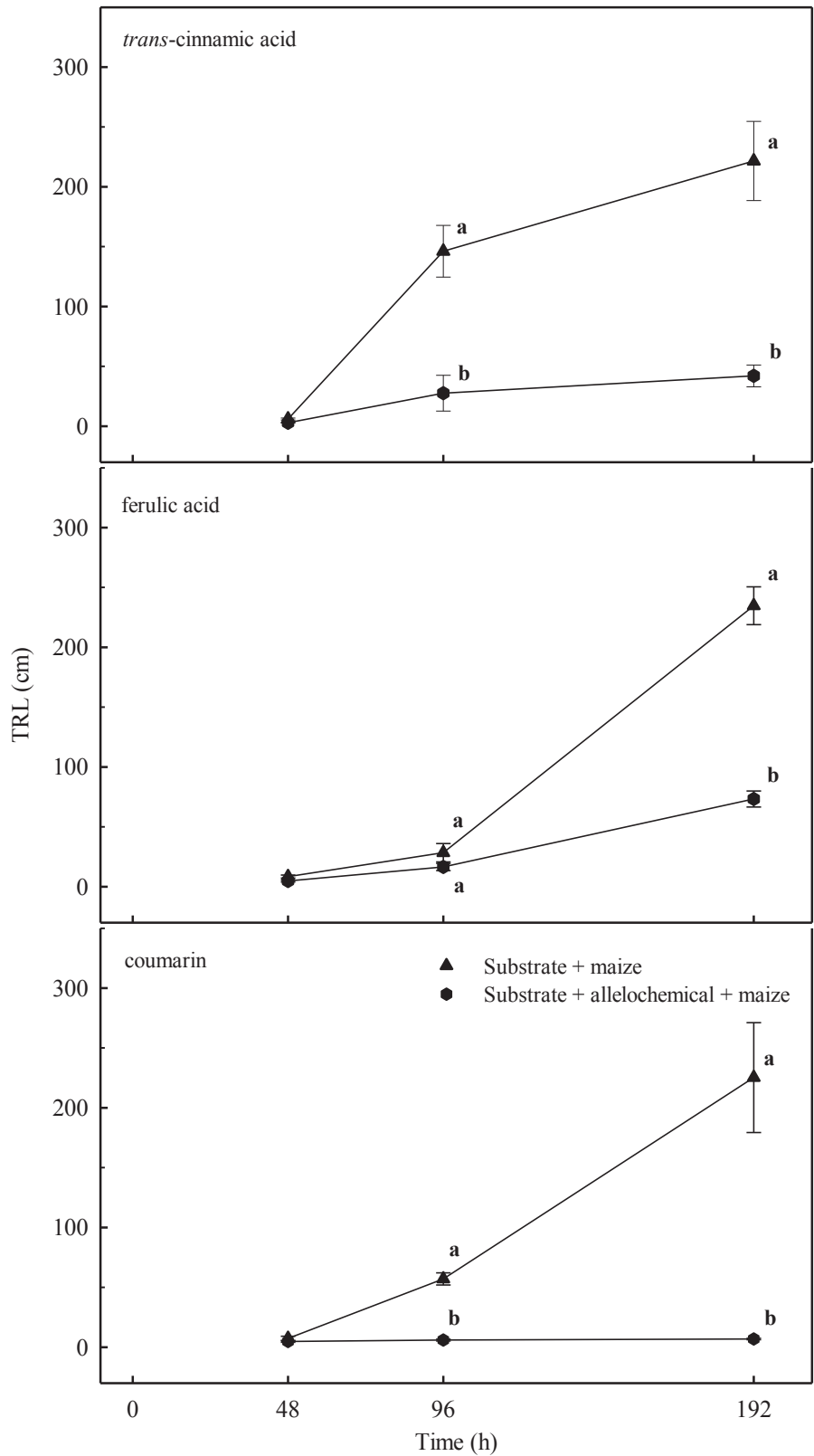


Figure 4. Changes in total root length (TRL) of maize seedling roots as induced by *trans*-cinnamic acid, ferulic acid and coumarin at three sampling times (48, 96 and 192 h) during the microcosm experiment. Values are means $\pm$ standard error bars ($n = 3$). At each sampling time different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Table 1. Freundlich parameters (K_f and $1/n$) and distribution coefficient (K_d) for the adsorption of *trans*-cinnamic acid, ferulic acid and coumarin on sterile 70/30 (v/v) clay arable loam soil/perlite. The coefficient of determination (r^2) and the significance level (as F -stat) for all curves are also shown.

Phenolic acid	K_f	$1/n$	r^2	F -stat [#]	K_d [§]
<i>trans</i> -cinnamic acid	0.03	0.96	0.99	2677.25***	0.02
ferulic acid	0.24	0.59	0.99	1644.32***	0.09
coumarin	0.05	0.88	0.99	2088.71***	0.04

[#] level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

[§] referred to a $C_e = 10 \mu\text{M}$.

at greater extent, coumarin could be explained by the hydrophobicity of the two molecules, which determines their binding affinity with the organic material. It also true that ionic interactions are also important. It was found that type and concentration of the added phenolic acids affected selectively the pH of the soil slurry. The addition of ferulic and *trans*-cinnamic acids caused a significant dose-response decline of the pH, which constantly decreased from 7.6 (control, 0 mM treatment) to approx 7.0 (3.2 mM treatment). Conversely, coumarin addition did not vary the pH of the soil slurry, which remained stable at 7.6 in all treatments. Because the pK_a value of ferulic and *trans*-cinnamic acids is somewhat lower (4.7) than the pH of the slurry (~7.0), this finding indicates that during the sorption experiment they both occur as phenolic acid anions. On the contrary, coumarin has no hydrolizable functional groups and therefore remains unchanged under the experimental conditions. Then, anionic exchange reactions cannot be invoked as a significant mechanism explaining the observed sorption magnitude between the allelochemicals and soil substrate surfaces. Since microorganisms or soil enzymes were excluded from the growing mixture by repeated sterilization, the decrease in recovery should be primarily attributable to an abiotic process due to physicochemical interactions with colloidal soil surfaces.

Depletion dynamics

Losses of *trans*-cinnamic acid, ferulic acid and coumarin from the growing mixture varied considerably with time and the particular type of allelochemical added (Fig. 2). In fact, the K_2SO_4 -extractable amount of *trans*-cinnamic acid and ferulic acid markedly decreased during the 96-h incubation assay and final recoveries were considerably low: -97 and -94%, respectively. Conversely, the amount of K_2SO_4 -extractable coumarin strongly decreased (-92%) soon after addition but then remained fairly unchanged over time (Fig. 2). The mathematical model of Eq. 3 was applied to the recovery data in order to describe the concentration decline of these

two cinnamic acids as a consequence of adsorption/desorption equilibrium processes occurring at the solid-liquid interfaces. If so, k_f and k_r would indicate the rate kinetic constants for the adsorption (k_{ad}) and desorption (k_{des}) process, respectively. After a certain period of time when the rate of adsorption equals the rate of desorption, the soluble concentration has reached a dynamic equilibrium (Fig. 2) that may be calculated using the kinetic parameters estimated according to the Eq. 3 (Table 2). Estimates of A_0 indicate that a major amount of the initially added phenolic compound is rapidly depleted from soil solution: i.e. 86 and 90% for ferulic and *trans*-cinnamic acids, respectively (Table 2). This finding is in line with what has previously been reported by Blum (1998) and Blum and Shafer (1988), who also used autoclaved soil systems to study phenolic acid interactions in soil. Nevertheless, occurring of complex action mechanisms including adsorption or oxidation processes, particularly acting during early stages (Dalton *et al.*, 1989; Cecchi *et al.*, 2004), or microbial utilization, cannot be ignored. Although a sterile soil-based substrate has been used to give prominence to abiotic processes occurring at the soil interface, the contribution of soil microbes to the allelochemical breakdown during the recolonization process could not been excluded. However, this contribution was equally acting on all microcosms with no difference among treatments. This assumption could help explain the different hierarchy of allelochemical availability observed in the sorption isotherm and in the depletion experiment. It is also worth noting that at longer times the bioavailable amount of ferulic and *trans*-cinnamics acid in soil solution reaches an equilibrium concentration that is consistent with what observed in other soil systems (Martens, 2002). However, the observed values are well below the $0.1 \mu\text{mol g}^{-1}$ soil threshold concentration needed to cause an appreciable allelopathic activity in soil (Blum *et al.*, 2000). Interestingly, even though 92% coumarin added to soil was rapidly retained by absorbing surfaces, then the available amount in soil solution had remained unchanged over time. Nevertheless, coumarin is fairly recalcitrant to microbial breakdown

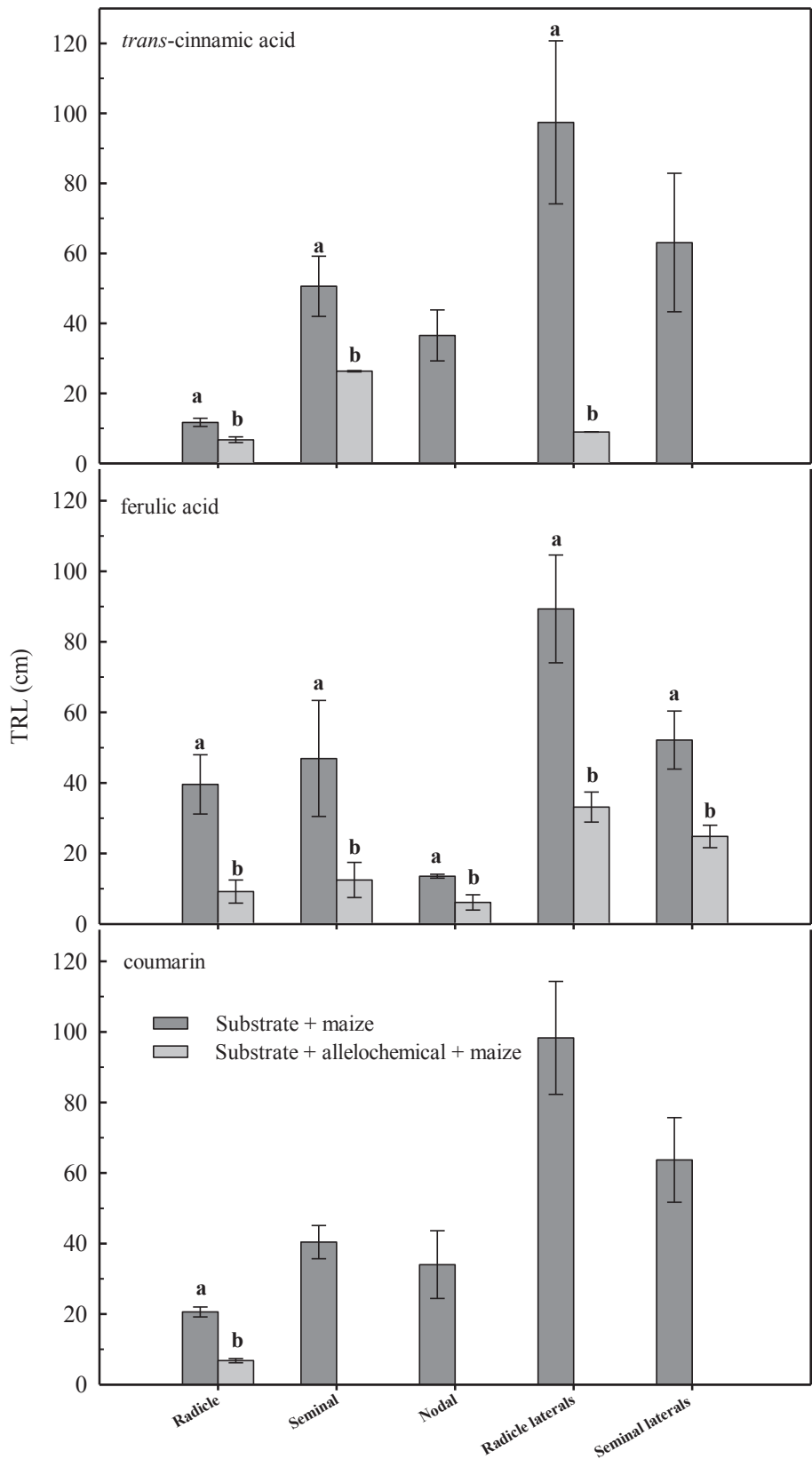


Figure 5. Changes in length of different root types of maize seedlings as induced by *trans*-cinnamic acid, ferulic acid and coumarin at the 192-h sampling time during the microcosm experiment. Values are means $\pm$ standard error bars ($n = 3$). Within each root type, different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Table 2. Parameters related to the depletion dynamics of *trans*-cinnamic acid, ferulic acid and coumarin from the sterile growing substrate as estimated according to the non-linear, first order equilibrium equation of Eq. 3.

<i>trans</i> -cinnamic acid	0.10	0.021	0.001	0.79	24.28***	0.005
ferulic acid	0.14	0.010	0.007	0.87	42.47***	0.061
coumarin	0.08	0.260	5.061	0.12	0.36	0.071

§ estimated concentration at time 0, in $\mu\text{mol g}^{-1}$ growing substrate.
* in h^{-1} .
level of significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
† estimated according to the relationship $C_{\text{eq}} = A_0 (k_{\text{des}} / (k_{\text{ad}} + k_{\text{des}}))$ Eq (4), in $\mu\text{mol g}^{-1}$ growing substrate.

and long persistence in soil environment has been reported (Kunc, 1974). This finding leads to the conclusion that the allelopathic potential of coumarin in soil remains stable over time after an equilibrium has been reached. To sum up, the following hierarchy of allelochemical availability can be inferred from the depletion kinetic parameters: coumarin > ferulic acid > *trans*-cinnamic acid. Differences from what it could be predicted by the sorption isotherm parameters are likely due to the prolonged incubation times used during the assay.

