Fungal Phytotoxins for Control of Cirsium arvense and Sonchus arvensis

Antonio Evidente* • Anna Andolfi • Alessio Cimmino

ABSTRACT

Perennial weeds, including Cirsium arvense and Sonchus arvensis, are a common problem in crop fields, especially in agricultural systems with reduced herbicide usage. Herbicides recommended for control of these perennials generally are restricted to only a few active ingredients that tend to have low selectivity, especially on dicot crops. Microbial phytotoxins or their synthetic analogues may be candidates for new weed-control options. Many plant pathogens, especially necrotrophic or hemibiotrophic fungi, produce a range of phytotoxins responsible for disease damage and may be a source of such useful metabolites. Several pathogens, including Stagonospora cirsii and Ascochyta sonchi, were found commonly on C. arvense and S. arvensis, and these fungi also produce phytotoxic metabolites. Phylllosticta cirsii and Phomopsis cirsii, belonging to two well-known toxin-producing genera, have also been proposed for biocontrol of C. arvense. Phytotoxins isolated from these fungal pathogens are metabolites belonging to several classes of natural compounds including enol pyruvic acid derivatives, cytochalasins, nonenolides, oxazatricycloalkkenones, pentasubstituted bicyclooctatrienyl ester of acetic acid, and beta-carboxylic acid methyl ester and beta-nitropropanoic acid. Some of these metabolites may be used as biomarkers, for studies on mode of action and development of structure-activity relationships.

Keywords: biomarkers, mycoherbicides, metabolites, natural compounds

INTRODUCTION

Microbial agents benign to the environment and highly specific to herbicide-resistant weeds offer potential advantages for weed management. More recently, microbial-based phytotoxins have been explored for potential weed biocontrol (Strobel et al. 1987; Graniti et al. 1989; Delfosse 1990; Koltin et al. 1993). Necrotrophic or hemibiotrophic
plant pathogens are good sources of phytotoxins (Abbas and Duke 1995), because they often need to kill host tissues prior to consuming them. Almost all fungal species produce phytotoxic metabolites, a group of compounds with different chemical structures, mechanisms of action, host specificity and biological and ecological impact. Many of these metabolites interfere with plant metabolism and are directly responsible for the pathogenesis of the fungus (Strobel 1989; Bottiglieri et al. 2000; Duke et al. 2000). Intensive research has been directed toward the isolation of phytotoxins produced by selected fungi pathogenic to weeds and belonging to important toxigen-producing genera e.g. Alternaria, Ascochyta, Drechslera, Fusarium, Phoma and Pyrenophora (Evidente and Motta 2001; Evidente 2006; Evidente and Abouzeid 2006; Rimando and Duke 2006). Phytotoxic metabolites are common weeds in crop production, especially in systems with reduced herbicide usage. Such weed species include Cirsium arvense (L.) Scop. and Sonchus arvensis (L.) in Asteraeae) commonly called Canada thistle and perennial sowthistle, respectively. Herbicides recommended for these perennial weeds are limited and tend to have low selectivity. Microbial phytotoxins or their synthetic analogues may be used for development of new herbicides against these weeds. Several pathogens, i.e. Stagonospora cirsii J.J. Davis, Ascochyta sonchi (Sacc.) Grove and related pathogens were common on these weed species and produce phytotoxic metabolites. Phyllosticta cirsii Desm. and Phomopsis cirsii Grove, belonging to genera well known for toxin production, have also been proposed for biocontrol of C. arvense. This review describes the isolation, structural elucidation and biological characterisation of the phytotoxins produced by fungi proposed as bioherbicides for control of C. arvense and S. arvensis, as potential natural herbicides or in combination with the producer fungus or low doses of chemical pesticides in integrated management strategies. The results of structure-activity relationship studies carried out on selected fungal toxins, as well as those on use of these toxins as biomarkers and for studying modes of action will also be discussed.

CANADA THISTLE AND PERENNIAL SOWTHISTLE

Canada thistle is a persistent perennial weed that spreads by roots growing horizontally, often forming infestation patches. Perennial sowthistle is native to southeastern Europe and the eastern Mediterranean area. It has spread to most temperate regions and is considered an important weed all over the world as it infests many habitats such as arable fields, roadsides, pastures and rangeland, railway embankments, and lawns. It infests at least 27 crops in 37 countries and thrives in temperate regions of the northern hemisphere (Mitch 1988). Managing these weeds can be a challenge, and combinations of mechanical, cultural, and chemical methods are more effective than any single method used alone (Trumble and Kok 1982). Herbicides recommended for both species are limited to clopyralid, diuron, bentazon, and phenoxy-acids, and weeds (Evidente 2006; Evidente and Abouzeid 2006) and many other weeds (Evidente 2006; Evidente and Motta 2001). Numerous surveys have been carried out to find pathogens on Canada thistle (Berestetsky 1997; Leth and Andrew 1999). Bailey et al. (1999) reported that the mycoparasite conidium of Alternaria, Ascochyta, Drechslera, Ophiobolus, Phoma and many other fungal pathogen cultures (Kenfield et al. 1989; Evidente and Motta 2001). Other species, such as A. caulina (P. Karst) v.d. Aa and V. Kest against Chenopodium album L. (Netland et al. 2001) and A. cypereicola R.K. Upadhyay, Kenfield, Strobel & W.M. Hess against Cyperus rotundus L. (Upadhyay et al. 1991). The ability of many of these pathogens to produce phytotoxins has been ascertained and their involvement in causing disease symptoms has been discussed (Evidente et al. 1993a, 1993b; Strange 1997). Recently, three novel toxins have been purified and identified from the liquid culture of A. caulina and proposed as natural herbicides to be utilized in addition to or as an alternative to living pathogen propagules (Evidente et al. 1999c, 2000; Vurro et al. 2001). Therefore, the production of toxic metabolites by A. sonchi is of interest. The culture of A. sonchi, showing high phytotoxicity on host leaves, were examined to ascertain the chemical nature of the responsible metabolites. Preliminary experiments in vitro revealed that the fungus produced hydrophilic phytotoxins, because the compounds remained in the aqueous phase after exhaustive extraction of the culture filtrates with organic solvents having increased polarity. The phytotoxic metabolites had molecular weights lower than 3500, and a TLC analysis of culture filtrates also suggested the presence of amines, amino acids or peptides. The main metabolite, isolated by cationic exchange and (medium pressure) silica gel column chromatography, was characterized by spectroscopic methods (essentially 1D and 2D 1H and 13C NMR and HREI MS) as (Z)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid and named ascosonchine (1, Fig. 1) (Evidente et al. 2004). This compound was further purified by high performance liquid chromatography and subsequently identified by 1H and 13C NMR spectroscopy. Its structure was determined by a combination of 1D and 2D NMR experiments. The IR absorption bands observed for the hydroxy, double bond and carboxylic groups in ascosonchine are in agreement with those reported for the Z-enol of phenylpyruvic acid derivatives, in which the enol OH group is intramolecularly hydrogen-bonded with the carboxyl group (Cassiedi et al. 1980; Lee et al. 1998). This Z-configuration was further supported by the difference in the vicinal coupling constants of the hydrogen bonded to the carboxyl group, which gives a positive value (JCH=CO=3.7 Hz) coupling, typical for a cis arrangement of the coupled nuclei in fragments C-C=CH=COOH of similar size of electron-deficient substituents (Stobbe and Kenyon 1971; Vogeli and von Philippsborn 1975; Sciacovelli et al. 1976). This result was in agreement with the stable green-blue colour yielded when ascosonchine dissolved in DMSO reacted with FeCl3, as already observed only for Z-enol tautomers of...
the p-hydroxy PPA (phenylpyruvic acid) treated in the same condition (Cassidei et al. 1980).

