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Fungal Phytotoxins for Control of *Cirsium arvense* and *Sonchus arvensis*

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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. *Phyllosticta 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, oxazatricycloalkenones, pentasubstituted bicyclooctatrienyl ester of acetic acid, pentasubstituted hexahydrobenzodioxine carboxylic acid methyl ester, and β-nitropropionic 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

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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

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Invited Review

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 1982; Graniti *et al*. 1989; Ballio and Graniti 1991; Evidente 1997; Evidente and Motta 2001).

Many fungal phytotoxins are not specific to one weed species and they may be considered as potential natural herbicides in native forms or as derivatives and analogues. The herbicidal activity may be increased by either direct application of derivatives or analogues, or in combination with the pathogen for optimal herbicidal efficacy or selectivity (Strobel 1982; Strobel *et al.* 1987; Delfosse 1990; Strobel *et al.* 1991; 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 *Pyrenephora* (Evidente and Motta 2001; Evidente 2006; Evidente and Abouzeid 2006; Rimando and Duke 2006).

Perennial weeds are common problems in crop production, especially in systems with reduced herbicide usage. Such weed species include *Cirsium arvense* (L.) Scop. and *Sonchus arvensis* (L.) (both in *Asteraceae*) commonly called Canada thistle and perennial sowthistle, respectively. Herbicides recommended for these perennials are limited and tend to have low selectivity. Microbial phytotoxins or their synthetic analogues may be used for development of new agrochemicals 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 cultivated 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 (Mitich 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 control of these perennials are limited to clopyralid, dicamba, chlorosulfuron, bentazon, and phenoxy-acids, and these herbicides are low in selectivity (Lemna and Messersmith 1990; Kloppenburg and Hall 1990; Grekul *et al*. 2005). Although there are commercial bioherbicide available at this time, the search for efficacious biocontrol microorganisms and natural herbicides has been active.

Microbial phytotoxins or their synthetic analogues may be used for development of new agrochemicals against weeds (Evidente 2006; Evidente and Abouzeid 2006) and many plant pathogens, especially necrotrophic or hemibiotrophic fungi capable of producing phytotoxins (Hoppe 1998). For instance, potent phytotoxins were isolated from culture filtrates or mycelia of *Alternaria*, *Ascochyta*, *Drechslera*, *Ophiobolus*, *Phoma* and many others fungal pathogen cultures (Kenfield *et al.* 1989; Evidente and Motta 2001).

Numerous surveys have been carried out to find pathogens on Canada thistle (Berestetsky 1997; Leth and Andreasen 1999; Bailey *et al.* 2000), while mycobiota on perennial sowthistle is less studied (Berestetski and Smolyaninova 1998). Several fungal pathogens, including *Stagonospora cirsii* and *Ascochyta sonchi* (syn. *Phoma exigua* Desm. var. *exigua*) were found to be common on both weed species and produce phytotoxic metabolites (Berestetskiy *et al.* 2005), whereas *Phyllosticta cirsii* and *Phomopsis cirsii* have been isolated only from diseased Canada thistle plants.

PHYTOTOXINS PRODUCED BY *ASCOCHYTA SONCHI*

Isolation and structural characterization

Ascochyta sonchi is a natural pathogen isolated from necrotic leaves of sowthistle. Pathogens belonging to the genus *Ascochyta* are responsible for diseases on many plant species, typically with necrotic lesions on leaves and stems (Mel'nik 1971). Some *Ascochyta* spp. have also been proposed as mycoherbicide agents for the biological control of noxious weeds, including *A. caulina* (P. Karst) v.d. Aa and v. Kest against *Chenopodium album* L. (Netland *et al.* 2001) and *A. cypericola* 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.* 1998c, 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 ^{1}H and ^{13}C NMR and HREI MS) as (*Z*)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid and named ascosonchine (**1**, **Fig. 1**) (Evidente *et al.* 2004).