Microcosm experiment: recovery of K₂SO₄-extractable allelochemicals from the sterile growing substrate after repeated additions

The amount of K₂SO₄-extractable phenolic compound occurring in amended soil microcosms was strongly dependent on the allelochemical and was considerably lower than the amount totally added. In particular, the bioavailable amount of *trans*-cinnamic and ferulic acids recovered soon after the initial addition was fairly consistent with what observed in the sorption experiment: the amount of adsorbed *trans*-cinnamic was always greater than that of ferulic acid (Fig. 2; Table 2). Despite the total addition of approx 4.0 $\mu\text{mol trans-cinnamic acid g}^{-1}$, the amount recovered had never exceeded 0.102 $\mu\text{mol g}^{-1}$, the lowest value was 0.026 $\mu\text{mol g}^{-1}$ and the mean value was 0.063 $\mu\text{mol g}^{-1}$ (~1.6% of total amount added) (Fig. 3). Whereas, the amount of ferulic acid recovered slightly increased over time without exceeding 0.152 $\mu\text{mol g}^{-1}$, the lowest value was 0.081 $\mu\text{mol g}^{-1}$ and the mean value was 0.114 $\mu\text{mol g}^{-1}$ (~2.8% of total amount added) (Fig. 3). Coumarin showed a completely different trend. Even though the available amount strongly decreased soon after addition (-92%, as could be predicted according to the depletion experiment, Fig. 2 and Table 2), then the amount recovered remained constant over time at 0.074 $\mu\text{mol g}^{-1}$, which corresponds to 7.4% of the totally added amount (Fig. 3). This finding further confirms that coumarin is a rather stable molecule

in soil and the available amount remains almost unchanged after the sorption/desorption processes have reached a dynamic equilibrium. Because *trans*-cinnamic and ferulic acids were rapidly depleted from soil solution by sorption processes or abiotic surface reactions, additional amounts of these two compounds were added to the soil system to maintain a consistent range of soil solution concentrations over the experimental period. The experimental approach with pulsed applications was already reported in previous studies (Blum and Gerig, 2006; Inderjit *et al.*, 2008). During the microcosm experiment the resident soil concentration of each allelochemical ranged between 0.06 and 0.15 $\mu\text{mol g}^{-1}$ for ferulic acid and coumarin, respectively; while a 0.02-0.10 $\mu\text{mol g}^{-1}$ range was found for *trans*-cinnamic acid. Even though these values appear rather low when compared to similar studies (Djurdjevic *et al.*, 2004; Qu and Wang, 2008), they can be considered still able to determine allelopathic interactions at the soil-plant interface (Blum, 1998; Martens, 2002; Blum and Gerig, 2006). Lastly, no detectable amount of *trans*-cinnamic and ferulic acids or coumarin was found in unamended (control) soil (Fig. 3).

Microcosm experiment: effects of the allelochemicals on maize root system

The most frequently observed effect of allelochemicals, and in particular of phenolic compounds, on plants is the inhibition of root growth (Rice, 1984). *Trans*-cinnamic acid and structurally related compounds such as ferulic, caffeic and *p*-coumaric acids are able to inhibit the root growth in many plant species (Fujita and Kubo, 2003). Also coumarins, which are lactones of *o*-hydroxycinnamic acid, have been shown to strongly affect both root morphology and function (Svensson, 1971; Aliotta *et al.*, 1993; Kupidowska *et al.*, 1994; Abenavoli *et al.*, 2001 and 2004).

The total root length of maize seedlings was strongly affected by exposition to *trans*-cinnamic and ferulic acids and coumarin, and root responses

Table 3. Changes in morphological and structural parameters of maize root seedlings as induced by *trans*-cinnamic acid, ferulic acid and coumarin at 192-h sampling during the microcosm experiment. Within each root type, different letters indicate significant differences between treatments (Tukey's test, $P < 0.05$).

Allelochemical	Treatment	DWr [§]	Fr [‡]	TMDr [#]
<i>trans</i> -Cinnamic acid				
Radicle	Substrate	0.0150 ^a	247.90 ^a	0.2288 ^a
	Substr + allelol	0.0064 ^b	174.30 ^b	0.1610 ^b
Radicle laterals	Substrate	0.0027 ^a	1084.44 ^a	0.0330 ^a
	Substr + allelol	0.0003 ^b	288.71 ^b	0.0089 ^b
Seminal	Substrate	0.0084 ^a	416.51 ^a	0.0694 ^a
	Substr + allelol	0.0028 ^b	369.88 ^a	0.0471 ^a
Seminal laterals	Substrate	- [§]	-	-
	Substr + allelol	-	-	-
Nodal	Substrate	-	-	-
	Substr + allelol	-	-	-
Ferulic acid				
Radicle	Substrate	0.0155 ^a	231.54 ^a	0.0746 ^a
	Substr + allelol	0.0067 ^a	113.08 ^b	0.0969 ^a
Radicle laterals	Substrate	0.0047 ^a	817.69 ^a	0.0430 ^a
	Substr + allelol	0.0029 ^a	600.55 ^a	0.0526 ^a
Seminal	Substrate	0.0092 ^a	224.47 ^a	0.2242 ^a
	Substr + allelol	0.0064 ^b	232.17 ^a	0.2256 ^a
Seminal laterals	Substrate	0.0031 ^a	802.84 ^a	0.0476 ^a
	Substr + allelol	0.0024 ^a	215.03 ^b	0.0486 ^a
Nodal	Substrate	0.0020 ^a	677.75 ^a	0.1570 ^a
	Substr + allelol	0.0009 ^b	425.76 ^a	0.1356 ^a
Coumarin				
Radicle	Substrate	0.0177 ^a	137.35 ^a	0.0530 ^a
	Substr + allelol	0.0132 ^b	139.22 ^a	0.1091 ^a
Radicle laterals	Substrate	-	-	-
	Substr + allelol	-	-	-
Seminal	Substrate	-	-	-
	Substr + allelol	-	-	-
Seminal laterals	Substrate	-	-	-
	Substr + allelol	-	-	-
Nodal	Substrate	-	-	-
	Substr + allelol	-	-	-

[§] root dry weight, in g; [‡] root fineness, in cm cm⁻³; [#] root tissue mass density, in g cm⁻³;

[§] not revealed

varied with time and the allelochemical considered (Fig. 4). In details, *trans*-cinnamic acid and coumarin markedly inhibited the root elongation already after 96 hours of exposure, causing a reduction by 81 and 89%, respectively. The inhibitory activity of *trans*-cinnamic acid remained fairly constant over the following 96 hours, whereas a 97%-inhibition of root growth was reached in coumarin-treated microcosms (Fig. 4). After 288 hours of exposure, either compounds had completely inhibited the root growth (*data not shown*). Conversely, in ferulic acid-treated microcosms a significant reduction

(-69%) of the root growth has been noticed after 192 h of exposure (Fig. 4), and this effect has been maintained over the whole time course (*data not shown*). Coumarin appeared to be the most phytotoxic compound followed by *trans*-cinnamic and ferulic acids. The different phytotoxic activity of tested allelochemicals can be attributable to the chemical structure of the compounds. It is well known that the occurrence of a substituent on the aromatic ring may change the potential phytotoxicity of allelochemicals. For example, hydroxylation decreased the inhibitory activity of *trans*-cinnamic on ion uptake (Yu and

Matsui, 1997), while reduced the negative effect of coumarin on root elongation (Kupidlovska *et al.*, 1994). Furthermore, the position and/or the isomerization of the substitution group defined allelopathic activity. Indeed, *cis*-cinnamic acid was ten times more active than *trans*-cinnamic acid in root growth inhibition of *Arabidopsis thaliana* (Wong *et al.*, 2005). Finally, the lactonization increased the inhibitory effect, *i.e.* coumarin was a stronger inhibitor on the germination compared to *trans*-cinnamic acid (Reynolds, 1989). According to Glass and Dunlop (1974), the results indicated that the presence of a hydroxyl group reduced the inhibition activity of the compounds, as observed with ferulic acid, while in disagreement with Maffei (1999), the methoxyl substituent seemed to play a minor role in defining the allelochemical phytotoxicity. Indeed, ferulic acid was less inhibitory of *trans*-cinnamic acid. Furthermore, in agreement with Reynolds (1989), coumarin, derived through the process of lactonization of *trans*-cinnamic acid, showed a greater inhibitory activity compared to its linear chain precursor. The strong inhibitory activity of coumarin respect to other simple phenolic compounds has already been reported (Kupidlovska *et al.*, 1994).

Several hypotheses have been drawn to explain the inhibition of the root elongation process induced by these allelochemicals. *trans*-Cinnamic acid may block the pumping of protons mediated by the H⁺-ATPase of the plasma membrane, resulting in a reduction of the internal pH (Fujita and Kubo, 2003). Coumarin is supposed to act through several action mechanisms: interference with the auxin metabolism through the activation of peroxidase and IAA oxidase activities (Goren and Tomer, 1971), changes in the distribution of micro-fibrils in the radial direction (Svensson, 1971), alteration of the mitotic process and/or the cell cycle (Kupidlovska *et al.*, 1994).

However, the data on the whole root system provided no information on the specific responses of different root types to each allelochemical. In fact, root systems are composed of several root types, each characterized by different anatomical, morphological, and physiological features that make it able to differentially respond under stress conditions (Waisel and Eshel, 2002). Abenavoli *et al.* (2008) demonstrated a selective and species-specific effect of coumarin on single root types: in *Arabidopsis* coumarin inhibited the primary root length whereas stimulated the lateral root formation. By contrast, in *Zea mays* coumarin inhibited the seminal and nodal root growth and left unchanged the primary root (Abenavoli *et al.*, 2004). Maize represents a useful model plant to study the effects of allelochemicals on root system, since its complex root system is characterized by

radicle, seminal, and nodal roots, differing in origin, development, physiology, and anatomy (McCully, 1999). To better understand the effects of *trans*-cinnamic acid, ferulic acid and coumarin on maize root system, morphological responses of different root types (*i.e.* radicle, seminal, nodal roots together with their laterals) were monitored. A 192 h exposure to *trans*-cinnamic acid was long enough to significantly inhibit the elongation process of radicle, lateral radicle and seminal roots; whereas the formation of nodal and the seminal lateral roots was completely inhibited (Fig. 5). Conversely, amendment with ferulic acid determined an even reduction (ranging from 50 to 70% inhibition *vs* control) in the elongation of all root types (Fig. 5). Finally, in coumarin-treated microcosms the radicle root elongation was markedly inhibited whereas no other root types were found after 192 h exposure. Root length readings confirmed the different sensitivity of maize root types to allelochemicals suggesting the following order of response: nodal > seminal > radicle roots, as previously reported by Abenavoli *et al.* (2004).

Since root length is known to be dependent on both biomass allocation (dry weight) and structural parameters (fineness and tissue density), they were also evaluated. In fact, plants can produce longer roots either by increasing the allocation of biomass towards the root or by increasing the fineness (Fr) and/or by reducing the density of tissue (TMDr). After 192 h of exposure, *trans*-cinnamic acid significantly reduced the root structural parameters: that is dry weight of the radicle and both fineness and tissue density (Table 3). In addition, the dry weight of the seminal root was affected, whereas structural parameters remained unchanged. Ferulic acid inhibited the fineness of radicle whereas the biomass allocation (dry weight and tissue density) was not affected (Table 3). Conversely, biomass allocation of seminal roots was reduced whereas the two structural parameters were not modified. On the other hand, the reduction of nodal roots may be attributable to the variation of the tissue density. In lateral roots of the seminal, fineness was sensitive to ferulic acid showing a significant inhibition. Finally, coumarin showed a reduction of biomass allocation (dry weight), but not in the structural parameters (Table 3). Therefore, plants may produce longer roots either by increasing biomass allocation to the roots, as also demonstrated under a low supply of nitrogen (Ryser and Lambers, 1995; Sorgonà *et al.*, 2005), or by increasing the root fineness and/or reducing root tissue density, thus leaving the biomass allocation unchanged (Ryser, 1998).

After 192 h of exposure, *trans*-cinnamic acid significantly reduced the radicle dry weight, and both fineness and tissue density, structural parameters.

In addition, the dry weight of the seminal root was affected, whereas structural parameters were left unaffected (Table 3). Ferulic acid inhibited the fineness of radicle leaving unchanged the biomass allocation (dry weight) and tissue density. Conversely, biomass allocation of seminal roots was reduced whereas the two structural parameters were not modified. On the other hand, the reduction of nodal roots after contact with ferulic acid may be attributable to tissue density variation. In lateral seminal roots, fineness was sensitive to ferulic acid showing a significant inhibition (Table 3). Finally, coumarin showed a reduction of biomass allocation (dry weight), but not in the structural parameters (Table 3).

Taken together these results indicate that each root type may adopt a different strategy in response to allelochemicals both in terms of biomass allocation and/or fineness and tissue density. Hence, the question of which 'morphological component' among biomass allocation and/or structural parameters mainly contributes to the variation in root length of each root order in response to allelochemicals still remains open.

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Algicide activity of rosmarinic acid on *Phaeodactylum tricornutum*

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ABSTRACT

Rosmarinic acid is a natural phenolic compound commonly found in land and aquatic plant species. Although the antibacterial, antiinflammatory, antimutagenic and antiviral activity of rosmarinic has been already demonstrated, its phytotoxic activity has been poorly investigated nor in land or aquatic ecosystems. Therefore, the effects of this secondary metabolite on growth and physiology of the diatom *Phaeodactylum tricornutum* Bohlin were analyzed in this study. Cell density and chlorophyll fluorescence were recorded along the treatment at different times. After five days, the distribution of polar and neutral lipids was observed by confocal microscopy with the fluorochrome Nile Red and photosynthetic pigments content was quantified by spectrophotometry. The results revealed a strong phytotoxic potential of rosmarinic acid with a decrease on cell density and chlorophyll content, and diminution of neutral lipids, which suggest the effect of rosmarinic acid on *P. tricornutum* plasma membrane permeability and survival.

Keywords: Secondary metabolite, phenolic compound, algicide, membrane permeability, phytotoxicity, microalgae.