Ascosonchine belongs to the group of α-ketoacids and in particular to that of the heteroarylpyruvic acids. In several cases, as also for ascosonchine, they exist exclusively under the enolic form (Sciacovelli et al. 1976; Dalla et al. 1997). The α-ketoacids, such as PPA, are metabolic products that are biologically important (Sakurai 1956; Meister 1997). The /g302-ketoacids, such as PPA, are metabolic products (Amalfitano et al. 2002), which are pyridylcarboxylic acid toxins closely related to ascosonchine, the use of a high density C18 stationary phase drastically reduced this phenomenon. Attempts were made to find the best elution conditions using this stationary phase. Satisfactory peak shape was obtained by eluting with an isocratic mixture of methanol and HPLC grade water (1:1, v/v, pH 6.2) at a flow rate of 1 mL/min over 15 min. The recovery of ascosonchine added to the culture filtrate was nearly 100%. These results indicated that a simple extraction with chloroform: iso-propanol (9:1 v/v) was adequate for the quantitative analysis of metabolites in culture filtrates (Evidente et al. 2006).

Relation between in vitro production of ascosonchine and virulence of A. sonchi strains

The optimised HPLC method was used to quantify the ascosonchine content in the culture filtrates of different A. sonchi strains from C. arvensis and S. arvensis. The HPLC chromatogram of the chloroform: iso-propanol soluble culture filtrate of A. sonchi (strain C-240) showed a peak sample, which coincided with the retention time of the ascosonchine standard at 4.60 min. The retention time was highly reproducible, varying by less than 0.5 min. Using the HPLC conditions described, ascosonchine could be quantitatively detected at 10 ng. Poor reproducibility was observed only at levels lower than 10 ng.

The ascosonchine content in culture filtrates of seven of the nine strains ranged between 0.5 and 2.7 mg/L (strain S-10 and C-240, respectively), whereas two strains (S-9 and C-177) did not produce any measurable amount of ascosonchine (Fig. 2) (Evidente et al. 2006).

There were significant differences in virulence for each C. arvensis strain (Fig. 3) when tested on both intact and wounded leaf disks. The most virulent strains were C-177, C-216 and C-240, causing necrotic lesions up to 45% the total leaf surface on wounded leaf disks, whereas strains S-7 and C-180 were almost avirulent on wounded disks and completely avirulent on unwounded leaf disks of C. arvensis. None of the strains were virulent on leaf disks of S. arvensis, regardless of wounding. Although the condition was designed to favour infection (high inoculum concentration and long period of leaf wetness), all the strains tested on whole plants of C. arvensis and S. arvensis showed a low level of pathogenicity (data not shown). Only in a few cases the lesion size reached 25% of the total leaf area with pre-wounding.

Positive relationships between toxin production and strain virulence have been found in other cases (Kumar et al. 2002; Reino et al. 2004). In our study, this hypothesis seems not to be supported, considering that the strains S-9 and C-177 (non-toxin producers in vitro), or strains S-10

\[ \text{COOH} \]

**Fig. 1** Chemical structure of ascosonchine (1).

**Table 1** Effect of ascosonchine observed in leaf-disk bio assay.\(^a\)

<table>
<thead>
<tr>
<th>Common name</th>
<th>Scientific name</th>
<th>Family</th>
<th>Effect on leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alligatorweed</td>
<td>Alternanthera philoxeroides</td>
<td>Amaranthaceae</td>
<td>++</td>
</tr>
<tr>
<td>Artichoke</td>
<td>Cynara scolymus</td>
<td>Compositae</td>
<td>-</td>
</tr>
<tr>
<td>Bean</td>
<td>Phaseolus vulgaris</td>
<td>Leguminosae</td>
<td>-</td>
</tr>
<tr>
<td>Bindweed</td>
<td>Convolvulus arvensis</td>
<td>Convolvulaceae</td>
<td>++</td>
</tr>
<tr>
<td>Chickpea</td>
<td>Cicer arietinum</td>
<td>Leguminosae</td>
<td>+</td>
</tr>
<tr>
<td>Eggplant</td>
<td>Solanum melongena</td>
<td>Solanaceae</td>
<td>-</td>
</tr>
<tr>
<td>Four-o'clock</td>
<td>Mirabilis jalapa</td>
<td>Nyctaginaceae</td>
<td>+</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>Setaria italica</td>
<td>Poaceae</td>
<td>++</td>
</tr>
<tr>
<td>Lamb's lettuce</td>
<td>Valerianella locusta</td>
<td>Valerianaceae</td>
<td>+++</td>
</tr>
<tr>
<td>Melon</td>
<td>Cucumis melo</td>
<td>Cucurbitaceae</td>
<td>+++</td>
</tr>
<tr>
<td>Pepper</td>
<td>Capsicum annuum</td>
<td>Solanaceae</td>
<td>-</td>
</tr>
<tr>
<td>Potato</td>
<td>Solanum tuberosum</td>
<td>Solanaceae</td>
<td>-</td>
</tr>
<tr>
<td>Common sage</td>
<td>Salvia officinalis</td>
<td>Labiatae</td>
<td>++++</td>
</tr>
<tr>
<td>Sowthistle</td>
<td>Sonchus arvensis</td>
<td>Asteraceae</td>
<td>+++</td>
</tr>
<tr>
<td>Spinace</td>
<td>Spinacia oleracea</td>
<td>Chenopodiaceae</td>
<td>-</td>
</tr>
<tr>
<td>Sun spurge</td>
<td>Euphorbia helioscopia</td>
<td>Euphorbiaceae</td>
<td>+++</td>
</tr>
<tr>
<td>Tomato</td>
<td>Lycopersicon esculentum</td>
<td>Solanaceae</td>
<td>+++</td>
</tr>
<tr>
<td>Triticale</td>
<td>Triticum durum</td>
<td>Poaceae</td>
<td>++</td>
</tr>
<tr>
<td>Zucchini</td>
<td>Cucurbita pepo</td>
<td>Cucurbitaceae</td>
<td>-</td>
</tr>
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*a* Toxicity determined with the following scale: - = no symptoms; + = necrosis with diameter around 1-2 mm; ++ = necrosis 2-3 mm; +++ = necrosis 3-5 mm; ++++ = necrosis > 5 mm

\(^a\) Evidente et al. 2004
Ascochyta sonchi was all able to cause disease symptoms. The origin of the strains or host plant (Russia or Norway) did not seem to be related to the virulence or toxin production (Evidente et al. 2006). It is possible that the low ascosonchine producers also biosynthesize other toxic metabolites (Evidente et al. 2006).