The stereochemistry of the olefinic double bond present in ascosonchine was deduced by IR and NMR experiments. In fact, the IR absorption bands observed for the hydroxy, double bond and carboxyl 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 (Cassidei *et al.* 1980; Lee *et al.* 1998). This *Z*-configuration was further supported by the ^{1}H, ^{13}C coupling constants recorded in the undecoupled ^{13}C NMR spectrum. In particular, the value of the vicinal ^{1}H-C=C-^{13}COOH [$^{3}J_{C1H3}$=3.7 Hz] coupling, is typical for a *cis* arrangement of the coupled nuclei in fragments ^{1}C-C=C-^{13}C with similar sums of electronegative substituents (Stobbe and Kenyon 1971; Vögeli and Von Philipsborn 1975; Sciacovelli *et al.* 1976). This result was in agreement with the stable green-blue colour yielded when ascosonchine dissolved in DMSO reacted with $FeCl_3$, as already observed only for *Z*-enol tautomer of

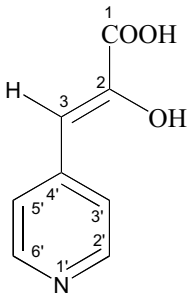

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Fig. 1 Chemical structure of ascosonchine (**1**).

Table 1 Effect of ascosonchine observed in leaf-disc bio assay.[a,b]

Common name	Scientific name	Family	Effect on leaves
Alligatorweed	*Alternanthera philoxeroides*	Amaranthaceae	++
Artichoke	*Cynara scolymus*	Compositae	-
Bean	*Phaseolus vulgaris*	Leguminosae	-
Bindweed	*Convolvulus arvensis*	Convolvulaceae	-
Chickpea	*Cicer arietinum*	Leguminosae	+
Eggplant	*Solanum melongena*	Solanaceae	-
Four-o'clock	*Mirabilis jalapa*	Nyctaginaceae	+
Foxtail millet	*Setaria italica*	Poaceae	++
Lamb's lettuce	*Valerianella locusta*	Valerianaceae	+++
Melon	*Cucumis melo*	Cucurbitaceae	-
Pepper	*Capsicum annum*	Solanaceae	-
Potato	*Solanum tuberosum*	Solanaceae	-
Common sage	*Salvia officinalis*	Labiatae	++++
Sowthistle	*Sonchus arvensis*	Asteraceae	+++
Spinac	*Spinacia oleracea*	Chenopodiaceae	+
Sun spurge	*Euphorbia helioscopia*	Euphorbiaceae	++++
Tomato	*Lycopersicon esculentum*	Solanaceae	-
Wheat	*Triticum durum*	Poaceae	+++
Zucchino	*Cucurbita pepo*	Cucurbitaceae	-

[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

[b] Evidente *et al.* 2004

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 1965; Casey and Dobb 1992) as an intermediate in the shikimic acid pathway (Ganem 1978; Haslam 1993).

Tested with a leaf-disc assay on the host plant, ascosonchine produced necrotic circular lesions 2 days after treatment and the speed of symptom development was similar to that caused by the pathogen. The diameter of the necrotic area could be up to 1 cm when 15 and 3 µg/droplet (around 6 and 1.2 10^{-3} M, respectively) were applied to the leaf surface. The lesions were still quite evident at a concentration five times lower (0.6 µg/droplet).

Assayed on several weedy and cultivated plants, including both monocots and dicots, at 15 µg/droplet, ascosonchine showed certain selectivity (**Table 1**). It was completely ineffective on all the solanaceous species assayed, slightly to almost non-toxic on cucurbitaceous or leguminous plants, but caused severe necrosis on several other species, including *Euphorbia helioscopia* L., *Salvia officinalis* L., *Valerianella locusta* L., and *Triticum durum* Desf. This semi-selectivity may have practical applications as a herbicidal compound because the toxin is still active when used at very low concentrations (Evidente *et al.* 2004).

In an antibiosis assay, ascosonchine at rates up to 50 µg/disk was completely inactive against the fungal pathogen *Geotrichum candidum* Link. The same result was observed when the toxin was tested on *Pseudomonas syringae* van Hall (Gram-) and *Lactobacillus plantarum* Orla-Jensen (Gram+). No effect was observed on brine shrimp (*Artemia salina* L.), when tested at concentrations up to 10^{-4} M (Evidente *et al.* 2004).