Introduction

The well-known persistence and toxicity of synthetic algicides as copper sulfate or diuron (Jančula and Maršálek, 2011) are in the origin of the search of more ecologically friendly natural compounds, which can be used as algicides, herbicides or insecticides. Although there are many organisms with algicidal activity, it is very difficult to isolate and characterize the active compound that is responsible of the biological activity. As an example, the secondary metabolite nostocarboline from the cyanobacteria *Nostoc* 78.12A showed an IC_{50} of 2.1 μ M in *Microcystis aeruginosa* PCC 7806, 5.8 μ M in *Synechococcus* PCC 6911 and 29.1 μ M in *Kirchneriella contorta* SAG 11.81 (Blom *et al.*, 2006). As well, ethyl-2-methylacetoacetate, commonly found in *Phragmites australis*, showed inhibitory effects on the growth of *Chlorella pyrenoidosa*, *Microcystis aeruginosa* and *Chlorella vulgaris*. (Li and Hu, 2005) Other natural compounds with algicide activity are 9,10-anthraquinone and derivatives, isoquinoline plant alkaloids (Jančula and Maršálek, 2011) or some toxins as cyanobacterine and fischerellin A and B, which can be found in many cyanobacteria (Srivastava *et al.*, 1998; Jüttner *et al.*, 2001; Berry *et al.*, 2008). However, these are limited examples in the algicidal research.

In the search of new biologically active compounds, it is essential to prospect new molecules with demon-

strated biological activity. Therefore, the effects of rosmarinic acid ((R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl) lactic acid), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic (Scarpati and Oriente, 1958) belonging to the group of the phenolic compounds, have been studied. Rosmarinic acid is commonly found in species of the families Boraginaceae and Lamiaceae (Litvinenko *et al.*, 1975), and it has also been described in other plant families, as in ferns of the Blechnaceae family (Häusler *et al.*, 1992), lower plants as hornworts (Takeda *et al.*, 1990), plants of the family Cannaceae (Petersen *et al.*, 2009), species of the Marantaceae family (Abdullah *et al.*, 2008), the marine monocotyls *Posidonia oceanica* or *Zostera marina* of the family Zosteraceae (Petersen *et al.*, 2009; Ravn *et al.*, 1994) or other aquatic plants of the family Potamogetonaceae. This phenolic compound has multiple biological activities, as astringent, antioxidant, antiinflammatory, antimutagenic or antiviral activities (Petersen and Simmonds, 2003; Tepe *et al.*, 2007; Nakamura *et al.*, 1998; Parnham and Kesseling, 1985). Phenolic compounds, as rosmarinic acid, can provide protection against cancer and contribute to the antioxidant activity of cosmetic products, which use plant species as *Rosmarinus officinalis* and *Sanicula europaea* (Petersen and Simmonds, 2003). This compound shows also a broad range of bactericide and fungicide

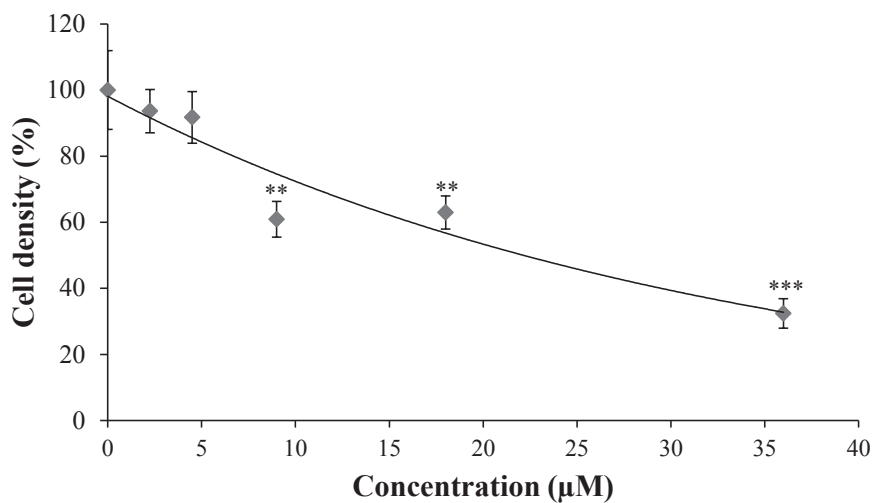


Figure 1. Dose-response curve for cell density of *Phaeodactylum tricornutum* treated with rosmarinic acid (2.25, 4.5, 9, 18 and 36 μM). Data are represented as percentage of the control (R2 = 0.9363). Asterisks indicate statistical differences compared to the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

activities, and it plays an active role in the regulation of symbiosis and the protection against microorganisms (Bais et al., 2004), i.e. once that basil is damaged by the fungi *Pythium ultimum*, the roots exude high amounts of rosmarinic acid, which inhibits the most part of soil pathogens (Bais et al., 2004).

Algae are commonly used in aquatic toxicity tests due to their easy growth and easy exposure to water soluble compounds. Besides, microalgae are sensitive to many chemicals or contaminants and have a short generation time, which allows the rapid study of the effects of toxins on growth (Choi et al., 2012).

Phaeodactylum tricornutum (Bohlin) (Lewin, 1958), is a diatom (Bacillariophyceae) belonging to the family Phaeodactylaceae. Diatoms represent an important group of eukaryotic algae found in marine and terrestrial ecosystems. These algae play a crucial role in the world primary production, having a key role in the marine food chain, where they are believed to be responsible for up to 25% of world primary production (Scala et al., 2002). These organisms are also of interest as food for the industrial cultivation of aquatic animals. Moreover, many diatoms are used as indicators of water quality (Domergue et al., 2003).

P. tricornutum represents a good biological model because is easy to grow, it has appreciated physiological and genetic characteristics (short generation time, short genome and easy genetic transformation; Scala et al., 2002; Soto et al., 2005) and its genome is fully sequenced (Bowler et al., 2008). This diatom is mainly known because is a potential source for the industrial production of eicosapentaenoic acid (EPA; 20:5^{Δ5,8,11,14,17},

Grima et al., 1996). EPA represents 30% of the fatty acids of *P. tricornutum*, while palmitoleic acid (16:1^{Δ9}), palmitic acid (16:0), hexadecatrienoic acid (16:3^{Δ6,9,12}) and miristic acid (14:0) represent 26, 17, 10 y 15% respectively (Domergue et al., 2003).

Material and methods

Phytotoxic bioassays of rosmarinic acid on microalgae

A dose-response experiment was carried out in order to determine the phytotoxic effects of rosmarinic acid in *Phaeodactylum tricornutum* cells.

The stock culture of the microalgae was obtained from the Marine Science Station of Toralla (ECIMAT, Pontevedra, Spain) and kept as batch culture in the laboratory. Guillard F/2 medium was used as growth medium. The culture medium was prepared in sea-water filtered through 1 μm filter and autoclaved. Nutrients were added in sterile conditions.

Cultures were oxygenated and maintained at 20 ± 1 °C with a photoperiod of 16/8 h light/darkness to allow the growth of *P. tricornutum*. The phytotoxic bioassays were performed for 6 days, being the inoculum of *P. tricornutum* added to fresh culture medium (500 mL per treatment) at day 0. The initial cell concentration was about 50,000 cells mL⁻¹, which was considered a good starting concentration to get the cells in exponential phase still after 6 days of growth. The dose-response curve of treated *P. tricornutum* cells was performed with 2.25, 4.5, 9, 18, 36, 75 and 150 μM rosmarinic acid.

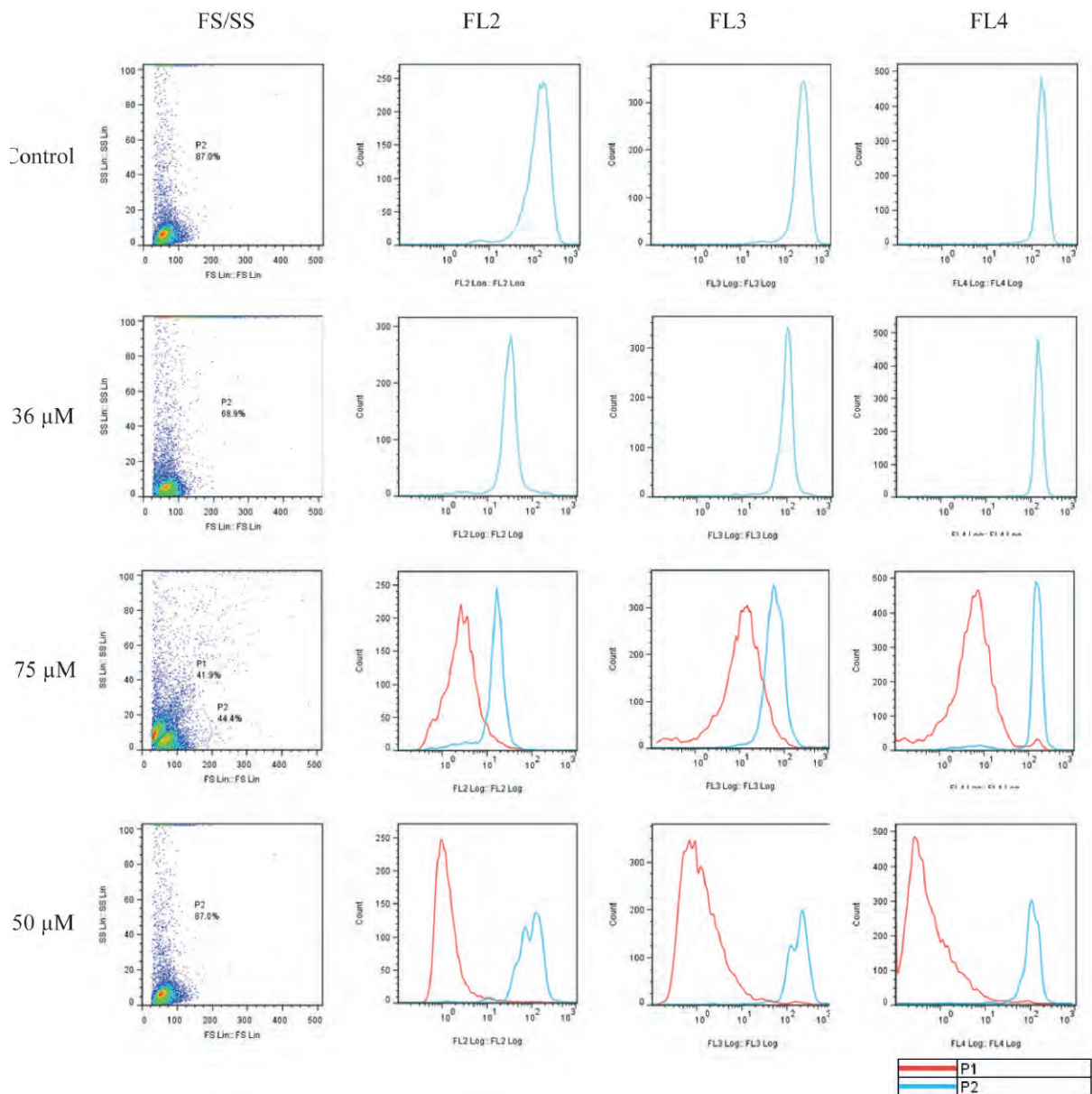


Figure 2. Flow cytometric histograms showing shifts in neural lipid (FL2), polar lipid (FL3) and Chlorophyll *a* (FL4) fluorescences. X and Y histogram axes represent fluorecence (log-transformed voltage units) and number of cells, respectively. Values shown correspond to control and concentrations of 36, 75 and 150 μM of rosmarinic acid.

Cell density was measured with a Neubauer chamber and a light microscope (Nikon ALPHAPHOT-2) 1, 3 and 5 days after treatment. Six complete chambers were counted per assayed concentration and the cell density was calculated ($\text{cel}\cdot\text{mL}^{-1}$). Non-pigmented or lysed cells were not considered for counting. The phytotoxic potential of the compound was determined by calculating the IC_{50} and the IC_{80} (rosmarinic acid concentrations that inhibit the growth of the algal population by 50% or 80%, respectively) of the dose-response curve after 5 days of treatment.

Measurement of chlorophyll a fluorescence by flow cytometry

The measurement of chlorophyll *a* fluorescence, previously used to assess the effect of heavy metals, herbicides, petrochemicals and nutrients on plant metabolism (Ralph et al., 2007), is an adequate technique to evaluate changes in the photosynthetic capacity of algae populations (Farah and Samuelsson, 1992; Samuelsson and Oquist, 1977). Moreover, this parameter is strongly affected by

the physiological status of the algae (Huot et al., 2007).

The chlorophyll *a* fluorescence, the cell size and the complexity were measured on days 1, 3 and 5 of the treatment, using FS and SS detectors, respectively, of FC 500 MPL flow cytometer (Beckman Coulter Instruments) equipped with an Argon laser with emission wavelength of 488 nm. Three samples of each rosmarinic concentration (0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM) were analyzed by flow cytometry. The chlorophyll *a* fluorescence was collected with a 675 nm dichroic filter (FL4), which corresponds to the emission peak of the chlorophyll. About 10,000 cells were analyzed per replicate, using a logarithmic amplification of the fluorescence signal. The results were expressed as fluorescence (log-transformed voltage units).

Detection of polar and neutral lipids by flow cytometry

As previously mentioned, *Phaeodactylum tricornutum* is mainly known as a potential source of EPA (eicosapentaenoic acid), a neutral lipid that is the most abundant fatty acid (30 %) of *P. tricornutum* (Domergue et al., 2003).

The relative abundance of polar and neutral lipids was determined using the solvatochromic fluorochrome Nile Blue A Oxazone (Nile Red; Sigma-Aldrich) that selectively stains cell lipids according to the protocol of Yu et al. (2009), with some modifications. Three replicates of each rosmarinic concentration (0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM) were analyzed by flow cytometry. Staining was performed by adding 25 μL of Nile Red (NR) stock solution in acetone (200 g mL^{-1}) per mL of algae suspension, to get a final concentration of 5 $\mu\text{g mL}^{-1}$. After adding the fluorochrome, the sample was vigorously stirred and incubated for 10 min at room temperature.

Flow cytometry can simultaneously measure the autofluorescence of chlorophyll *a* and the fluorescence of cells stained with a specific staining as NR (de la Jara et al., 2003). When NR is excited at 488 nm with the Argon laser of the FC 500 MPL flow cytometer, it exhibits an intense yellow-gold fluorescence if it is dissolved in neutral lipid, and a red fluorescence if it is dissolved in polar lipids (Alonzo and Mayzaud, 1999).