The main toxin produced by A. sonchi was named ascosonchine and characterized as a new enol tautomer of 4-pyridylpyruvic acid. It possesses primarily phytotoxicity, with less impact on fungi, bacteria or arthropods. This is an important feature from the practical point of view due to potentially less non-target effect. A simple and sensitive method has been developed for quantitative analyses of ascosonchine based on HPLC with UV detection and was used to evaluate the ascosonchine content in association with different A. sonchi strains. However, the toxin production is not correlated with strain virulence.

**PHOMA EXIGUA VAR. EXIGUA**

Phytopathogenic Phoma spp.

Ascochyta sonchi is synonymous to Phoma exigua Desm. var. exigua (Peve), a typical wound pathogen with a wide host range (van der Aa et al. 2000; Boerema et al. 2004). Our preliminary identification based on fungal morphology
agreed with the designation. This species has been recorded on a number of hosts in the Compositae family (Kubota and Abiko 2002; Widmer et al. 2002; Tunali et al. 2003), including C. arvense (Bithell and Stewart 2001) on which it proved to be a weak pathogen requiring a wound for successful infection (Bithell and Stewart 2001; Waipara 2003).

Even though ascosonchine was not produced by the strains S-9 or C-177, both were pathogenic on leaf disks of C. arvense (Fig. 3). Preliminary chemical and spectroscopic analyses (1H and EI and ESI MS) of additional metabolites produced by these two strains indicated that they were very different from ascosonchine and seemed to be closely related to those produced by other species of Phoma or Pyrenophora (Evidente and Motta 2001; Evidente and Abouzeid 2006). Ascosonchine has never been reported for P. exigua complex.

The genus Phoma includes many plant pathogens responsible for diseases on many plant species (Boerema et al. 2004). Although P. exigua is considered an opportunistic parasite of more than 300 plant species, it is continuously being reported as a potential biocontrol agent on weeds such as Taraxacum officinalis Weber (dandelion) (Stewart-Wade and Boland 2004), Gaultheria shallon Pursh (salal) (Zhao and Schamoun 2006), and C. arvense (Bithell and Stewart 2001; Waipara 2003; Bilder and Berestetskiy 2006). Analyses (1H and EI and ESI MS) of additional metabolites produced by these two strains indicated that they were very different from ascosonchine and seemed to be closely related to those produced by other species of Phoma or Pyrenophora (Evidente and Motta 2001; Evidente and Abouzeid 2006). Ascosonchine has never been reported for P. exigua complex.

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cola Durieu & Mont. against C. arvense (Guske et al. 2004), P. proboscis Heiny against Convolvulus arvensis L. (Heiny and Templeton 1995), and P. macrostoma Mont. against different dicotyledonous weeds (Bailey and Derby 2007). A number of Phoma species including P. lingam (Tode) Desm., P. herbarum, P. putaminum Spec., and P. macrostoma were found to produce phytotoxins, and their involvement in the disease symptom induction has been proposed (Evidente et al. 1995; Pedras et al. 1999; Rivero-Cruz et al. 2003; Graupner et al. 2006). Phytotoxins were also reported for Peve (cytochalasins A and B), the causal agent of potato gangrene (Scott et al. 1975).

The phytotoxins produced by two Phoma exigua var. exigua strains in vitro

From the organic extract of Peve strain C-177 (previously A. sonchi strain C-177) solid or liquid cultures, the well known toxins p-hydroxybenzaldehyde (2, Fig. 4) and cytochalasins, including cytochalasins B, F and deoxaphomin (3, 4 and 5, Fig. 4) were isolated. From the organic extract of solid Peve strain S-9 cultures, the cytochalasins B, Z2 and Z3 (6 and 7, Fig. 4) and deoxaphomin were isolated. Metabolites were identified by NMR spectroscopy (including COSY, HSQC and HMBC, spectra) and MS spectrometry (Cimmino et al. 2008).

Some of the cytochalasins (B, F, T, Z1 and deoxaphomin) are well known metabolites reported from several fungal species (Vurro et al. 1997), while cytochalasins Z2 and Z3 were reported for the first time from Pyrenophora seme-

Fig. 4 Chemical structure of p-hydroxybenzaldehyde (2) and cytochalasins B, F and deoxaphomin (3, 4 and 5) isolated from liquid and solid culture of P. exigua var. exigua strain C-177, and that of cytochalasins Z2 and Z3 (6 and 7) isolated from solid culture of P. exigua var. exigua strain S-9.
nipera (Brittlebank & Adam) Shoemaker, a pathogen proposed as bioherbicide for control of grass weeds (Evidente et al. 2002) and successively from S-9 Peve strain. The cytochalasins ZZ and Z3, which showed like Z1, an original structure among the 24-oxa[14]cytochalasan subgroup, were biologically characterized, as reported below by assaying their unique capacity to inhibit the germination of wheat and tomato seeds. Cytochalasins ZZ and Z3 were also isolated from various species of P. exigua (Peve) var. exigua reported as Ascochyta heteromorpha, grown in conditions common (Evidente et al. 2003). Peve is the causal pathogen of a foliar disease on oleander (Nerium oleander L.) and has been extensively studied for its capacity to produce phytotoxins in liquid cultures. In fact, many reported cytochalasins were isolated from this culture filtrate as cytochalasins A, B, 7-O-acetylcyclochalasin B, cyclochalasin F, T and deoxaphomin as well as new cytochalasins as cyclochalasin T, U, V, W and ascochalasin. Ascochalasin belong to [13]-carboxyclic subgroup of cyclochalasins. Cytochalasins U and V belong to the 25,26-dioxo[16]- and the 25-oxa[15]-subgroups of cytochalasins, while cyclochalasin T and W are close to cytochalasins B (Vurro et al. 1997). When grown on solid substrates, Peve showed an increased capacity to synthesize cyclochalasins. In fact, cyclochalasin B was isolated from the culture yields of S-9 and C-177, with similar yields of T212 cyclochalasins A, F, T and 7-O-acetyl cytochalasin B, while cyclochalasins ZZ and Z3 were isolated in lower amounts (Evidente et al. 2003). Three new cytochalasins, named Z4, Z5 and Z6, were isolated from the same organic extract, and chemically identified as different and novel member of the 24-oxa[14]cytochalasan subgroup (Evidente et al. 2003). Their activity was assayed for inhibition of tomato seedlings (Evidente et al. 2003).

p-Hydroxybenzaldehyde was a known phytotoxic metabolite of fungi pathogenic to important agrarian crops (e.g. apple, stone-lee and onion, and grapevine) (Venkatsawai et al. 1991; Guo et al. 1996; Tabacchi et al. 2000). p-Hydroxybenzaldehyde was also isolated as a toxin from Ceratoctis spp., associated with blue stain of pine (Ayer et al. 1986), from a phytopathogenic Monilia sp. (Arinbasarov et al. 1988) and as a metabolite from Pythium aphanioides (Edson) Fitzp., which is the causal agent of Pythium red blight, a serious disease of benggrass (Shimada et al. 1999). p-Hydroxybenzaldehyde and the cytochalasins B, F, ZZ and Z3 and deoxaphomin were tested on C. arvenses and S. arvenses using a leaf-disk bioassay. Deoxaphomin (5) demonstrated the highest levels of toxicity to leaves of S. arvenses. Other cytochalasins showed less activity. on C. arvenses. The presence of the hydroxybenzaldehyde moiety residue were important features (Cimmino et al. 2008).