Method for ascosonchine quantification in complex samples

We attempted to correlate the *in vitro* production of ascosonchine with its ability to cause disease to better understand primary mode of biocontrol. An HPLC method was developed for rapid analysis of ascosonchine in culture filtrates of nine *A. sonchi* strains, as well as for optimization of ascosonchine production.

Preliminary tests using various elution conditions with an ascosonchine standard on reverse phase C18 and C8 stationary phases produced unresolved and asymmetric peaks. This was due to a strong adsorption on the stationary phase. On the basis of earlier experience in developing a HPLC method for the analysis of fusaric and 9,10-dehydrofusaric acids (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. arvense* 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 ($P<0.05$) among *C. arvense* strains (**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. arvense*. 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. arvense* and S. *avernsis* 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

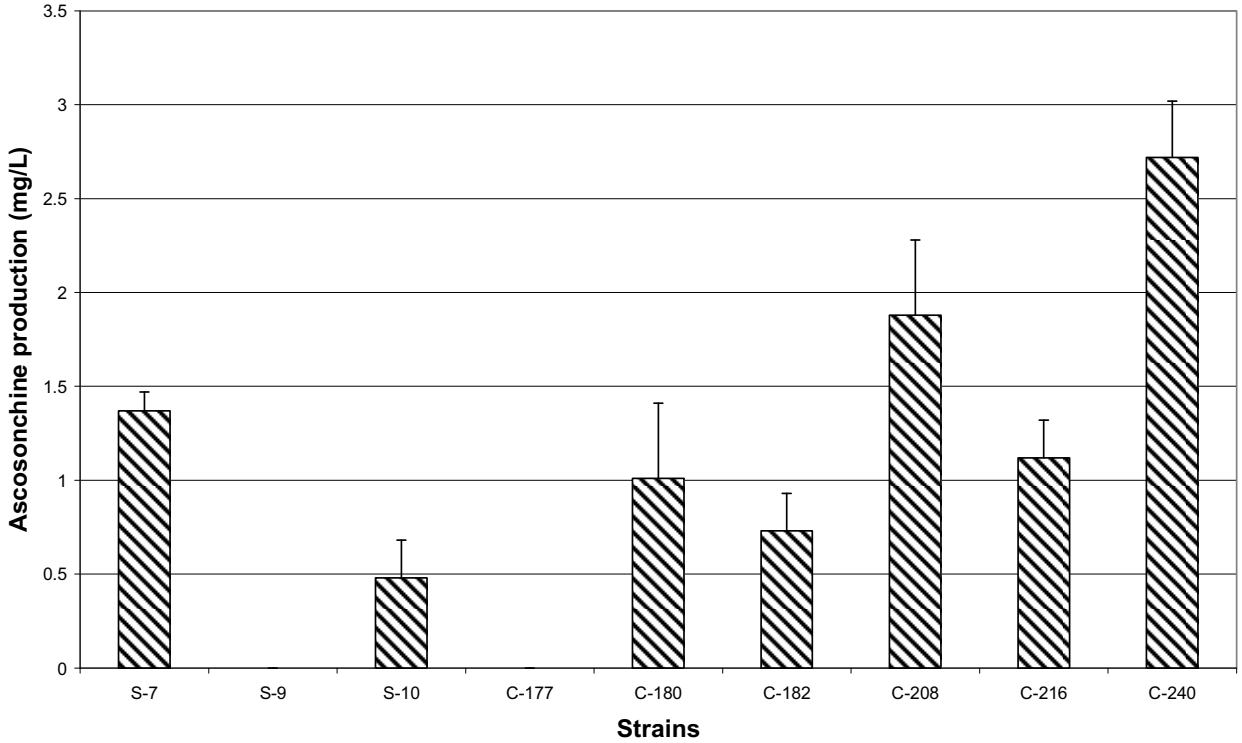


Fig. 2 The concentration of ascosonchine in culture filtrates of nine *Ascochyta sonchi* strains with different geopgraphical origins. The toxin amount was determined by HPLC on high density C_{18} stationary phase column eluted with an isocratic mixture of methanol and water (1: 1, v/v, pH 6.2).