The optical system of this equipment collects yellow fluorescence emission (575 nm dichroic filter) in the channel FL2 and the red fluorescence (620 nm dichroic filter) in the channel FL3. Over 10,000 cells were analyzed using the logarithmic amplification of the fluorescence signal. Results were expressed as fluorescence (log-transformed voltage units) (Mendoza Guzmán et al., 2010).

Detection of polar and neutral lipids by confocal microscopy

Polar and neutral lipids were detected in 0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM rosmarinic acid treated-cells by using a Leica TCS SP5 confocal microscope (Wetzlar, Germany) with a hybrid detector. Nile red (NR) stained cells were excited at 514 nm for the emission of yellow fluorescence at 580 nm, corresponding to neutral lipids, and at 543 nm for the emission of red fluorescence at 610 nm, corresponding to polar lipids. The emission of *P. tricornutum* chlorophyll between 673 and 678 nm avoided interferences with the detection of neutral or polar lipids.

Statistical analysis

The data were statistically analyzed with SPSS Statistics 19.0 software, testing normality by Kolmogorov-Smirnov and homoscedasticity by Levene's test. The statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests in the case of homoscedastic normally distributed data, by Tamhane's T2 test in the case of heteroscedastic normally distributed data, and by the Kruskal-Wallis test in the case of non-normally distributed data.

Fluorescence data were analyzed by student *t* tests.

Results

Phytotoxicity bioassays

Microalgae growth tests were used to measure the toxicity of rosmarinic acid. The treatment with the different rosmarinic acid concentrations resulted in a strong inhibition of algal growth. The dose-response curve obtained with 0, 2.25, 4.5, 9, 18 and 36 μM treated cells showed IC_{50} and IC_{80} values of 22.5 μM and 53 μM respectively (Fig. 1).

Chlorophyll *a* fluorescence and lipid content

The treatment with the highest concentrations of rosmarinic acid, produced an important change in the morphology of the algae population. At concentrations of 75 and 150 μM two different cell populations are founded (P1 and P2) (Fig. 2) with different fluorescence emission peaks in the FL2, FL3 and FL4 channels. One of the populations (P2) was similar to the single population presented in the control and at the lower concentrations of rosmarinic acid. However, P1 showed lower fluorescence values for all the channels

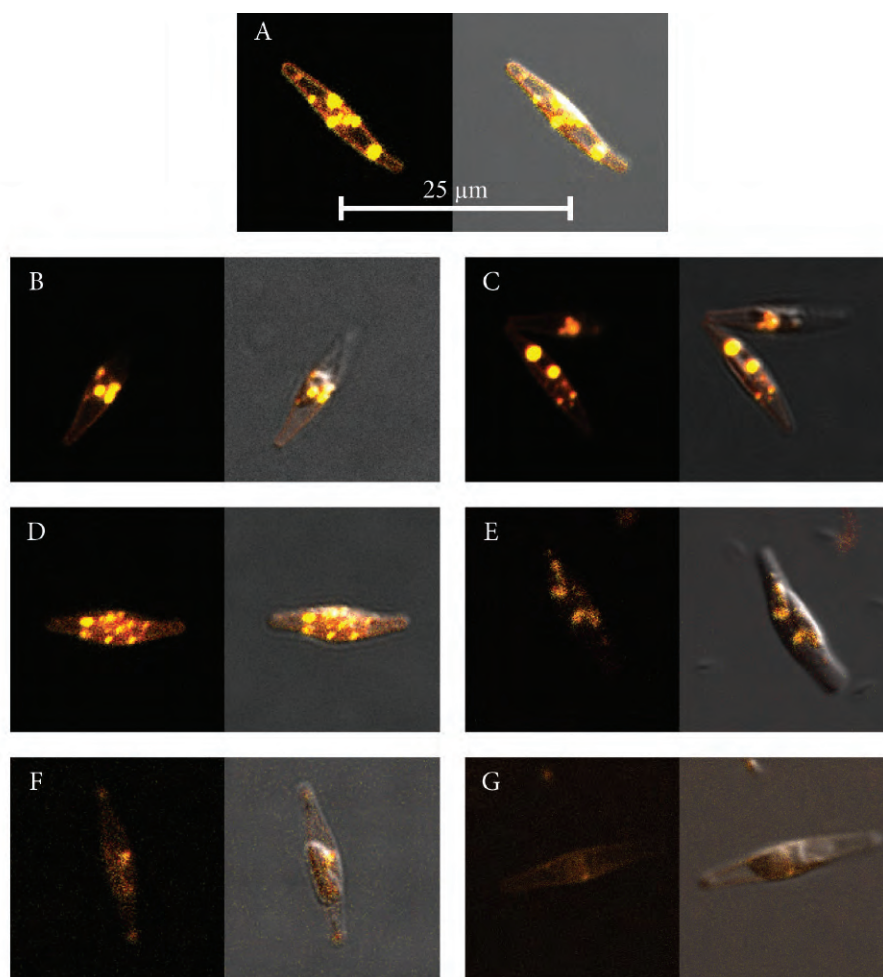


Figure 3. Confocal microscopy images of Nile Red (NR) stained *Phaeodactylum tricornutum* after 6 days of growth in the presence of (A) 0 μM , (B) 2.25 μM , (C) 4.5 μM , (D) 9 μM , (E) 18 μM , (F) 36 μM , and (G) 75 μM rosmarinic acid. Images on the left are overlay images of red and yellow channels while images on the right are brightfield images. Yellow drops indicate neutral lipids while red color corresponds to polar lipids.

which means that the treatment produces a decrease in polar and neutral lipids and in chlorophyll *a* fluorescence.

The strong effects of rosmarinic acid on neutral lipids, detected by flow cytometry, were confirmed by confocal microscopy with Nile Red fluorochrome. Stained cells showed an accumulation of neutral lipids in form of yellow drops (Fig. 3) (Alonzo and Mayzaud, 1999). As low concentrations as 4.5 or 9 μM rosmarinic acid, induced an increase in the number of yellow drops, although higher concentrations showed a decrease in size and number of neutral lipids in form of yellow drops (Fig. 3) and concentrations higher than 18 μM rosmarinic acid showed a strongly decreased staining intensity and lipid drops could not be observed in the samples. Moreover, the typical yellow staining of neutral lipids appears more

dispersed inside and even outside the cells, which could be associated with a release of lipids (Fig. 4). At higher concentrations of this secondary metabolite, the staining intensity is almost zero. These results are in concordance with the cytometer data where a new population of algae with lower fluorescence emission in the channels FL2, FL3 and FL4 appeared at higher concentrations of treatment suggesting that the alga membrane is damaged and there is a release of pigments and lipids out of the cells.

Discussion

The results here obtained show a decrease in the growth of *Phaeodactylum tricornutum* after rosmarinic acid treatment. As already mentioned in the introduction, other compounds, as nostocarboline,

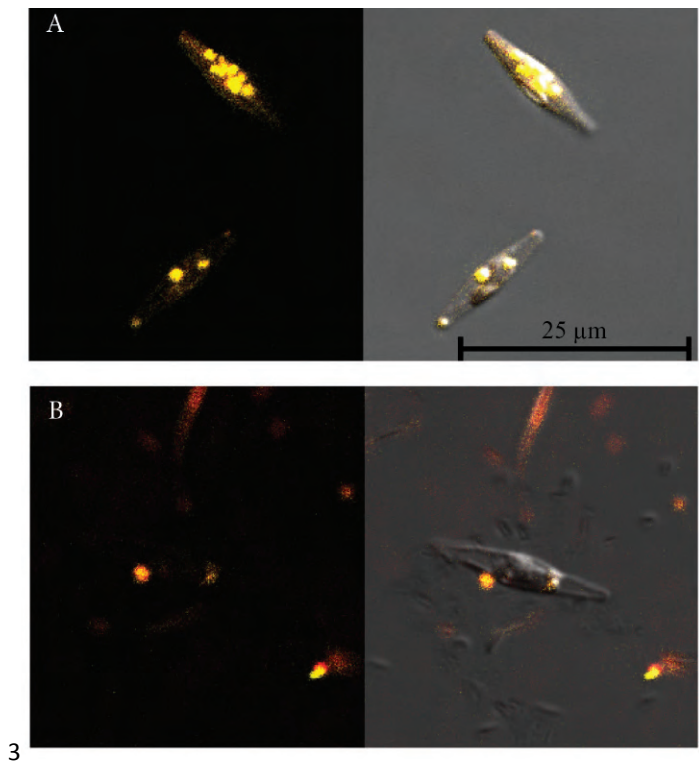


Figure 4. Confocal microscopy images of NR stained *P. tricornutum* after 6 days of growth with (A) 0 μM and (B) 18 μM rosmarinic acid. Images on the left are overlay images of red and yellow channels while images on the right are brightfield images. Yellow drops correspond to neutral lipids in and out of the cell.

cyanobacterine or ethyl-2-methylacetoacetate, have been previously found to show algicide activity, but this is the first time that rosmarinic acid is reported as algicide.

Rosmarinic acid treatment produced a significant decrease in the content of both polar and neutral lipids. Microorganisms, particularly microalgae, are the first organisms affected by chemicals in aquatic ecosystems, as they are in direct contact with the medium from which are only separated by the cell wall and the cytoplasmic membrane, which consists mainly of polar lipids. Cell membranes are selective dynamic barriers that play an essential role in the regulation of biochemical and physiological events, so that any change produced in the medium will produce changes in the membranes of the microorganisms (Cid et al., 1996). Therefore, the altered contents of polar lipids in rosmarinic treated cells suggest altered membrane permeability after the treatment with this phenolic compound.

Furthermore, this secondary metabolite also affected the content of neutral lipids. *P. tricornutum* is a diatom widely known to be a potential source of eicosapentaenoic acid (EPA, 20:5^{Δ5, 8,11,14,17}) for indus-

trial production, as this neutral lipid represents the 30% of total neutral lipids in this species (Domergue et al., 2003). It has been shown that in diatoms, the damage of the membranes causes the release of polyunsaturated fatty acids, particularly of EPA as a defense reaction (Budge and Parrish, 1999; Jüttner, 2001; Pohnert, 2005). Similarly, the mechanical damage caused by osmotic stress caused also the release of fatty acids (Jüttner, 2001; Jüttner and Dürst, 1997). It has been found that most allelochemicals reduce the algal cell membrane integrity and cause a leakage of cytoplasm. For example, salcolin B, extracted from *Hordeum vulgare*, was found to directly attacks the *Mycrocistis aeruginosa* cell membrane causing a significant increase in cell membrane leakage after 5-days exposure (Xiao et al., 2014). Therefore, the effect of rosmarinic acid on the lipid content and the observed leakage of lipids out of the treated cells could be related to a direct damage of rosmarinic acid on the plasma membrane of *P. tricornutum*.

Although chlorophyll *a* fluorescence has been considered to be a good indicator for the toxicity of water soluble compounds (Choi et al., 2012) and it has been previously used to evaluate the effects of heavy met-

Table 1. Neutral lipids (FL2), polar lipids (FL3) and chlorophyll a (FL4) fluorescence values (arbitrary units in percentage of the control). The superscripts indicate statistically significant differences among treatments. Shaded cells indicate values with significant differences compared to the control ($p \leq 0.05$).

	Neutral lipids	Polar lipids	Chlorophyll a
Control	100.00 ^{ac}	100.00 ^a	100.00 ^{ab}
2.25 μ M	120.85 ^{abc}	109.17 ^{ab}	100.44 ^{ab}
4.5 μ M	160.00 ^{ab}	138.76 ^b	110.40 ^a
9 μ M	168.72 ^b	125.59 ^{abc}	95.57 ^{abc}
18 μ M	82.21 ^c	83.14 ^{ac}	95.35 ^{abc}
36 μ M	20.59 ^d	44.97 ^d	94.91 ^{abc}
75 μ M	8.03 ^e	15.18 ^d	65.88 ^c
150 μ M	0.97 ^f	0.59 ^e	0.34 ^d

als, herbicides, petrochemicals and nutrients on plant metabolism (Ralph et al., 2007); the treatment caused no change in the *in vivo* chlorophyll *a* fluorescence of *P. tricornutum* cells, hinted that the target is not on the reaction centre of photosynthesis. However at the highest concentration of rosmarinic the chlorophyll *a* fluorescence decreased due to the disintegration of the algal cells.

As it was said before, the release of lipid suggest cell membrane damage, This effect, together with the strong decline in the growth of algae suggests that rosmarinic acid triggers automortality processes in cells of the algae, as at the late stage of the events associated with the automortality, cells lose membrane integrity resulting in a complete disintegration of the algal cells (Kroemer et al., 1995; Naganuma, 1996). It has been demonstrated that non-viable algal cells still possess their photopigments and are capable of photosynthesis but the loss of membrane integrity will accelerate the efflux of small metabolites across the cell wall (Veldhuis et al., 2001).

The strong phytotoxicity of this phenolic compound on the diatom *Phaeodactylum tricornutum*, together with the extensive knowledge of its biosynthesis convert rosmarinic acid in an interesting microalgal growth regulator. A deeper study on their effects at the cellular level and its assay on other algal species, could allow us to better elucidate the mode of action of this compound.

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A chemical basis for differential allelopathic potential of root exudates of *Sorghum bicolor* L. (Moench) cultivars on companion weeds

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ABSTRACT

Study was conducted to test if the variation in weed population and biomass between the stands of Enkath and Rabeh sorghum cultivars, which was observed in the field, was due to differences in their allelopathic potential. Field experiment revealed that Enkath cultivar significantly suppressed weed density and dry weight biomass over Rabeh cultivar by 34 and 29% after 35 days after sowing (DAS), and 22 and 24% after 65 DAS. Stair case experiment indicated that root exudates of Enkath cultivar showed more suppression to weeds than Rabeh giving additional evidence for the superiority of Enkath indicated that in its allelopathic weed suppression. Chemical analysis revealed that sorgoleone and several phenolic acids were present in higher concentrations in root exudates of Enkath compared to Rabeh. The results recommend screening more sorghum cultivars in order to offer a potential source of allelopathic germplasm that could be manipulated to enhance weed suppression in an effective and environmentally sustainable approach.

Keywords: Allelopathy, sorghum cultivars, weeds, root exudates, phytotoxins.