These results are in agreement with those previously described in the structure-activity-relationship studies carried out by some of us while testing the phytotoxicity of several cytochalasins and their derivatives on different plants (Bottalico et al. 1990; Capasso et al. 1991; Evidente et al. 2003; Vurro et al. 1997; Berestetskii et al. 2008).

The activity of p-hydroxybenzaldehyde was in agreement with the absence of inhibitory activity observed towards bentgrass (Shimada et al. 1999), but in contrast with the toxicity observed by leaf bioassay on 17 apple cultivars (‘Classic Red’, ‘Empire’, Firm Gold’, ‘Gala’, ‘Golden Delicious’, ‘Jonathan’, ‘Ida Red’, ‘Low Rome’, ‘Mcintosh’, ‘Oregon II’, ‘Paula Red’, ‘Red Chief’, ‘Red Delicious’, ‘Silverpur’, ‘Smoothie’, ‘Stayman’, ‘Super Gold’) and eight weed species. Only the cultivars ‘Super Gold’ and ‘Silverpur’ were highly sensitive, while three cultivars showed moderate resistance. Among the weeds species, prickly sida (Sida spinosa L.) and morning glory (Ipomoea) were highly sensitive (Venkatasawai et al. 1991). The different effects may be due to the metabolite concentration range used and to the difference in plant sensibility as previously observed for several fungal phytotoxins (Evidente and Motta 2001; Evidente and Abouzeid 2006).

Characterization of Phoma exigua var. exigua in vitro

The 7-day old colony dimensions of strains C-177 and S-9 were in accordance to the description of Peve. Both strains demonstrated E+ reaction (green following by red staining of the agar media) to a drop of 6N NaOH applied to colony margins, which is an important species feature of P. exigua (Boerema et al. 2004). ITS sequences from our strains of Peve were identical to those of GenBank (Müle and Vurro, pers. comm.) (Cimmino et al. 2008).

A representative culture of Peve, the type species of the section Phyllotrichoides of the genus Phoma, was reported to produce both cytochalasins A and B, and antibiotic E (van der Aa et al. 2000; Boerema et al. 2004). A strain of P. herbarium was found to produce cytochalasins C, D and E (El-kady and Mostafa 1995). Furthermore, the isolation of cytochalasins from cultures of Peve (Vurro et al. 1997), P. multiforma (Zhor and Swaber 1994), and Phoma spp. (Graf et al. 1974; Wyss et al. 1980) demonstrated these metabolites to be typical for several species belonging to the genus Phoma whereas cytochalasins have not been reported with Ascochyta spp. This may be the additionally information that supports re-designation for the strains C-177 and S-9 to Peve, which synthesize the cytochalasins and antibiotic E. Furthermore, a comparison of ITS sequences from our strains of P. exigua var. exigua with those uploaded in GenBank showed their identity (Müle G, Vurro, M, pers. comm.).

Several authors proposed Peve, in particular the strain C-177 (Berestetskii 2005; Beresteskii et al. 2005), as a potential mycoherbicide against Canada thistle. However, this species is capable of producing high amounts of known cytochalasins that possess both phytotoxic and cytotoxic property. This latter activity would restrict usefulness of the fungus as a biocontrol agent because cytochalasin are considering as potential myco toxin. If high level of toxins were really produced in vivo, this could in practice make it hazardous to use the fungus to control Canada thistle (Cimmino et al. 2008).

The strain C-177 and S-9, grown in liquid and solid cultures, produced the p-hydroxybenzaldehyde and cyclochala san B, F, ZZ, and Z3, and deoxaphomin. On the leaves of both C. arvenses and S. arvenses, p-hydroxybenzaldehyde appeared inactive, deoxaphomin and cyclochalasin ZZ showed the highest and reduced toxicity, respectively. ITS sequences indicate that the strain C-177 and C-9 are similar to Phoma exigua var. exigua. This taxonomic designation was further supported by the capability of these strains to synthesize the cytochalasins and antibiotic E, typically produced by Peve.

STAGONOSPORA CIRSI PHYTOTOXINS

Stagonolide, the main phytotoxin produced by Stagonospora cirsii

The pycnidial fungus Stagonospora cirsii is another foliar pathogen of C. arvenses, with the potential as a mycoherbicide (Berestetskii et al. 2005). In a preliminary study, this fungus was capable of producing phytotoxins killing leaves and roots of the weed (Mitina et al. 2005). The main toxin produced in liquid cultures was named stagonolide (8, Fig. 5) and characterized as a new nonenolide (Yuzikin et al. 2007). Stereo-chemistry was assigned according to MM2 and MOPAC calculation. It was found that the structure 8 satisfied experimental spectroscopic data (Gordon and Ford 1976; Dneprovskyi and Temikovka 1991). This configuration was also supported by observations in NOESY spectrum. Stagonolide can be described as (8R, 9R)-8-hydroxy-7-oxo-9-propyl-5-monens-9-oxide (Yuzikin et al. 2007).

When assayed on the host plant, stagonolide caused first
symptoms (necrotic) about 10 h post application. The minimal concentration for causing the symptoms was about 1 × 10^{-4} M. At 5 × 10^{-3} M, necrotic spots reached ca. 4 mm in diameter 48 h after treatment. At 5 × 10^{-3} M, stagonolide did not show selectivity among host and non-host Asteraceae members. Besides C. arvense, hollyhock, sunflower, lettuce, sowthistle and peppermint were also highly sensitive to the toxin. However, two Solanaceae species were insensitive. Stagonolide inhibited growth of seedling roots of C. arvense at concentrations ≤ 1 × 10^{-5} M, decreasing the length by more than 30% when compared to untreated controls (Yuzikhin et al. 2007). However, it was low toxic to Colpoda steinii Maupas (Protozoa) while weakly suppressive against the fungus Candida tropicalis Cast. and bacteria (Yuzikhin et al. 2007).

Stagonolides B-F, five new nonenolides

S. cirsii grown on a solid medium produced five new phytotoxic nonenolides named stagonolides B-F (9-13, Fig. 5). Their structures were determined using spectroscopic methods, including NMR and MS techniques (Evidente et al. 2008d).