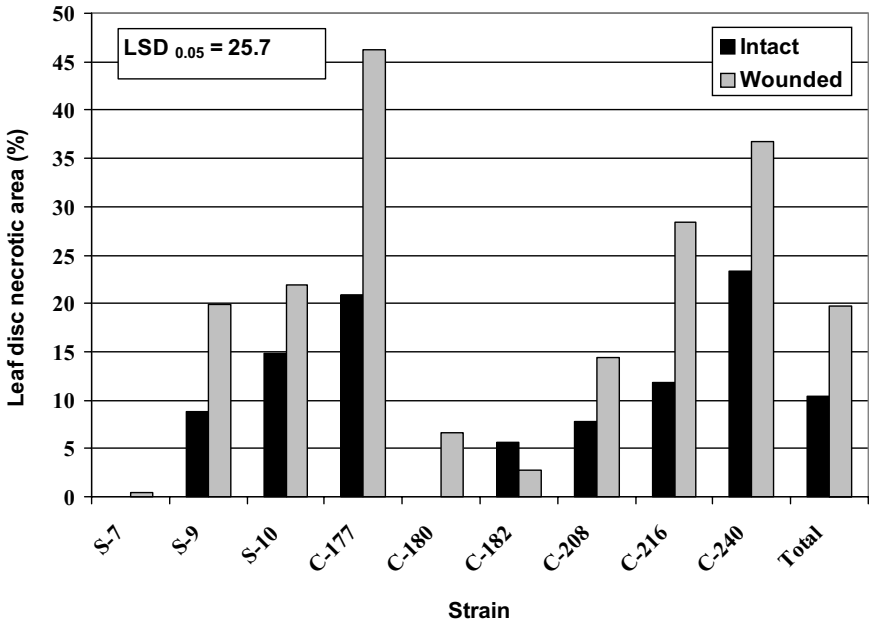


Fig. 3 Virulence of *Ascochyta sonchi* strains to intact and wounded leaf disks of *Cirsium arvense*. Conidial suspension was applied on 10 mm diameter leaf discs cut from expanded leaves of *C. arvense* (10 µL/disc, 12 disks/treatment). Before inoculation, half of the discs were wounded in the centre with a sharp needle. Leaf disks were incubated in plastic containers at 25°C under continuos light. Symptoms and disease severity were assessed 7 days after inoculation.

and C-182 (low toxin producers), were 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 posseses 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 (^{1}H 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). Several *Phoma* species were also proposed and/or patented as mycoherbicide agents for biological control of noxious weeds. This is the case for *P. herbarum* Westendorp against *T. officinalis* (Neuman and Boland 2002), *P. destructiva* Plowrigth, *P. nebulosa* (Pers.: Fr.) Berkeley and *P. hedericola* 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* Speg., *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-*