Introduction

During the past four decades considerable research work has been focused on the possible use of allelopathic crops in weed control with the aim of reducing the rely on synthetic herbicides (Alsaadawi et al., 2007; Cheema et al., 2002; Dilday et al., 1998; Weston, 1996). Different strategies have been adopted including the use of root exudates of allelopathic plants (3, 26). Sorghum Allelopathy has been well documented, and studies performed in the field have revealed that sorghum phytotoxicity varies with genotype, plant part, plant age, environmental factors, and the target weed species (25,27). The residues of sorghum provide short-term weed suppression, which appears to be due to phenolic compounds released from decomposing sorghum shoot residues (4,14,26). Most of these phenolic compounds are relatively simple in structure and are quickly metabolized in the soil rhizosphere (26). Also, sorghum produced root exudates containing phytotoxic chemicals. The exudates were

reported to contain two major components, which were identified as 2-hydroxy-5-methoxy-3-[(80Z, 110Z)-80,110,140-pentadecatriene]-p-benzoquinone (sorgoleone) and its p-hydroquinone reduced form named xenognosin (6,8). Sorgoleone was found to inhibit photosynthesis by blocking the flow of electron in electron transport system and reported to be more effective as photosystem II inhibitor than the herbicide atrazine (11,13,24).

Field observations indicated that growth and population of companion weeds are variable between the stands of two local sorghum cultivars (Enkath and Rabeh). The suppressive ability increased with increased planting density (1). This suggests that allelopathy might be the mechanism responsible for the variation in weed growth and population, and the differences among stands could be attributed to the variable allelopathic potential of the two cultivars. The aim of this study was to determine the suppressive potential of root exudates of these cultivars and to isolate, identify and quantify allelochemicals in their root exudates.

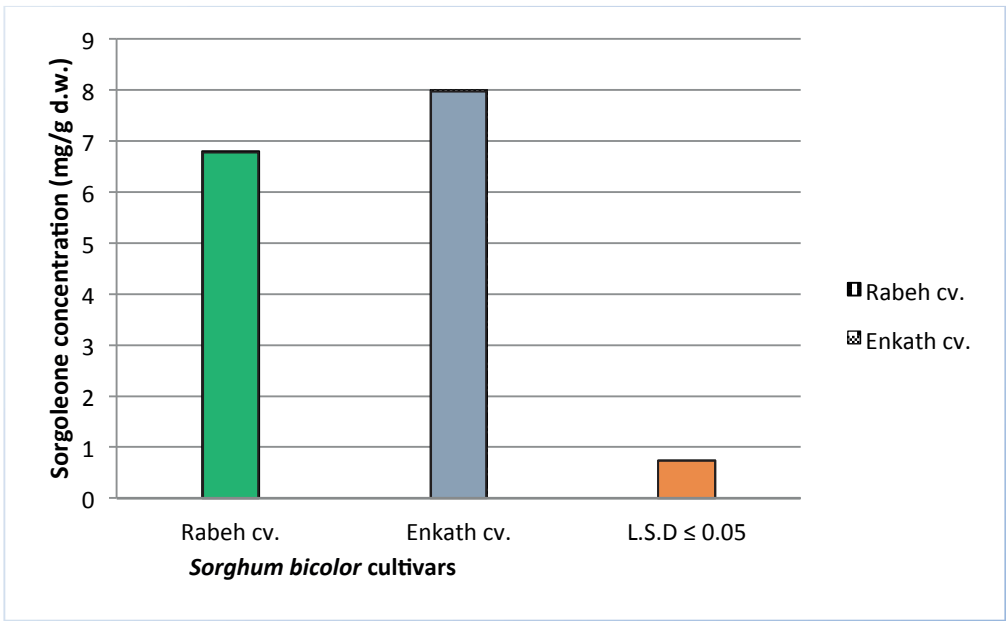


Figure 1. Sorgoleone collected from root exudates of two *Sorghum bicolor* cultivars.

Materials and methods

Field experiment

Field experiment was conducted in 2011 at Experimental Station of Field Crop Department in Abu-Ghraib, State Board of Agricultural Researches, Ministry of Agriculture, Baghdad, Iraq to determine the allelopathic potential of the test sorghum cultivars on companion weeds. The site was located at 33°19' 29.12" N, 44° 12' 56" E. The field is characterized by calcareous silt clay loam soil with pH 7.4 and electrical conductivity 2.7 dS m⁻¹; average annual rainfall was less than 50 mm and day/night temperatures during the growing season were 17-45/ 14-38 °C. Plots (7 × 9 m) were randomly made in the first of April 2011 in a field where previous field history showed heavy weed infestation. Seeds of sorghum cultivars were sown in their respective plots in 75 cm apart rows with a distance of 20 cm between seeds in order to achieve target density of 6.6 plants per m². Plots without crops were used as a control. The experiment was laid down in a randomized complete block design (RCBD) with three replicates. Fertilizers used were nitrogen as urea (46% N) at 240 kg ha⁻¹ and phosphorus as triple super phosphate (46% P₂O₅) at 160 kg ha⁻¹, which were applied as recommended for this crop; the whole phosphorous and half of the nitrogen were applied at planting while the remaining half of the nitrogen was applied at flowering stage.

For weed measurements, weeds density and dried biomass were taken after 35 and 65 days of sowing of sorghum crop. Weeds biomass was recorded after clipping the weeds at the soil surface and oven dried at 70°C for 72 h.

The statistical analysis was performed using analysis of variance test (ANOVA) by averages with GENISTAT computer software package. Differences among treatment averages were compared using LSD at 0.05 probability level (22).

Root exudates bioassay

This experiment was run under greenhouse conditions to test the differential allelopathic potential of the test sorghum cultivars against weeds by eliminating the competition for light, moisture and minerals between sorghum and weeds. The assay was made using stair case devise described by Alsaadawi and Rice (2) with little modification. Seeds of sorghum cultivars and the weed *Echinochloa colonum* L., one of the companion weeds found in sorghum fields, were planted separately in trays filled with acid washed sand to prepare seedlings for the assay devise. Two uniform seedlings of Rabeh and Enkath cultivars and *E. colonum* L. were transplanted in their respective plastic pots each containing 1.5 kg acid washed sand. Treatment series consisted of pots of sorghum cultivars alternated with pots of *E. colonum* L. in the stair case system. A control series consisted of pots

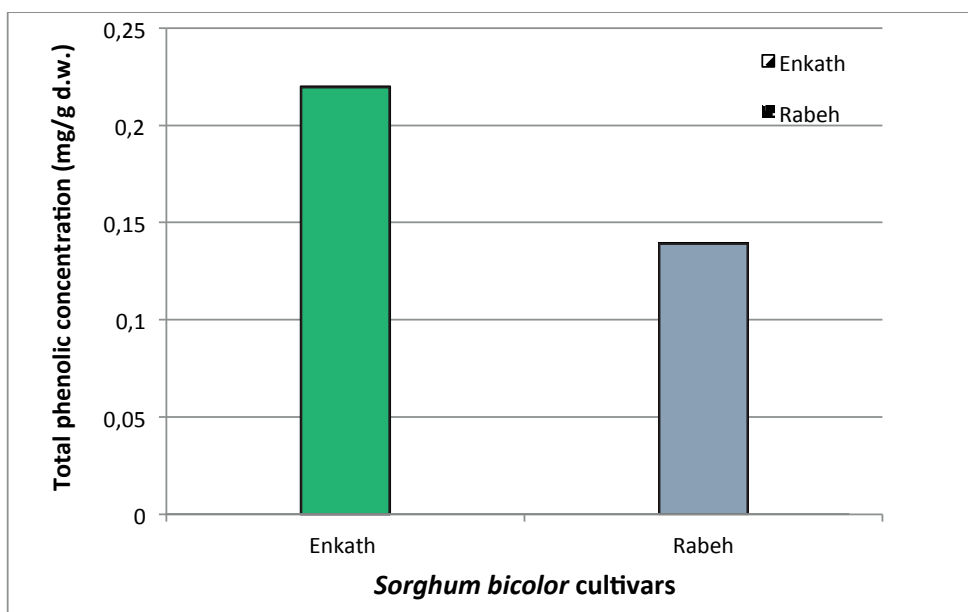


Figure 2. Total phenolics collected from root exudates of two *Sorghum bicolor* cultivars. Difference between the test cultivars was statistically significant at 0.05 level by ANOVA test.

of *E. colonum* L. alternated with pots containing acid washed sand only. Complete nutrient solution (15) was stored at reservoirs fixed at the top of each series and allowed to pass the pots of each series and finally accumulated at the bottom reservoirs of each series, where it recycled every day. The solution was changed biweekly. *Echinochloa colonum* plants were allowed to grow for 60 days, then harvested, separated to roots and shoot and compared on the basis of oven dried weight. All treatments were replicated three times and were arranged in a randomized design. The data were normal and there was homogeneity of variance. The average values were compared using Least Significant Differences (LSD) at 0.05 probability level.

Isolation and characterization of root exudates

Collection of sorgoleone

Seeds of Enkath and Rabeh sorghum cultivars were surface-sterilized by soaking for 20 min in 10% bleach (0.6% sodium hypochlorite) and rinsed with copious amount of deionizer water (9). Twenty seeds were placed in 20 × 100 mm sterile Petri dishes over the surface of sterile Whatman #1 filter paper (90 mm diameter). Three milliliters of sterile water were added to each dish, and the seeds were covered with a second sterile filter paper. The top lid was placed upside down to provide some mechanical

pressure over the top of the developing seedlings. The dishes were sealed by paraffin film and incubated in the dark at 30 °C. Each treatment was replicated three times. Sorgoleone was extracted from 7-day-old seedlings. All steps of procedure were executed under low-intensity light (9) to prevent the formation of anthocyanins by sorghum roots and photo degradation of the samples during extraction.

For sorgoleone extraction, roots were excised from sorghum seedlings and immersed in 12 ml of CHCl_3 and stirred the roots for three minutes. Roots and root debris were removed and the organic solvent was collected in a clean tube and concentrated under N_2 jet at 40 °C. The weight of the total crude extract was recorded prior to compositional analysis by HPLC. The roots were transferred to pre-weighted pieces of aluminum and oven-dried three days at 60°C. The roots with the aluminum were weighted, and then the dry weight of roots was calculated.

Identification of sorgoleone by HPLC

Sorgoleone in the root exudates for the two cultivars was identified and quantified by HPLC system (Shimadzo HPLC system, a Model LC-2010A HT autosampler) equipped with a column of Hyper Clone 5 μm CN 120A (4.6 × 250 mm I.D) phenomenex C_{18} reversed phase; the column temperature was set at 25°C. Mobile phase consisted of 0.1% phosphoric acid and acetonitrile (50:50 v/v); the flow rate was 0.5

Table 1. Effect of root exudates of sorghum cultivars on total dry biomass and population of companion weedsat 35 and 65 days after sowing (DAS).

Sorghum cultivars	Total weeds dry weight	Reduction (% of control)	Total weeds number (plant/ m ²)*	Reduction (% of control)
35 DAS				
Enkath	214.3	41.2	51.2	46
Rabeh	302.0	17.1	78.1	17
Control	364.3	0.0	94.6	
LSD ≤ 0.05	7.6		8.0	
65 DAS				
Enkath	74.11	51.0	56.3	42
Rabeh	98.15	35.1	72.0	26
Control	151.26	0.0	97.2	0
LSD ≤ 0.05	11		7.3	

mlmin⁻¹. The injection volume for each sample was 1 µl and each injection lasted for 20 min. The amount of sorgoleone in each extract was quantified at280 nm based on a calibration curve obtained with standard kindly provided by Dr Franck Dayan, US Department of Agriculture, Agricultural Research Service (USDA-ARS), Natural Products Utilization Research Unit. Each treatment was replicated three times.

Collection and quantification of total phenolics

Total phenolics of root exudates were collected and extracted from living sorghum seedlings of the test cultivars using a direct resin adsorption method described by Kong et al. (16). Five uniform seedlings at the 3rd leaf stage were transplanted into previously autoclaved pot (10 × 7 cm) containing 75 g XAD-8 resin and 300mL of 1mM Mes-Tris buffer (pH 5.5) +0.5mM CaSO₄ solution, and the

pots were placed in a sterile environment growth chamber. The seedlings were grown at 25±1°C with a 12h photoperiod (light intensity), and the pots were watered once a day to maintain the volume of the solution the same during the course of the experiment. After 14 days, the seedlings were carefully separated from the resin, and then the resin and the solution were transferred to a column (5×20 cm). The water was first allowed to drain from the column and the resin was washed with distilled water and then eluted three times with 50 ml methanol. The methanol fractions were pooled and evaporated to dryness under vacuum by rotary evaporator at 40°C and the residues were taken up by2 ml methanol and kept in deep freeze until use.

For determination of total phenolics, the residues collected from elution of XAD-8 resin were used to quantify phenolics by the Folin-Denis method as described by Ben-Hammouda et al.(4).

Table 2. Effect of root exudates of sorghum cultivars on growth of *Echinochloa colonum* weed.

Treatments	Dry weight (g)		
	Roots	Shoot	Whole plant
Control	0.191	0.126	0.317
Enkath	0.093	0.038	0.131
Rabeh	0.164	0.064	0.228
LSD ≤ 0.05	0.03	0.024	0.102

Table 3. Isolation and quantification of phenolic acids collected from root exudates of two *Sorghum bicolor* cultivars.

Compounds	Concentration µg/ml	
	Cultivars	
	Enkath	Rabeh
protocatecheuic acid	54.66	33.16
p-hydroxybenzoicacid	79.20	15.55
vanillic acid	129.66	44.81
syringic acid	254.64	209.81
p-coumaric acid	191.17	108.74
ferulic acid	400.35	84.11
sinapic acid	350.40	172.72
Total	1460.08	668.9

Identification of phenolic acids by HPLC analysis

Separation and quantification analysis of allelochemicals in root exudates was also performed using Fast Liquid Chromatographic Apparatus, Shimadzu 2010 LC equipped with binary delivery pump model 2010 Shimadzu with a C₁₈ reversed-phase column (50 × 2.0 mm I.D), 3 µm particle size. Mobile phase was methanol-deionized water (30:70, v/v), eluted at a flow rate of 1.0 mlmin⁻¹. The injection volume of samples was 20µl. All samples were filtered through a 0.25-µm nylon syringe before analysis, and the eluted peaks were monitored by UV-Vis 2010 SPD spectrophotometer detected at 352 nm.