The relative configuration of the epoxy group in stagonolide D as well as that of the double bonds of all nonenolides was assigned by comparison NMR data with those reported in literature for suitable 1,2-disubstituted cis- and trans-oxirans (Batterham 1972; Pretsch et al. 2000) and the olefinic systems (Pretsch et al. 2000). The double bonds between C(5)-C(6) in stagonolides B-D and between C(4)-C(5) and C(6)-C(7) in stagonolide E and F are trans, while the double bond between C(2)-C(3) in stagonolide E is cis.
Further new nonenolides, stagonolides G-I, and modiolide A from *S. cirsii*

Further investigation on the organic extract of solid *S. cirsii* cultures allowed the isolation of four additional nonenolides. Three of them appeared to be new, and were therefore named stagonolides G-I (14-16, Fig. 5). Their structures were determined with NMR and MS techniques (Evidente et al. 2008c). The fourth one was designated as modiolide A (17, Fig. 5), previously isolated from a *Paraphaeosphaeria* sp., a fungus associated with the horse mussel *Modiolus auriculatus* Krauss (Tsuda et al. 2003).

The relative configuration of the stereogenic carbons was determined using the method described previously by comparing NMR data with those reported in literature for modiolide A and herbarumin I (Rivero-Cruz et al. 2000; Tsuda et al. 2003), and was assigned to stagonolides G-I. This designation was in agreement with the NOE effects observed. In fact, a significant NOE effect was observed with the stagonolides G and H between H-8 and H-9, and H-7 and H-8, respectively (Evidente et al. 2008c).

Nonenolides G-I and modiolide A at 1 mg/mL had different phytotoxic activities; Stagonolide H was most toxic to leaves of *C. arvense*, while stagonolide I and modiolide A were significantly less toxic, and stagonolide G non-toxic. In *A. ochraceus* bioassay at a concentration of 2 mg/mL, stagonolide G exhibited strong phytotoxicity on chicory seedlings (85% reduction compared to control), while other compounds were inactive at the concentration used. Stagonolide H appeared to have less inhibitory activity to chicory seedlings than stagonolide G, which showed similar activity at 1 μg/mL (Yuzikhin et al. 2007). Leaves of eight plant species showed different sensitivity to stagonolide H; *C. arvense* was most sensitive while tomato was only affected slightly. With the high phytotoxicity and selectivity, stagonolide H may be a potential natural herbicide candidate (Evidente et al. 2008c). Modiolide A exhibited strong phytotoxicity on radish leaves but showed significantly less effect on other plants tested.

**Natural fungal nonenolides**

Macrolides and, in particular, nonenolides, are common natural phytotoxins from *Phoma putaminum* (Evidente et al. 1995, 1997, 1998a) and *P. herbarum* (Rivero-Cruz et al. 2000, 2003). However, the host range of these herbarumins was generally not clear. Other phytotoxins produced by fungi include pinolidoxins and aspinolides A-C isolated from *Ascochyta pinnodes* (Evidente et al. 1993a, 1993b) and *A. ochraceus* (Fuchs and Zeeck 1997). Pinolidoxin and putaminoxin (18 and 19, Fig. 6) are potent inhibitors of phenylalanineammonio lyase (PAL), an enzyme that plays an important role in plant defensive mechanism (Vurro and Ellis 1997).

Except stagonolide H, neither other stagonolides nor putaminoxin display host selectivity. However, the host of the pathogens tend to show higher sensitivity to the respective toxins than non-hosts (Evidente et al. 1995, 1998b; Yuzikhin et al. 2007; Evidente et al. 2008c, 2008d). Herbarumin I and stagonolide demonstrated high potency as root growth inhibitors at low concentrations (1 × 10⁻⁸ M) on *Amaranthus hypochondriacus* L. (Rivero-Cruz et al. 2000) and *C. arvense*, respectively. Because the main task in control of *C. arvense* is to prevent root and underground shoot growth, both toxins may be considered natural herbicide candidates for this weed.

The main phytotoxic metabolite produced by *S. cirsii* in liquid cultures was stagonolide, a new nonenolide. It appears to be a non specific phytotoxin with a low antimicrobial activity. Eight new stagonolides, named stagonolides B-I and modiolide A, were isolated from solid cultures of *S. cirsii* and their phytotoxic and zootoxic activity was evaluated. Stagonolide H appeared to be the most potent and selective toxin.

**STRUCTURE-ACTIVITY RELATIONSHIPS AMONG SELECTED PHYTOXINS**

Stagonolide from *S. cirsii* (Yuzikhin et al. 2007) is structurally related to several known phytotoxins including herbarumins from *P. herbarum* West. (Rivero-Cruz et al. 2000), pinolidoxins from *A. pinnodes* (Evidente et al. 1993a, 1993b), and putaminoxins from *P. putaminum* (Evidente et al. 1995, 1997, 1998a). Although the structure-activity relationships were studied with several of the phytotoxins (Evidente et al. 1998b), stagonolide was not compared with other nonenolides against *C. arvense* and *S. arvensis*.

The Peve strains C-177 and S-9 produce, in both liquid and solid cultures, cytochalasins B, F, Z2 and Z3 and deoxaphomin. Numerous cytochalasins are known for plant...


pathogenic fungi (Capasso et al. 1991; Natori and Yahara 1991; Vurro et al. 1997; Evidente et al. 2003) including weed pathogens (Evidente et al. 2002). The structure-activity relationship was studied to assess 15 natural analogues and derivatives belonging to two groups of organic compounds, nonenolides and cytochalasins, against both C. arvense and S. arvensis. The nonenolides (stagonolide, putaminoxin, pinolidoxin) and cytochalasins [deoxaphomin, cytochalasins A (22, Fig. 6), B, F, T (26, Fig. 6), Z2 and Z3] were tested together with 7,8-O,O'-diacetyl- and 7,8-O,O'-isopropylidene-pinolidoxin (20 and 21, Fig. 6) and 7-O-acetyl-, 7,20-O,O'-diacetyl- and 21,22-dihydro-cytochalasin B (23-25, Fig. 6) derivatives (Berestetskiy et al. 2008).

Phytotoxic activity on leaves of Canada thistle and perennial sowthistle

Among the 15 compounds tested with the leaf-disk bioassay, stagonolide demonstrated the highest level of toxicity to C. arvense. Other nonenolides, i.e. putaminoxin or 7,8-O,O'-isopropylidene-pinolidoxin were less toxic (Fig. 7). Among cytochalasins, only cytochalasin A was highly toxic to the weed. Deoxaphomin was the most toxic compound to S. arvensis, while stagonolide, cytochalasin A and cytochalasin B also showed a high level of phytotoxicity. Other cytochalasins were moderately toxic but the pinolidoxin and diacetylpinolidoxin were non-toxic to either weed (Fig. 7) (Berestetskii et al. 2008).

The results demonstrated different responses of C. arvense and S. arvensis to these compounds. The natural nonenolides were generally more toxic than cytochalasins on C. arvense. Furthermore, a marked modifications in respect to stagonolide and putaminoxins in both functional groups and conformational freedom of the nonenolide ring showed a strong decrease or total loss of toxicity. These results are in agreement with data from a study of structure-activity relationship performed previously on putaminoxin, pinolidoxin (Evidente et al. 1998b), and herbarumins (Rivero-Cruz et al. 2000).