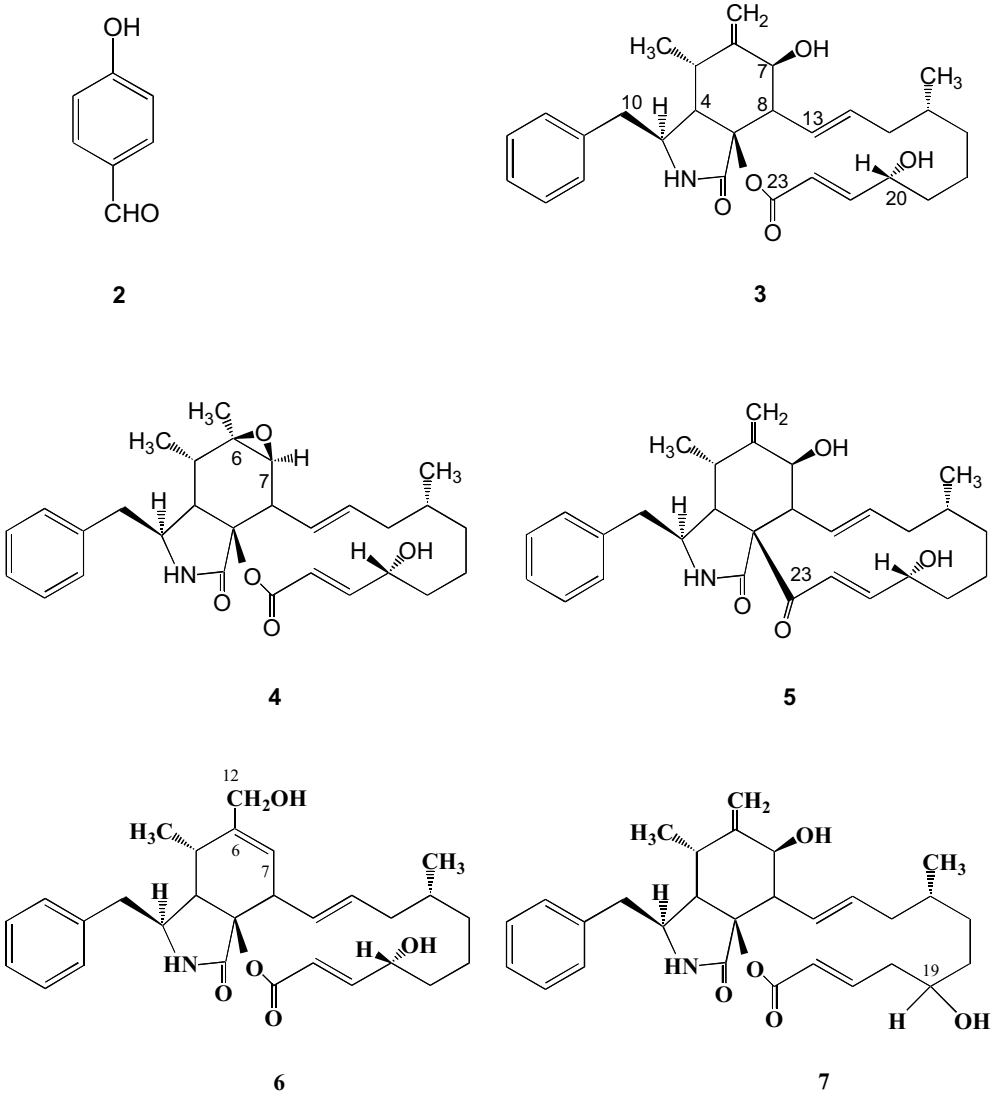


Fig. 4 Chemical structure of *p*-hydroxybenzaldehyde (**2**) and cytochalasins B, F and deoxaphomin (**3**, **4** and **5**) isolated from liquid and solid culture of *P. exigua* var. *exigua* strain C-177, and that of cytochalasins Z2 and Z3 (**6** and **7**) isolated from solid culture of *P. exigua* var. *exigua* strain S-9.

niperda (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 Z2 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 Z2 and Z3 were also isolated from *Phoma exigua* var. *heteromorpha* (Pevh), previously reported as *Ascochyta heteromorpha*, grown in same conditions (Evidente *et al.* 2003). Pevh 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*-acetylcytochalasin B, cytochalasins F, T and deoxaphomin as well as new cytochalasins as cytochalasin T, U, V, W and ascochalasin. Ascochalasin belong to [13]-carbocyclic subgroup of cytochalasin. Cytochalasins U and V belong to the 25,26-dioxa[16]- and the 25-oxa[15] subgroups of cytochalasans, while cytochalasin T and W are close to cytochalasins B (Vurro *et al.* 1997). When grown on solid substrates, Pevh showed an increased capacity to synthesize cytochalasins. In fact, cytochalasins B was isolated with very high yields (2.12 g kg^{-1}) together with cytochalasins A, F, T and 7-*O*-acetyl cytochalasin B, while cytochalasins Z2 and Z3 were isolated in very 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-leek and onion, and grapewine) (Venkatasuwaiah *et al.* 1991; Guo *et al.* 1996; Tabacchi *et al* 2000). *p*-Hydroxybenzaldehyde was also isolated as a toxin from *Ceratocystis* 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 aphanididermatum* (Edson) Fitzp., which is the causal agent of *Pythium* red blight, a serious disease of bentgrass (Shimada *et al.* 1999).