Results and discussion

Weed flora appeared in the sorghum field during the study consisted mainly of *Sorghum halepense* L., *Echinochloa colonum* L. *Convolvulus arvensis* L. *Portulaca oleracea* L., *Xanthium strumarium* L., *Amaranthus retroflexus* L., *Malva rotundifolia* L., and *Chenopodium album* L. Both sorghum cultivars significantly suppressed total number and dry biomass of these weeds at 35 and 65 DAS (Table 1). However, Enkath recorded the highest inhibition of total weed number and dry biomass with a reduction of 46 and 41% of control, respectively at 35 DAS and 42 and 51% at 65 DAS. The differential inhibition of weeds between the two cultivars could be attributed mainly to the difference in exudates production and not to competition on light, moisture or nutrients since field measurements exhibited no differences in plant height and above ground biomass between the test cultivars (data not shown). Differential allelopathic potential among cultivars of several allelopathic crops

including grain sorghum have been documented previously (4,5,20). The staircase experiment supports the results of field experiment as the root exudates of both cultivars significantly inhibited root and shoot growth of the test weed (Table 2), with the superiority of Enkath over Rabeh in the suppression of roots, shoots and whole plant of the test weed by 43, 40 and 43%, respectively.

Chemical analysis indicated the presence of sorgoleone compound in root exudates of both cultivars with significant increase in Enkath cultivar (8 mg/g d.w.) compared to cultivar Rabeh (6.8 mg/g D.W) (Fig. 1). Other studies have indicated differences in root exudates production among different sorghum accessions grown under the same conditions (19,24). This compound has been reported to be phytotoxic to broadleaf and grass weeds at concentrations as low as 10 µM in hydroponic assays (11,19), and broadleaf weed species are more susceptible than grass weed species (23). Sorgoleone exerts both pre-emergence and post-emergence effects on different weeds (24,8).

The results of this study showed that sorgoleone was not the only phytotoxin exists in root exudates but phenolics are also present in sizeable amounts (Fig.2). These compounds appeared to be higher in Enkath (0.22 mg/g) than in Rabeh (0.14 mg/g) giving additional evidence for the higher allelopathic potential of Enkath. Chemical analysis on these phenolics by HPLC indicated the presence of several allelochemicals as protocatecheuic, hydroxybenzoic, vanillic, syringic, p-coumaric, ferulic and sinapic acids and four unidentified compounds (Table 3). Almost all these compounds were drastically higher in Enkath than in Rabeh cultivar. The inhibitory activity of the identified allelochemicals against

weeds has been reported and most of them are water soluble and when imbibed by the germinating weed seeds, hampered their germination and subsequent seedling growth, thus contributing to overall decline in the density, vigor and stand establishment of the weed community (12,16).

In conclusion, the variation in weed suppressive ability between the test sorghum cultivars was attributed to their differences in the allelochemicals content of root exudates. The phytotoxicity of root exudates was not restricted to sorgoleone alone but with phenolic acids. These results encourage conducting more work in order to offer a potential source of germplasm that could be manipulated to enhance weed suppression in an effective and environmentally sustainable approach.

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First approach to enhance the physicochemical properties of 2-amino-3*H*-phenoxazin-3-one (APO)

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ABSTRACT

Benzoxazinones and their degradation products are naturally occurring allelochemicals that are present in wheat, corn, rye, other members of the Gramineae, and some non-Gramineae species. 2,4-Dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) is the major hydroxamic acid in *Secale cereal* L. This compound is unstable in solution and soils and is transformed into 2-benzoxazolinone (BOA) and other degradation products. Further degradation of this compound yields the tricyclic structures 2-amino-3*H*-phenoxazin-3-one (APO). The synthesis of a wide variety of compounds with an aminophenoxazine skeleton has been studied extensively, mainly due to their pharmaceutical interest rather than for their agronomical use. In this sense, we have prepared a new generation of chemicals that includes eighteen derivatives with the aminophenoxazine skeleton with the aim of improving their phytotoxicity. These compounds were tested in a wheat coleoptile bioassay and with selected standard target species (STS) in the search for the optimal levels for phytotoxicity.

Keywords: Allelochemicals, Aminophenoxazines, Benzoxazinoids, 2-Benzoxazolinone, SAR.

Introduction

In recent years the scientific interest in selective biological weed management has increased significantly (Niemeyer, 1988; Einhelig, 1995). Allelopathy could be an alternative approach to solve this problem and allelochemicals are considered to be promising new resources for agricultural pest management. Moreover, allelochemicals potentially have more specific interactions with target organisms and they may be less environmentally damaging, features that make them attractive agrochemicals (Rimando Agnes and Duke Stephen, 2006). In the past, research into allelochemicals has been mainly focused on their isolation from natural sources, their structural characterization, and the evaluation of their phytotoxic bioactivity in germination and growth bioassays as the final result of bioguided isolation procedures (Vyvyan, 2002). In addition, these compounds may have novel or multiple modes of action, a possibility that could further enhance their effectiveness (Duke et al., 2000).

Benzoxazinones and their degradation products are naturally occurring allelochemicals that are present in wheat, corn, rye, other members of the Gramineae (Niemeyer, 1988) and insome non-

Gramineae species (Pratt et al., 1995; Baumeler et al., 2000). Several types of bioactivity have been described and evaluated for these compounds, including fungistatic (Bravo and Lazo, 1996; Friebe et al., 1998), antifeedant (Klun et al., 1967; Argandoña et al., 1983), and phytotoxic (Barnes and Putnam, 1987; Perez and Ormeno-Nunez, 1991).

2,4-Dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIBOA) is the major hydroxamic acid in *Secale cereal* L. (Honkanen and Virtanen, 1960). This compound can be detected within the plants as the β -D-glucosides. Upon plant injury the glucoside is enzymatically converted into DIBOA by a β -glucosidase. This aglycone is unstable in solution and soil and is transformed to 2-benzoxazolinone (BOA) and other degradation products. Further degradation of this compound yields the tricyclic structure 2-amino-3*H*-phenoxazin-3-one (APO) (Fomsgaard et al., 2004; Macias et al., 2005b). This metabolite has been reported to be more phytotoxic than 2-benzoxazolinone (BOA) (Gagliardo and Chilton, 1992; Friebe et al., 1996). Transformations in environments such as aqueous solutions, crop soils and microbial cultures have been widely studied (Gagliardo and Chilton, 1992; Zikmundova et al., 2002; Macias et al., 2005b).

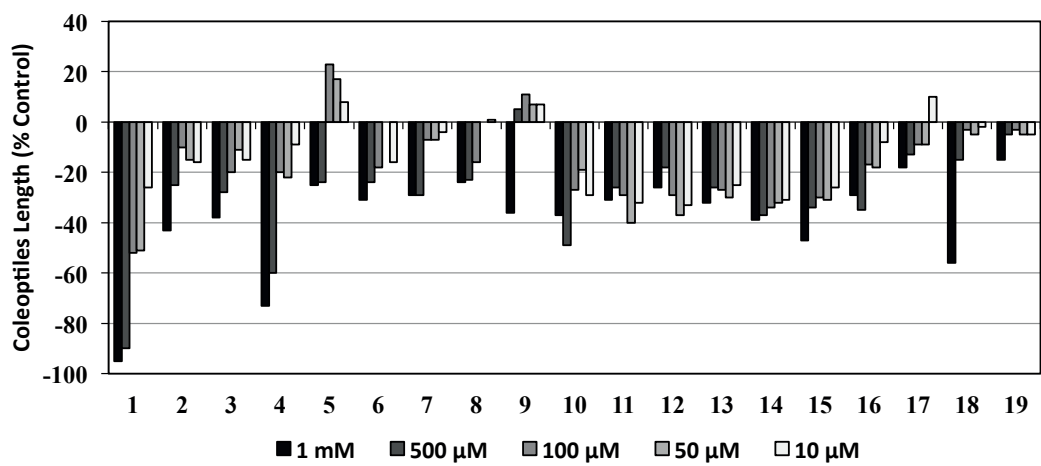


Figure 1. Effects of the assayed compounds in the wheat coleoptile bioassay.

APO displays a wide variety of interesting properties – especially with regard to its phytotoxic activity and the influence that this has on the allelopathic phenomena observed for Poaceae plants. Previously, we evaluated the phytotoxicity of benzoxazinones and related compounds (Tables 1 and 2) on STS and weeds in the search for new compounds that could be applied as part of a strategy directed towards weed control in affected crops (Macias et al., 2005a; 2006). Statistical treatment of the acquired data would enable structure-activity relationships to be highlighted, and these could be useful in the further development of highly phytotoxic compounds based on natural product structures. The results of these studies indicated that 2-amino-3*H*-phenoxazin-3-one (APO) seems to be an optimal candidate for the development of further natural herbicide models for weed management.

The synthesis of a wide variety of compounds with an aminophenoxazine skeleton has been carried out, mainly due to the pharmaceutical interest in these compounds (Tomoda et al., 2011; Takemura et al., 2012) rather than for their agronomical use (Fomsgaard et al., 2004). All of the synthetic methodologies employed to date involve the dimerization of two phenol units to access the tricyclic structure (Kehrmann, 1906; Buckley et al., 1982; Szeverenyi et al., 1991) and they mainly concern the synthesis of 2-amino-3*H*-phenoxacin-3-one (APO).

As part of our ongoing research into the structure-activity relationships of benzoxazinones and their degradation products, a new generation of chemicals that includes eighteen derivatives with the aminophenoxazine skeleton was developed in an effort to enhance the phytotoxicity and aqueous solubility. These compounds were tested in the wheat coleoptile

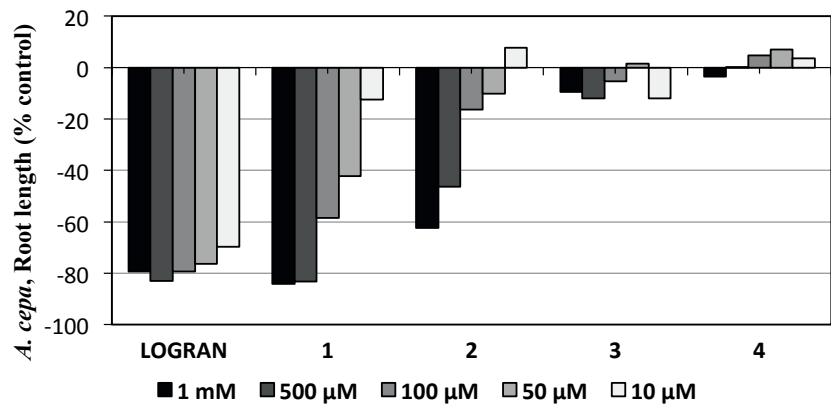


Figure 2. Phytotoxic effects of tested halogenated compounds.

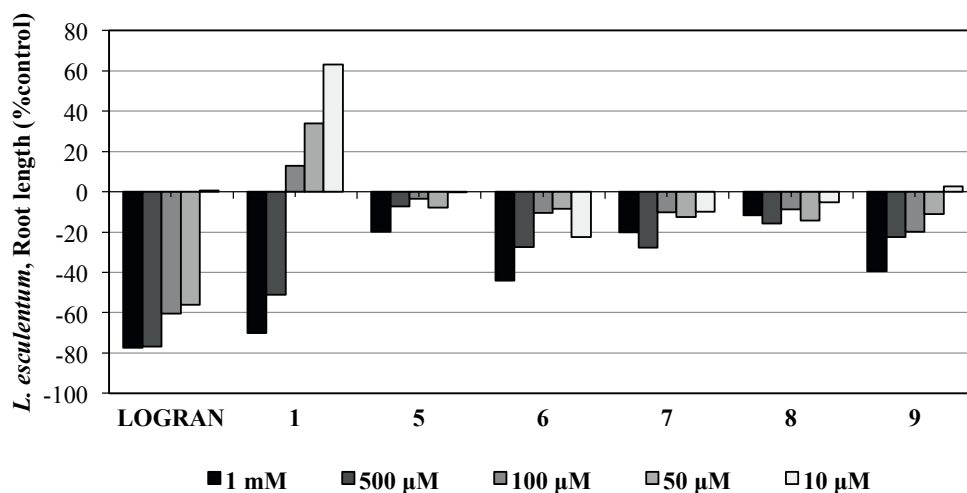


Figure 3. Phytotoxic effects of tested alkenoxy compounds on the root length of the species *Lycopersicon esculentum* W.

bioassay and with selected standard target species (STS) in the search for the optimal aqueous solubility/lipophilicity levels to obtain phytotoxicity. The lipophilicity was increased by incorporating alkyl and alkene groups into the structure. Moreover, an improvement in the aminophenoxazine skeleton by modification of the steric and electronic features was also carried out. These modifications included the addition of halogen substituents to the tricyclic structure.

Materials and methods:

General methods

The purities of the isolated and synthetic compounds were determined by NMR and HPLC analyses and were found to be >98%. ^1H and ^{13}C NMR spectra were recorded using CD_3SOCD_3 as solvent on

a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual dimethyl sulfoxide (DMSO) was set to δ 2.49. The solvent peak for ^{13}C was set to δ 39.5 as an internal reference. Mass spectra were recorded on a WATERS SYNAPT G2 spectrometer using methanol as solvent. FTIR spectra were obtained on a BX Perkin-Elmer FTIR spectrophotometer. Frequency values are given in cm^{-1} .

Chemicals

The chemicals employed for this study are listed in Table 3. Eighteen derivatives were synthesized and these included aromatic ring modifications at positions C-4, C-6 and C-8 plus the addition of side chains at position C-7 of the aminophenoxazine skeleton. The spectroscopic data are shown in Tables 4, 5 and 6.