In our trials, cytochalasins were more toxic than nonenolides on S. arvensis, particularly deoxaphomin, cytochalasins A and B which possess a [13]carbocyclic or a [14]lactonic macrocyclic ring joined with an unaltered perhydroysoindolyl residue. In the latter moiety, the presence of the secondary hydroxyl group on C-7, which is missing in the compound 4, 6 and 26 or acetylated in 23 and 24, appeared to be an important feature to impart toxicity. Furthermore, the significant decrease of toxicity observed with the 21,22-dihydro derivatives of cytochalasin B and cytochalasins Z3 also indicated the importance of the functionality on C-20 and the conformational freedom of the macrocyclic ring. These results are consistent with those re-
caused by cytochalasin A 2 hours post treatment. The ability to control, significant changes in the light absorption were observed within this region are characterized by peak absorption at 632.8 nm. The development of lesions on leaf disks treated with stagonolide was similar to untreated controls, where the conductivity of water extracts obtained from leaf tissues was reversed and diminished to minimal values after 150 seconds. Leaf disks treated with cytochalasin B did not express substantial change in resistance while those treated with stagonolide showed a linear decline similar to the boiled leaf discs with even a faster speed. It appeared that stagonolide had little effect on the permeability of cell membranes, whereas cytochalasin B caused electrolyte leakage from leaf cells (Berestetskiy et al. 2008).

Effect of selected toxins on conductometric properties

Electrical resistance in C. arvense leaves was measured after treatment of phytotoxins in vivo using the protocol introduced by Lisker (1991). Under electrical tension, cell ions tend to accumulated along electrodes, thus interfering with the current. For boiled leaf tissues, initial increase in the resistance was reversed and diminished to minimal values after 150 seconds. Leaf disks treated with cytochalasin B did not express substantial change in resistance while those treated with stagonolide showed a linear decline similar to the boiled leaf discs with even a faster speed. It appeared that stagonolide had little effect on the permeability of cell membranes, whereas cytochalasin B caused electrolyte leakage from leaf cells (Berestetskiy et al. 2008).

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This hypothesis was supported by another experiment where the conductivity of water extracts obtained from leaf disks treated with stagonolide was similar to untreated controls but lower than that of the extracts from disks treated with cytochalasin B. These results were not surprising due to the well-known effects of cytochalasins such as cytochalasin B. This cytochalasin inhibited cytoplasmatic streaming, organelle movement, cell division, pollen germination, cell wall metabolisms and auxin transport (Natori and Yahara 1991). In particular, it regulates plasma membrane Ca2+ channels activity via effect on actin microfilaments (Wang et al. 2004). Interestingly, stagonolide did not significantly increase the membrane permeability in the leaf tissue of C. arvense (Berestetskiy et al. 2008).

Among the 15 compounds tested, stagonolide was most phytotoxic to leaves of C. arvense whereas deoxaphomin leaves in near infrared region. At > 700 nm, leaves of healthy plants have minimal or no light absorption, while under stress conditions leaves can start to absorb radiation in the near-infrared region possibly due to appearance of chlorophyll degradation products (Kräutler 2002; Merzlyak et al. 2002). Therefore, increased absorption in the near infrared region can be related to the level of leaf damage caused by phytotoxins.

The results of photometric assays performed with different equipment provided similar trends. In fact, the nonenolides, in particular stagonolide and putaminoxin, appear to affect the light absorption at multiple wavelengths. Stagonolide seems to inhibit photosynthesis of C. arvense leaves, possibly chlorophyll synthesis as well. Most known herbicides affecting photosynthesis inhibit electron transport in the photosystem II by damaging cell membranes (Fedtke and Duke 2004; Wakabayashi and Böger 2004; Duke et al. 2005). The structural features discussed above for each group of compounds may be important to impar- ting this activity (Berestetskiy et al. 2008).

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Among the 15 compounds tested, stagonolide was most phytotoxic to leaves of C. arvense whereas deoxaphomin
demonstrated the highest herbicidal effect on *S. arvensis* leaves. The phytotoxic nonanolides were stronger inhibitors of photosynthesis on *C. arvense* leaves than cytochalsins A and B. Although photometric data indicated that neither stagonolide nor cytochalasin B inhibited electron transport, stagonolide had much less effect on cell membrane permeability than did cytochalasin B, implying different modes of action.

**PHYLOSTICTA CIRSI TOXINS**

New phytotoxic oxazatricycloalkenones

Recently, the fungus *Phyllosticta cirsii* has been evaluated as a possible biocontrol agent of Canada thistle (Beresteskiy et al. 2005). Species belonging to the genus *Phyllosticta* are known to produce bioactive metabolites, including non-host-specific phytotoxins, e.g. phyllosinol, brefeldin and PM-toxin (Sakamura et al. 1969, Comstock et al. 1973, and Entwistle et al. 1974). *Phyllosticta* spp. have been shown to produce phytotoxic metabolites in liquid cultures. Four new oxazatricycloalkenones, namely phyllostictines A-D (27 and 32-34, Fig. 8), were isolated and characterized with extensive uses of NMR and MS techniques. The structure assigned to phyllosticine A was further supported by converting the toxin into the 15-O-acetyl- and 11,15-O,O'-diacetyl derivatives (28 and 29, Fig. 8) via the usual reaction with pyridine and acetic anhydride. The spectroscopic data of both derivatives were consistent with the structure of phyllosticine A assigned to the toxin. The phyllostictines B-D appeared to be closely related to phyllosticine A and to each other, but differed from phyllosticine A in the size of macrocyclic ring. Phyllosticine C also differed for the substituent at C-5. Phyllosticine D showed the presence of a δ-lactam instead of the β-lactam present in phyllostictines A-C and differed for the functionality of both the lactam and macrocyclic rings. The absolute configuration to phyllosticine A was assigned by combination of the application of Mosher’s method (Dale et al. 1969; Dale and Mosher 1973; Ohtani et al. 1991) through the comparison of the ‘H-NMR spectra of its 15-O-SMTTPA and 15-O-RMTPA esters (30 and 31, Fig. 8) and NOESY spectroscopy. The absolute stereochemistry of phyllostictines B-D was assigned after comparing their NMR data with those of phyllosticine A (Evidente et al. 2008a).

**Biological activity of phyllostictines**

Tested at about $6 \times 10^{-3}$ M on Canada thistle using the leaf-disk assay, phyllosticine A was particularly active, causing...
the fast appearance of large necrotic spots (about 6-7 mm in diameter). Phyllostictines B and D were slightly less toxic, whereas phyllostictine C was almost non-toxic. These results may indicate that the size, conformational freedom and functionalization of the macrocyclic ring are important features for the activity. The N-ethyl β-lactam ring appears to be less important while the importance of other rings remains to be ascertained (Evidente et al. 2008a).