p-Hydroxybenzaldehyde and the cytochalasins B, F, Z2 and Z3 and deoxaphomin were tested on *C. arvense* and *S. arvensis* using a leaf-disk bioassay. Deoxaphomin (**5**) demonstrated the highest level of toxicity to leaves of *S. arvensis.* Other cytochalasins showed less activity. On *C. arvense* all cytochalasins showed a medium level of activity. This meant that [13]carbocyclic or a [14]lactonic macrocyclic ring fused to with an unchanged perihydroisoindolyl 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; Berestetskiy *et al.* 2008).

The inactivity 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', 'Smoothee', 'Stayman', 'Super Gold') and eight weed species. Only the cultivars 'Super Gold' and 'Silverspur' 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 (Venkatasuwaiah *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 (Múle and Vurro, pers. comm.) (Cimmino *et al.* 2008).

A representative culture of Peve, the type species of the section *Phyllostictoides* 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. herbarum* was found to produce cytochalasins C, D and E (El-kady and Mostafa 1995). Furthermore, the isolation of cytochalasins from cultures of Pevh (Vurro *et al.* 1997), *P. multipora* (Zhori and Swaber 1994), and *Phoma* spp. (Grafa *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 (Mulè G, Vurro, M, pers. comm.).

Several authors proposed Peve, in particular the strain C-177 (Berestetskiy 2005; Bereteskiy *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 mycotoxin. 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).

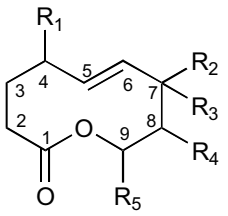
The strain C-177 and S-9, grown in liquid and solid cultures, produced the *p*-hydroxybenzaldehyde and cytochalasin B, F, Z2, and Z3, and deoxaphomin. On the leaves of both *C. arvense* and *S. arvensis*, *p*-hydroxybenzaldehyde appeared inactive, deoxaphomin and cytochalasin Z2 showed the highest and reduced toxicity, respectively. ITS sequence indicates that the strain C-177 and S-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 CIRSII PHYTOTOXINS

Stagonolide, the main phytotoxin produced by *Stagonospora cirsii*

The pycnidial fungus *Stagonospora cirsii* is another foliar pathogen of *C. arvense*, with the potential as a mycoherbicide (Berestetskiy *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; Dneprovskiy and Temnikova 1991). This configuretion was also supported by observations in NOESY spectrum. Stagono-lide can be described as (8*R*, 9*R*)-8-hydroxy-7-oxo-9-propyl-5-nonen-9-olide (Yuzikin *et al.* 2007).

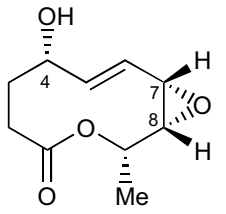
When assayed on the host plant, stagonolide caused first



8 R_1=H, R_2+R_3=O, R_4=α-OH, R_5=β-$CH_2^{10}CH_2^{11}CH_3^{12}$

9 R_1=β-OH, R_2=β-H, R_3=α-OH, R_4=α–OH, R_5=β-$CH_2CH_2CH_3$

10 R_1=α-OH, R_2=α-H, R_3=β–OH, R_4=H, R_5=β-Me



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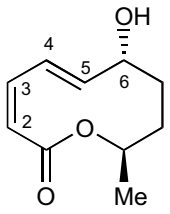

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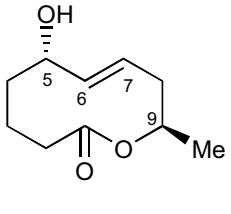

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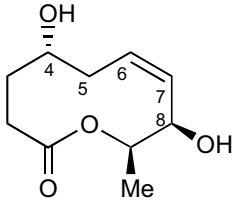

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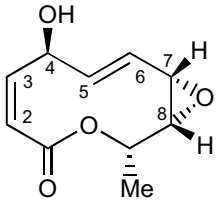