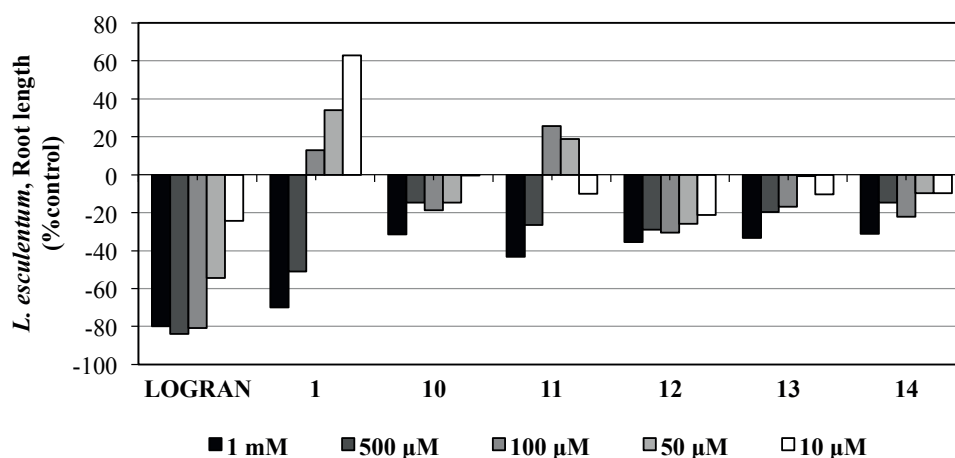


Figure 4. Phytotoxic effects of tested alkyloxy compounds on the root length of the species *Lycopersicon esculentum* W.

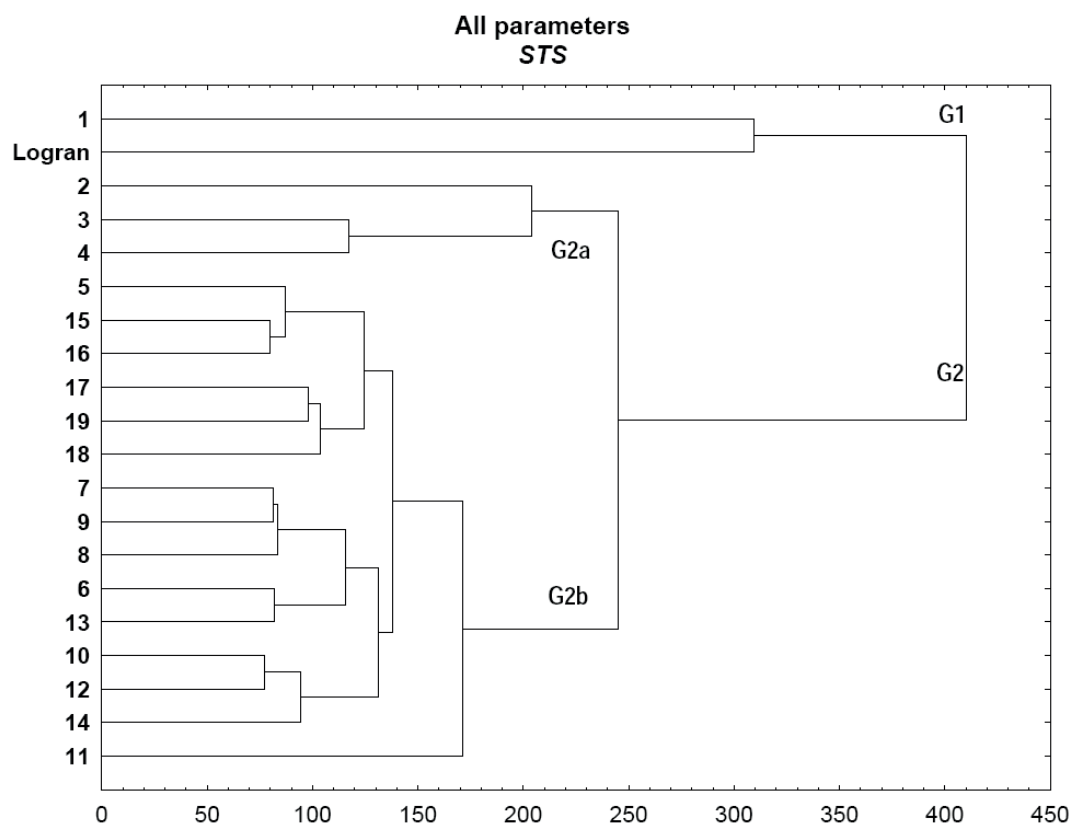


Figure 5. Cluster analysis (phytotoxicity data, germination, and growth parameters, all tested species) of the tested chemicals.

General synthesis procedures

Preparation of 2-amino-3H-phenoxazin-3-one (1). This compound was synthesized according to the literature procedure (Gagliardo and Chilton, 1992).

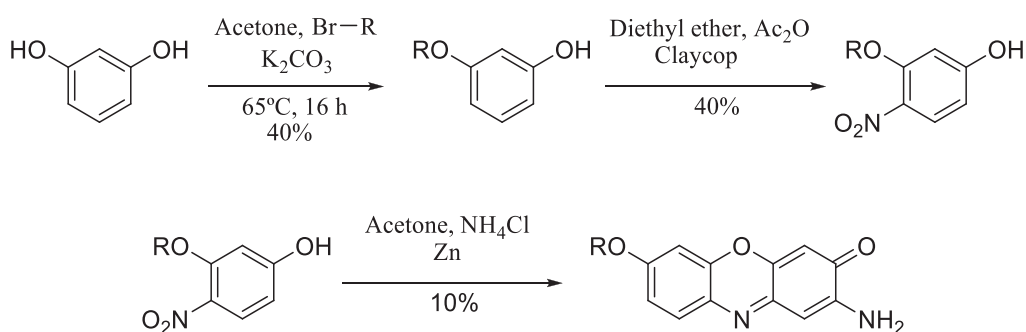
C-7 derivatives. The C-7 derivatives were obtained in a three-step sequence: nucleophilic substitution (Moreno-Manas et al., 2001), nitration employing claycop as the nitrating agent (Dwyer and Holzapfel, 1998) and dimerization to achieve aminophenoxazine ring formation (Scheme 1).

General procedure for nucleophilic substitution: resorcinol (15.1 mmol) was dissolved in acetone. The reaction mixture was stirred under argon at room temperature. The mixture was then heated and K_2CO_3 (23.1 mmol) was added. When the temperature was 68 °C the allyl bromide/iodide/chloride was added. The reaction mixture was stirred at 68 °C for 16 h. After this time the reaction mixture was quenched. The solvent was removed under reduced pressure. The crude product was redissolved in Sørensen buffer and was treated with HCl_{aq} . The product was extracted with dichloromethane (4 × 100 mL). The organic layers were combined and

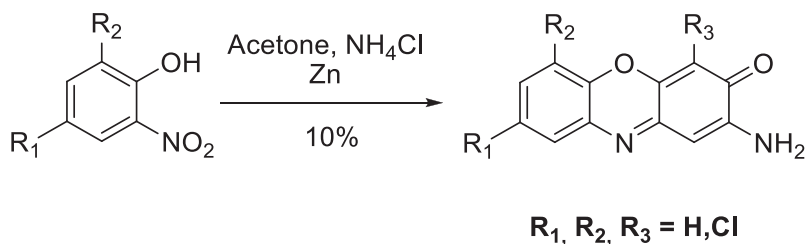
dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure. The product was purified by chromatography (CC, ethyl acetate/hexane).

General procedure for nitration: 3-allylphenol (5 mmol) was dissolved in diethyl ether (25 mL) and acetic anhydride (50 mmol) and the reaction mixture was stirred. Claycop (2.5 g) was added and the reaction mixture was stirred for 2 h. Once the reaction was complete, the suspension was filtered through Celite to remove the claycop and the solvent was removed under reduced pressure. The crude product was purified by chromatography (CC, ethyl acetate/hexane) to give the corresponding derivatives.

General procedure for aminophenoxazine ring formation: 5-allyl-2-nitrophenol (1 mmol) was dissolved in acetone (4.5 mL), and saturated aqueous ammonium chloride (6 mL) and zinc metal (4 mmol) were added. The reaction mixture was stirred for 5 min under ultrasound until the reaction was completed. The evolution of the reaction was monitored by TLC. Once the reaction was complete, the suspension was filtered to remove the zinc metal and the solvent was removed under reduced pressure.



Scheme 1



Scheme 2

The crude product was dissolved in acetone and the solution was allowed to stand at room temperature during 7 days. A brown precipitate was obtained. The suspension was filtered to obtain the corresponding pure derivatives.

Chlorinated derivatives. The chemicals were obtained in one step, as shown in Scheme 2. 4-, 6- or 4,6-(di)chloro-2-nitrophenol (1 mmol) was dissolved acetone (4.5 mL) and saturated aqueous ammonium chloride (6 mL) and zinc metal (4 mmol) were added. The reaction mixture was stirred for 5 min under ultrasound until the reaction was complete. The evolution of the reaction was monitored by TLC. Once the reaction was complete, the suspension was filtered to remove the zinc metal and the solvent was removed under reduced pressure. The crude product was dissolved in acetone and the solution was allowed to stand at room temperature for 7 d. A brown precipitate was formed. The suspension was filtered to obtain the corresponding pure derivatives.

Calculation of IC_{50}

The phytotoxicity data were fitted to a sigmoidal dose-response model (constant slope) using the Graphpad prism v.4.00 software package (Graphpad, 2003).

General Activity Bioassays

Wheat coleoptile bioassays. Wheat seeds (*Triticum aestivum* L. cv. Catervo) were sown in 15 cm diameter Petri dishes moistened with water (15 mL) and grown in the dark at 22 ± 1 °C for 3 days (Hancock et al., 1964). The roots and caryopses were removed from the shoots and placed in a *Van der Weij* guillotine. The apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for the bioassay. All manipulations were performed under a green safelight (Nitsch and Nitsch, 1956). Test solutions of each compound were prepared in a phosphate-citrate buffer solution containing 2% sucrose adjusted to pH 5.6. Parallel controls were also run. Five coleoptiles were placed in each test tube containing 2 mL of test or control solutions (three replicates per dilution). The test tubes were kept in constant rotation at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were then digitally photographed and measured. Data were statistically analyzed using Welch's test (Martín Andrés and Luna del Castillo, 1990). Results are presented as percentage differences from control. Thus, zero represents the control, positive values represent growth stimulation and negative values represent growth inhibition.

Table 1. Natural benzoxazinones and synthetic derivatives mentioned in this study.

R1c1ccc2c(c1)oc(R2)c(=O)n2R3

	R ₁	R ₂	R ₃	Acronym
Natural Allelochemicals (A)	H	O-β-D-Glc	OH	DIBOA-Glc
	H	OH	OH	DIBOA
	OCH ₃	OH	OH	DIMBOA
Degradation Products (B)	H	OH	H	HBOA
	OCH ₃	OH	H	HMBOA
Synthetic analogs (C)	H	H	H	D-HBOA
	OCH ₃	H	H	D-HMBOA
	H	H	OH	D-DIBOA
	OCH ₃	H	OH	D-DIMBOA
	H	H	OAc	ABOA
	OCH ₃	H	OAc	AMBOA

Phytotoxicity bioassays

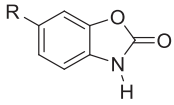
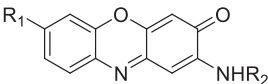
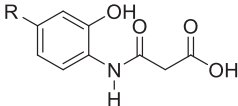
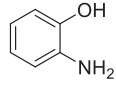
Target Plants. The selection of target plants was based on an optimization process carried out by us in the search for a standard phytotoxicity evaluation bioassay (Macias et al., 2000). This process led to several standard target species (STS) being proposed, including the monocot *Allium cepa* L. (onion) and dicots *Lycopersicon esculentum* Will. (tomato), *Lepidium sativum* L. (cress) and *Lactuca sativa* L. (lettuce), which were assayed for this study.

Methodology. Bioassays were carried out using Petri dishes (90 mm diameter) with one sheet of Whatman No. 1 filter paper as substrate. Germination and growth were conducted in aqueous solutions at controlled pH using 10⁻² M 2-(N-morpholino)ethane-sulfonic acid (MES) and the addition of 1 M NaOH to give a pH of 6.0. Solutions (0.2, 0.1, 0.02, 0.01 and

0.002 M) of the compounds to be assayed were prepared in dimethylsulfoxide (DMSO) and then diluted with buffer (5 μL DMSO mL⁻¹ buffer) to give the test concentrations for each compound (1000, 500, 100, 50, 10, 5, 1 μM). This procedure facilitated the solubility of the assayed compounds. Twenty-five seeds were placed in each Petri dish. The treatment control (or internal reference solution) (5 mL) was added to each Petri dish. Four replicates were used for each species.

After the addition of seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled-environment growth chamber in the absence of light. Bioassays took 4 days for cress, 5 days for lettuce and tomato, and 7 days for onion. After growth, plants were frozen at -10 °C for 24 h to avoid subsequent

Table 2. Benzoxazinone degradation products mentioned in this study.

Benzoxazolinones	R		Acronym
	H		BOA
	OCH ₃		MBOA
Aminophenoxazines	R ₁	R ₂	Acronym
	H	H	APO
	OCH ₃	H	AMPO
	H	OAc	AAPO
	OCH ₃	OAc	AAMPO
	OH	H	AHPO
Malonamic acids	R		Acronym
	H		HPMA
	OCH ₃		HMPMA
Miscellaneous	Acronym		
	APH		

growth during the measurement process. This helped in the handling of the plants and allowed a more accurate measurement of root and shoot lengths.

The commercial herbicide Logran[®], a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]benzenesulfonamide (triasulfuron, 0.6%), was used as the internal reference in accordance with a comparison study reported previously (Macias et al., 2000).

Bioassay data acquisition. The evaluated parameters (germination rate, root length, and shoot length) were recorded using a Fitomed system (Castellano et al., 1999), which allowed automatic data acquisition and statistical analysis by its associated software.