The antimicrobial and the zootoxic activities were assayed for phyllostictines A and B, at up to 100 μg/disk, were completely inactive against G. candidum and Esche- richia coli Migula (Gram-). Phyllostictine A at 5 μg/disk inhibited Lactobacillus sp. (Gram+). When tested on brine shrimp (Artemia salina L.) larvae, only phyllostictine A caused total larval mortality at 10⁷ M, whereas phyllostictine B had only negligible effect. The integrity of oxazatri-cycloalkenone structure in phyllostictine A appears important to antimicrobial and zootoxic activities (Evidente et al. 2008a).

Phyllostictines A-D are the first fungal metabolites described for the oxatricycloalkanenones group as natural compounds with interesting biological activities. The phyllostictine A also showed both herbicidal and antibacterial (Gram+) properties. Compounds containing macrocyclic rings as well as furan derivatives are quite common and are often biologically active (Turner and Aldridge 1983; Trin-gali 2001). Phyllostictine A was further studied to: a) develop a rapid analytical method to estimate phyllostictine A content in culture preparations; b) to assess the phytotoxic effect for potential weed control (Zonno et al. 2008).

On C. arvense leaf disks, the strongest toxicity was observed with filtrates from 12- to 16-day-old P. cirsii cultures grown in the Fries shake-flask medium while no toxicity was observed for filtrates from M-1-D medium cultures (Table 2). In contrast, the fungus produced the highest amount of phyllosticine A in the static M-1-D medium after 4 weeks (Table 3). The phytotoxicity of culture filtrate seemed to correlate to production of specific metabolites by the fungus under certain cultural conditions (Zonno et al. 2008).

### Toxin quantification

A rapid, sensitive HPLC method for qualitative and quantitative analysis of phyllostictine A was developed using high-density reversed-phase Nucleosil 100-5 C₁₈ HD col- tective analysis of phyllostictine A was developed using A rapid, sensitive HPLC method for qualitative and quanti-

### Table 2 Toxicity of culture filtrates produced by Phylllosticta cirsii in shake-flask media on Canada thistle leaf discs.⁴,⁵

<table>
<thead>
<tr>
<th>Culturing duration (days)</th>
<th>Medium</th>
<th>M-1-D</th>
<th>Fries</th>
<th>Malt</th>
<th>Kent-Strobel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

⁴ Scale of toxicity: - not toxic; +: necrotic lesion < 1 mm; ++: 2-3 mm; +++: 3–5 mm; ++++: > 5 mm

⁵ Zonno et al. 2008

### Table 3 Production of phyllostictine A (mg L⁻¹) by Phylllosticta cirsii under static cultural conditions.⁶

<table>
<thead>
<tr>
<th>Culturing duration (weeks)</th>
<th>M-1-D</th>
<th>Fries</th>
<th>Malt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.12</td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>2</td>
<td>3.59</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>13.72</td>
<td>0.08</td>
<td>0.35</td>
</tr>
<tr>
<td>4</td>
<td>28.83</td>
<td>0.96</td>
<td>0.59</td>
</tr>
<tr>
<td>5</td>
<td>23.69</td>
<td>0.52</td>
<td>0.38</td>
</tr>
<tr>
<td>6</td>
<td>21.93</td>
<td>0.22</td>
<td>0.36</td>
</tr>
<tr>
<td>7</td>
<td>18.18</td>
<td>0.11</td>
<td>0.30</td>
</tr>
<tr>
<td>8</td>
<td>16.91</td>
<td>0.06</td>
<td>0.26</td>
</tr>
<tr>
<td>9</td>
<td>15.23</td>
<td>0.02</td>
<td>0.22</td>
</tr>
</tbody>
</table>

⁶ Zonno et al. 2008

### Protoplasts assay

In comparison to the use of whole plants, bioassays performed on isolated protoplasts offer the advantage of using lower amounts of toxins and avoid cell wall interference thus allowing observation of host response at cellular levels. In a bioassay in which protoplasts of Canada thistle was used, phyllostictine A at 10⁻⁷ M was highly effective even 1 hour after application, killing most of the protoplasts completely. At 5 × 10⁻⁸ M, the toxicity was time dependant, with around 50% kill 1 h after treatment and 100% after 6 h. At 10⁻⁷ M, however the toxin was almost completely ineffective. For comparison, fusaric acid showed a time-dependant toxicity at 10⁻⁶ M, causing 60% mortality 1 h after application, and higher mortality after 3 and 6 h. Similarly, glyphosate at a label rate was highly toxic but time dependant, causing total mortality of thistle protoplasts 3 h after treatment but at reduced rates it caused 60% mortality regardless of the time of exposure (Zonno et al. 2008).

Flow cytometry, which measures protoplast size, chlorophyll auto-fluorescence (FL2) and fluorescence due to uptake and subsequent esterase cleavage of FDA (FL1), may be used to analyze the auto-fluorescence of unstained protoplasts. After staining with FDA, another population was obtained, and this would be active protoplasts exhibiting both high chlorophyll auto-fluorescence level and fluorescence from FDA. Only protoplasts were analyzed while chloroplasts and debris were excluded. Viable protoplasts would have brighter fluorescence during flow cytometric analysis, whereas dead protoplasts were non-fluorescent. An example of citogram obtained using the flow cytometer to analyze tobacco protoplasts treated with phyllostictine A is given in Fig. 9. At 10⁻⁷ M phyllostictine A acted quickly, killing almost all the protoplasts within an hour. At 5 × 10⁻⁷ M the toxin was still fast acting, causing around 80% mortality 1 h after the treatment, although the effect was time dependant as more than 3 h, whereas at 10⁻⁸ M no toxicity was observed. Fusaric acid was highly toxic at 10⁻⁷ and 5 × 10⁻⁸ M, causing almost 100% mortality after 6 h, whereas at 10⁻⁷ M, about 60 to 70% of protoplasts remained alive after 6 h. Glyphosate at a label rate was highly toxic but much less toxic at reduced rates (Zonno et al. 2008). The tobacco protoplasts seemed more sensitive and convenient to use than those of Canada thistle, but the real question is which is more relevant to weed control efficacy.
Fungal phytotoxins for weed biocontrol. Evidente et al.

Besides phyllostictines A-D, two additional metabolites, phyllostoxin and phyllostin (35 and 36, Fig. 10) were also found in cultural filtrates of P. cirsii. They are new penta-substituted bicyclooctatrienyl ester of acetic acid and penta-substituted hexahydrobenzodioxine carboxylic acid methyl ester, respectively. Their structures were determined using NMR and MS spectroscopy (Evidente et al. 2008b). The relative stereochemistry of chiral centers of phyllostin were assigned based on a X-ray diffrattometric analysis. Phyllostoxin appeared to be the diastereomer of 5-lactyl shikimate lactone previously isolated from a Penicillium sp. (Isogai et al. 1985) for which the absolute stereostructure was established by two independent enantioselective syntheses (Muralidharan et al. 1990; Alberg et al. 1992).

X-ray crystallographic analysis of phyllostin

Phyllostin, obtained as a crystalline solid, was re-crystallized from toluene and resulted in colorless crystals, which were suitable for an X-ray analysis. An ORTEP (Farrugia 1997) view of phyllostin with atomic labelling is shown in Fig. 11A.

Bond lengths and angles in phyllostin are in the normal range (Rendle and Trotter 1975; Schweizer and Dunitz 1982; Ayer et al. 1993). The molecule contains two 6-membered rings. In the 2-oxo-1,4-dioxane ring (ring A) which assumes a distorted half chair conformation with C3/C2/O1/C8a atoms nearly coplanar below and above this mean plane respectively. Ring A is trans fused with the tetrasubstituted cyclohexene ring (ring B) through the atoms C4a and C8a. This confirmed the stereochemistry of junction between the two rings that was previously assigned on the basis of NMR analysis (Evidente et al. 2008b). Bond lengths and angles around C6 and C7 confirmed the presence of the cyclohexene double bond. The conformation of ring B is half chair, in fact, C5/C6/C7/C8 atoms are strictly coplanar, C8a and C4a deviate from this plane. Four chiral centres are present in the molecule at C3/C4a/C8/C8a, whose relative configuration is R/S/R/S.

The methyl-ester group attached to cyclohexene ring is all planar and in plane with the double bond C6=C7. This feature, together with the distorted half chair conformation of ring A, give a flat shape to the whole molecule, whose mean plane disposes quite parallel to crystallographic ac plane (Fig. 11B).

The crystal packing is characterized by strong intermolecular head to tail O-H···O hydrogen bonds involving the hydroxyl H atom and carbonyl oxygen atom of methyl-ester group (see Fig. 11C). Chains are packed together to form parallel layers of molecules stacking in the [0 1 0] direction (see Fig. 11C). The packing is also stabilized, between layers, by weak C-H…O interactions involving O5 carbonyl oxygen atom as acceptor (Tuzi et al. 2010).

Biological assays of phyllostoxin and phyllostin

When tested using C. arvense leaf-disc assay at 10⁻³ M (20 μL/droplet), phyllostoxin was highly phytotoxic, causing severe necrosis rapidly, similar to those caused by phyllostictine A. At the same concentration, however, phyllostin was completely ineffective. Neither phyllostoxin nor phyllostin (up to 10⁻³ M) suppressed G. candidum (fungus), E. candidum inoculated on the leaf discs (Fig. 9).
coli and Lactobacillus sp. (bacteria), or brine shrimps larvae.

Phyllostoxin appeared to be a new bicyclooctatrienyl derivative (Grieco et al. 1980; Kobayashi et al. 1992a, 1992b) with a strong phytotoxic activity but little impact on microbes and animals. Therefore, this toxin may represent a potential new natural herbicide opportunity. Further studies are in progress in order to produce the active compound in larger amounts to allow a more complete biological characterization. Phyllostin, with no toxicity in any of the assays performed, is likely one of the many possible stereoisomers with the same molecular structure. Only one of the stereoisomers is a fungal metabolite (Isogai et al. 1985) while others are all synthetic compounds (Chen and Low 1966; Alberg et al. 1992; Evidente et al. 2008b).

Four new oxazatricyclic alkenones, named phyllostictines A-D, were isolated from Phyllosticta cirsii cultures together with a new pentasubstituted bycyclo-octatrienyl acetic acid ester and pentasubstituted hexahydrobenzodioxine carboxylic acid methyl ester named phyllostoxin and phyllostin, respectively. On leaf disks of the host plant, phyllostictine A proved to be highly toxic while the toxicity was lower with phyllostictines B or D, and non-detectable for phyllostictine C. Phyllostictine A showed antibiotic activity against Gram+ bacteria and a noticeable zootoxic at a high concentration. Phyllostoxin turned out to be highly phytotoxic in the same bioassay, while phyllostin was ineffective.

**TOXINS FROM PHOMOPSIS CIRSI**

Phomopsis cirsii is another fungal pathogen proposed as a bioherbicide for the control of these two perennial weeds. It belongs to a well-known genus with many toxin producers including P. foeniculi Du Manoir & Vegh (Evidente et al. 1994), P. viticola (Randazzo et al. 1980), P. amaranthicola (Wyss et al. 2004). Studies on culture filtrates of P. cirsii showed that the fungus produced, in vitro, toxic metabolites which were low-molecular-weight acidic compounds. The main toxin was identified as β-nitropropionic acid using spectroscopic methods. The same toxin was previously isolated from Septoria cirsii Niessl, another foliar pathogen of C. arvense (Herschenhorn et al. 1993). However, this toxin showed a broad activity spectrum and can also be produced by Aspergillus spp. (Wilson 1966) and Melanconis thelebola (Fr.) Sacc. (syn. M. marginalis). The latter is a fungus proposed for biocontrol of A. albus spp., a noxious weed for forestal plant (Evidente et al. 1992). Some close analogues of β-nitropropionic acids, including 3-methylthiopropanoic
and 3-methylthiopropenoic acids, have also been identified as the main phytotoxins in cultures of Xanthomanas campestris pv. vitianis (later reclassified as X. campestris pv. orthoceras), the causal agent of lettuce crown rot (Scala et al. 1996). β-Nitropropionic acid, a simple compound, demonstrated a potent herbicide activity in field trials for control of some troublesome weeds in forest (Dowworth and Glover 1991).

CONCLUSIONS

Phytopathogenic fungi belonging to the genera Ascochyta, Stagonospora, Phylllosticta and Phomopsis have been proposed for biocontrol of C. arvensis and S. arvensis. Investigations on nine A. sonchi strains of varying origins showed that two of them were atypical isolates based on the phytotoxins produced. From S. cirsii cultures, ten new nonenolides were identified, and nine of which are naturally occurring compounds. Among them only stagonolide and stagonolide H appeared to have a significant phytotoxic activity and may be considered for applications to manage these two weeds.

A structure-activity relationship study testing 15 compounds, cytochalasins, nonenolides and their derivatives showed that stagonolide was most phytotoxic to C. arvensis, while deoxaphomin appeared most toxic to S. arvensis. The conformational freedom is an important factor to impart the toxicity for the nonenolides, while the presence of two hydroxy groups at C-7 and C-20 as well as the conformational freedom of the macrocyclic ring are important for cytochalasins. Stagonolide was a strong inhibitor of photosynthesis on C. arvensis leaves, while cytochalasin B showed a strong effect on cell membrane permeability. P. cirsii produces four phytotoxins with an original carbon skeleton, being the first natural oxazatriciclochelones, which were named as phyllostictines A-D. Phylllostictine A was most phytotoxic against C. arvensis, but its effect would depend on the size and functionality of the macrocyclic ring. Due to a lack of zootoxic and antimicrobial activity, phylllostictine A appears to be a candidate for natural herbicide.

The isolation, chemical and biological characterization of known or new phytotoxins from different fungal species proposed for biocontrol of C. arvensis and S. arvensis represent an important opportunity to develop low-risk natural herbicides for management of these two problematic weeds. These toxins may be used alone or in combination with the fungal agents, in integrated management strategies.

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