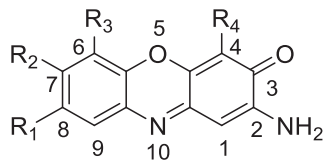
Statistical analysis. Data were statistically analyzed using Welch's test, with significance fixed at 0.01 and 0.05. Results are represented in bar charts in which the zero value represents control, negative values represent inhibition, and positive values represent stimulation of the studied parameter (Macias et al., 2000). Phytotoxic activities expressed in this way can be

found in the Supporting Information for all chemicals and species. Once the germination and growth data had been acquired, cluster analysis was used to group compounds with similar phytotoxicity behavior and associate the properties with the molecular structure. Complete linkage was used as an amalgamation rule and the distance measurement was based on squared Euclidean distances, given by the equation

$$d(x,y) = \sum_i (x_i - y_i)^2$$

where $d(x,y)$ is the squared Euclidean distance (i -dimensional), i represents the number of variables, and x and y are the observed values. The cluster was obtained using Statistica v.5.0 software (Statsoft Inc.). Germination rate, shoot length, and root length effects for all tested species were included in the analysis to provide an overall view of the phytotoxicity and its relationship with chemical structure. EC₅₀ values were obtained after the phytotoxicity data had been adjusted to concentration (logarithmic scale), to a constant slope sigmoidal dose-response curve,

Table 3. Derivatives prepared.



R ₁	R ₂	R ₃	R ₄	COMPUESTO	
H	H	H	H	2-amino-3H-phenoxazin-3-one	1
Cl	H	H	H	2-amino-8-chloro-3H-phenoxazin-3-one	2
H	H	Cl	Cl	2-amino-4,6-dichloro-3H-phenoxazin-3-one	3
Cl	H	Cl	Cl	2-amino-4,6,8-trichloro-3H-phenoxazin-3-one	4
H	-O-CH ₂ -CH ₂ =CH ₂	H	H	7-allyloxy-2-amino-3H-phenoxazin-3-one	5
H	-O-(CH ₂) ₃ =CH ₂	H	H	2-amino-7- butenyloxy-3H-phenoxazin-3-one	6
H	-O-CH ₂ -CH ₂ =CH ₂ -CH ₃	H	H	2-amino-7-crotyloxy-3H-phenoxazin-3-one	7
H	-O-CH ₂ -CH=CH(CH ₃) ₂	H	H	2-amino-7-dimethylallyloxy-3H-phenoxazin-3-one	8
H	-O-CH ₂ -CH(CH ₃)=CH ₂	H	H	2-amino-7-methylallyloxy-3H-phenoxazin-3-one	9
H	-O-CH ₃	H	H	2-amino-7-methoxy-3H-phenoxazin-3-one	10
H	-O-CH ₂ -CH ₃	H	H	2-amino-7-ethoxy-3H-phenoxazin-3-one	11
H	-O-CH ₂ -CH ₂ -CH ₃	H	H	2-amino-7-propoxy-3H-phenoxazin-3-one	12
H	-O-(CH ₂) ₃ -CH ₃	H	H	2-amino-7-butoxy-3H-phenoxazin-3-one	13
H	-O-(CH ₂) ₄ -CH ₃	H	H	2-amino-7-pentyloxy-3H-phenoxazin-3-one	14
H	-O-(CH ₂) ₅ -CH ₃	H	H	2-amino-7-hexyloxy-3H-phenoxazin-3-one	15
H	-O-(CH ₂) ₆ -CH ₃	H	H	2-amino-7-heptyloxy-3H-phenoxazin-3-one	16
H	-O-(CH ₂) ₇ -CH ₃	H	H	2-amino-7-octyloxy-3H-phenoxazin-3-one	17
H	-O-(CH ₂) ₈ -CH ₃	H	H	2-amino-7-nonyloxy-3H-phenoxazin-3-one	18
H	-O-(CH ₂) ₉ -CH ₃	H	H	2-amino-7-decyloxy-3H-phenoxazin-3-one	19

defined by the equation

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{\log EC_{50} - X}}$$

where X indicates the logarithm of concentration, Y indicates the response (phytotoxicity), and Y_{max} and Y_{min} are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r²). The adjustment and r² were obtained using GraphPad Prism software v.4.00 (Graphpad, 2003).

Results and discussion

General Bioactivity Profiles

The effects of the assayed compounds on wheat coleoptiles are shown in Figure 1. All of the compounds showed inhibitory profiles for the parameter evaluated, but in general the substitutions performed on APO (1) did not improve the activity. The most

active compound was 4, which showed inhibition values of around 70–75% at a concentration of 1 mM. Compounds 14 and 15 also showed good inhibitory profiles.

Phytotoxic bioactivity profiles

All of the compounds that were active showed inhibitory profiles for all evaluated parameters. In general terms, growth parameters (root and shoot lengths) were more markedly affected than germination rates. Among the growth parameters, root growth was more affected than shoot length, as one would expect for aminophenoxazine derivatives. In general terms, these compounds had a less pronounced phytotoxicity in comparison to 2-amino-3H-phenoxazin-3-one (1). None of the species were significantly affected by the tested chemicals. There was a general decrease in the phytotoxic effects of these compounds in comparison to 2-amino-3H-phenoxazin-3-one (1).

Halogenated derivatives. Among the Standard Target

Table 4. ¹H NMR chemical shifts (400 MHz, signal of residual solvent centered at δ 2.49 ppm for DMSO).

H	2	3	4	5	6	7	8	9	10	11
1	6.36 s	6.41 s	6.98 s	6.34 s	6.41 s	6.47 s	6.35 s	6.34 s	6.35 s	6.35 s
4	6.34 s	--	--	6.32 s	6.37 s	6.40 s	6.32 s	6.32 s	6.32 s	6.33 s
6	7.52 d	--	--	7.11 d	7.16 d	6.87 d	7.10 d	7.10 d	7.11 d	7.10 d
7	7.46 dd	7.70 dd	8.15 d	--	--	--	--	--	--	--
8	--	7.42 t	--	7.02 dd	7.05 dd	6.95 dd	6.99 dd	7.02 dd	7.01 dd	7.01 dd
9	7.73 d	7.64 dd	8.11 d	7.63 d	7.68 d	7.65 d	7.62 d	7.62 d	7.64 d	7.64 d
1'	--	--	--	4.68 dt	4.20 t	4.54 d	4.63-4.58 m	4.59 s	3.85 s	4.14 q
2'	--	--	--	6.05 ddt	2.57 m	5.95-5.88 m	5.47-5.43 m	--	--	1.36 t
3'	--	--	--	5.29 dq 5.43 dq	5.95 ddt	5.95-5.88 m	--	5.07-4.94 m	--	--
4'	--	--	--	--	5.24 ddd	1.78 dd	1.74-1.71 m	--	--	--
5'	--	--	--	--	5.15 dd	--	1.74-1.71 m	1.77 s	--	--

* s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet

Table 5. ¹H NMR chemical shifts (400 MHz, signal of residual solvent centered at δ 2.49 ppm for DMSO).

H	12	13	14	15	16	17	18	19
1	6.36 s	6.34 s	6.35 s	6.45 s	6.45 s	6.45 s	6.46 s	6.46 s
4	6.33 s	6.31 s	6.33 s	6.38 s	6.38 s	6.38 s	6.39 s	6.39 s
6	7.10 d	7.10 d	7.10 d	6.84 d	6.83 d	6.84 d	6.84 d	6.84 d
8	7.01 dd	6.99 dd	7.0 dd	6.92 dd	6.92 dd	6.92 dd	6.93 dd	6.93 dd
9	7.63 d	7.62 d	7.63 d	7.63 d	7.63 d	7.63 d	7.64 d	7.64 d
1'	4.05 t	4.07 t	4.07 t	4.02 t	4.01 t	4.02 t	4.02 t	4.02 t
2'	1.76 tq	1.70 tt	1.74 tt	1.82 tt	1.81 tt	1.81 tt	1.81 tt	1.81 tt
3'	0.99 t	1.43 m	1.38 m	1.46 tt	1.46 tt	1.46 tt	1.46 tt	1.46 tt
4'	--	0.93 t	1.38 m	1.37-1.32 m	1.36-1.31 m	1.38-1.23 m	1.37-1.24 m	1.37-1.24 m
5'	--	--	0.9 t	1.37-1.32 m	1.36-1.31 m	1.38-1.23 m	1.37-1.24 m	1.37-1.24 m
6'	--	--	--	0.9 t	1.36-1.31 m	1.38-1.23 m	1.37-1.24 m	1.37-1.24 m
7'	--	--	--	--	0.88 t	1.38-1.23 m	1.37-1.24 m	1.37-1.24 m
8'	--	--	--	--	--	0.88 t	1.37-1.24 m	1.37-1.24 m
9'	--	--	--	--	--	--	0.87 t	1.37-1.24 m
10'	--	--	--	--	--	--	--	0.87 t

* s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet

Table 6. ¹³C NMR chemical shifts (400 MHz, signal of residual solvent centered at δ 39.5 ppm for DMSO).

C	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	103.7	104.2	104.6	103.3	103.2	104.0	103.2	103.3	103.3	103.3	103.2	103.2	103.1	104.0	104.0	104.0	104.0	104.0
2	149.1	148.9	148.9	148.5	184.5	160.2	148.6	148.6	148.6	148.7	148.6	148.6	148.3	149.2	149.2	149.2	149.2	149.3
3	180.2	176.0	177.4	179.8	179.8	207.0	179.8	179.8	179.8	179.9	179.8	179.8	179.8	184.0	180.1	180.0	180.0	180.1
4	98.0	113.5	113.0	98.8	98.8	100.6	98.8	98.8	98.8	98.9	98.8	98.8	98.5	100.2	100.2	100.2	100.2	100.2
4a	147.8	150.1	149.7	145.7	145.6	145.1	143.2	145.7	145.7	145.6	145.5	145.6	145.6	145.1	145.1	145.1	145.1	145.1
5a	143.2	135.0	139.3	143.1	143.2	144.1	138.0	143.1	143.2	143.3	143.2	143.2	143.2	144.1	144.1	144.1	144.1	144.2
6	115.2	119.8	126.2	100.8	100.5	101.3	100.6	100.8	100.0	100.4	100.4	100.4	100.4	101.3	101.3	101.3	101.3	101.3
7	122.8	128.9	128.2	158.8	159.2	180.1	159.2	158.9	160.0	159.3	159.4	159.4	159.2	160.7	160.7	160.7	160.7	160.7
8	126.6	126.9	127.0	113.9	113.8	114.2	113.9	112.8	113.4	113.8	113.8	113.8	114.0	114.1	114.1	114.1	114.1	114.1
9	117.6	125.7	123.6	128.4	128.3	122.5	120.7	128.4	128.4	128.3	128.3	128.3	128.2	128.7	128.7	128.7	128.7	128.7
9a	140.8	137.7	137.7	129.0	129.0	129.7	128.3	128.9	129.0	129.1	129.0	129.0	129.0	129.6	129.6	129.6	129.6	129.6
10a	148.8	147.2	149.3	146.6	146.5	149.2	146.5	146.6	146.6	146.6	146.5	146.5	146.5	145.8	145.8	145.8	145.8	145.8
1'	--	--	--	69.0	67.5	69.5	65.3	71.6	56.0	64.2	69.8	68.1	68.2	21.5	100.2	68.9	68.9	68.9
2'	--	--	--	133.0	32.8	131.6	119.2	140.3	--	14.5	21.8	30.6	28.0	29.0	31.7	29.3	29.0	29.0
3'	--	--	--	118.1	134.6	125.0	128.9	113.9	--	--	10.3	18.7	27.4	25.6	29.0	26.0	25.6	26.0
4'	--	--	--	--	117.2	17.9	25.4	--	--	--	--	13.6	21.8	22.6	29.0	29.2	29.2	29.3
5'	--	--	--	--	--	--	18.1	19.1	--	--	--	--	13.7	14.0	25.9	29.0	29.5	29.5
6'	--	--	--	--	--	--	--	--	--	--	--	--	--	0.9	22.6	31.8	29.3	29.5
7'	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14.1	22.6	31.8	29.3
8'	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14.1	22.6	31.9
9'	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14.1	22.6
10'	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14.1

Species, onion was the species that was affected the most, whereas lettuce and tomato were the most tolerant plants to the tested treatments. The results obtained indicate that the most active compound was 2-amino-8-chloro-3*H*-phenoxazin-3-one (**2**) on the root length of *Allium cepa* L (Figure 2).

Derivatives with an alkene group at C-7. The compounds considered in this section are 2-amino-7-butenyloxy-3*H*-phenoxazin-3-one (**6**) and 2-amino-7-methylallyloxy-3*H*-phenoxazin-3-one (**9**). Both chemicals caused inhibitions of 40% at 1000 μM for root length in *L. esculentum* W. (Figure 3).

Derivatives with an alkyl group at C-7. As in the case described above, only a slight effect of these compounds on the Standard Target Species was observed for the alkyloxy derivatives. The incorporation of an ether function did not lead to significant increases in the phytotoxicity in comparison to the lead compound. It can be pointed out that the most active compound was 2-amino-7-propoxy-3*H*-phenoxazin-3-one (**12**), which caused an inhibition of 40% at 1000 μM for root length of *Lycopersicon esculentum* L. (Figure 4).

Structure-Activity Relationship (SAR)

Taking into consideration the effects on all parameters and species, a cluster analysis was carried out to classify the tested chemicals according to their phytotoxicity. The results of this analysis are shown

in Figure 5. Cluster analysis of the data allowed us to classify the assayed compounds according to their phytotoxicity into two main groups: G1 and G2 (G1 for the highest activities and G2 for weaker effects). The most active group (G1) includes the compound APO (**1**) and the commercial herbicide Logran, which present the highest activities. Compounds with lower activity are included in G2 and this group includes all of the chemicals synthesized in this work. The chlorinated compounds (**2**, **3** and **4**) were the most active in this group.

Conclusions

The synthesis of derivatives of APO (**1**) has been carried out. Chains were added in the C-7 position with the aim of optimizing the solubility and lipophilicity of these compounds and modifications were also made on the rings by incorporating halogenated substituents in order to improve the stereoelectronic properties of the lead compound. Both the general and phytotoxic activities of these derivatives were evaluated for use in the rational development of model herbicides.

In this sense, the results obtained indicate that the most active compound was 2-amino-4,6,8-trichloro-3*H*-phenoxazin-3-one (**4**) on the root length of *Allium cepa* L. The work described here complements a previous study by examining the effects of further

modifications of the aminophenoxazine skeleton in the search for an accurate description of the chemical selectivity for action. These compounds constitute the first generation of compounds to be tested in this way and further modifications are required to enhance the bioactivity.

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