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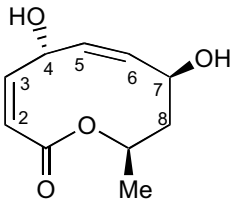

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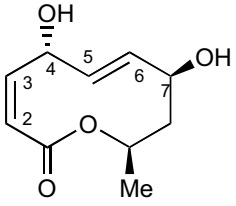

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Fig. 5 Chemical structure of stagonolide (**8**) and stagonolides B-I (**9**-**16**) and modiolide A (**17**) isolated from liquid and solid cultures of *Stagonospora cirsii*.

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 showed 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 $\geq 1 \times 10^{-5}$ 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*

(Pretsch *et al.* 2000). The relative configuration of the chiral carbons of the stagonolides B-F as depicted in the structural formulas was determined by comparison of the $^3J_{H,H}$ spin systems observed with those of herbarumin I and/or putaminoxin, for which the absolute stereochemistry was independently determined (Evidente *et al.* 1995; Rivero-Cruz *et al.* 2003). The relative configuration assigned to stagonolides B-E is in full agreement with the NOE effects observed in the NOESY spectra and with the inspection of Dreiding models (Evidente *et al.* 2008d).

Stagonolide F appears to be a diastereomer of aspinolide A, a fungal metabolite isolated with other nonenolides and polyketides from *Aspergillus ochraceus* Wilh. and for which no biological activity was reported (Fucsher and Zeeck 1997). This was confirmed by the similar spectroscopic data for Stagonolide F and the aspinolides (Fucsher and Zeeck 1997) and by the different optical properties such as the specific optical rotation and CD data (Evidente *et al.* 2008d).

When tested by the leaf-disk assay at a concentration of 1 mg/mL, stagonolides B-F showed no toxicity to *C. arvense* and *S. arvensis*, whereas stagonolide was highly toxic to both plants. Stagonolide and stagonolide C at 0.05 mg/mL (*ca*. 2×10^{-4} M) were practically non-toxic to *Colpoda steinii*, but inhibited movement in 50-60% of infusorium cells after 3-h exposure. Stagonolides B, D-F were non-toxic (Evidente *et al.* 2008d). Toxicity data on stagonolide were in agreement with those from a previous study (Yuzikin *et al.* 2007).

The results reported by Rivero-Cruz *et al.* (2003) indicated that in herbarumin I hydroxylation of the lactone core at C-2 decreased the resultant phytotoxic activity. Possibly, the hydroxylation of the lactone core at C-4 led to the loss of phytotoxicity for stagonolide B in the leaf-disk bioassay. Stagonolide C demonstrated the same level of zootoxicity as stagonolide but showed the loss of phytotoxicity. The latter observation can be connected with a change of propyl group at C-9 in stagonolide to a methyl group in stagonolide C. These results confirm that modifications at the C(2)-C(4) moiety of the nonenolide ring induces a decrease or total loss of phytotoxicity. Furthermore, the stagonolides C-F differ from the phytotoxic herbarumins I-III (Rivero-Cruz *et al.* 2003) or putaminoxins (Evidente *et al.* 1995, 1997, 1998a) in having a methyl group at C-9 instead of a *n*-propyl group, which appears to be an important structural feature for the latter two groups of phytotoxins. The functionality and conformation of the nonenolide ring are important for the activity of putaminoxins as well as closely related pinolidoxins which are phytotoxic metabolites isolated from cultures of *Ascochyta pinodes* Jones, a fungal pathogen for pea (*Pisum sativum* L.) (Evidente *et al.* 1993a, 1993b, 1998b). The total loss of phytoxicity for stagonolides D and E compared to the stagonolide was in agreement with these results. Stagonolides D and E possess marked changes of both substituents groups and conformational freedom of the nonenolide ring with a methyl instead of a *n*-propyl group at C-9 (Evidente *et al.* 2008d).

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. The minimum concentration of stagonolide H causing leaf lesions in *C. arvense* was about 30 μg/mL (~1.5×10^{-4} M). It is similar to the level of activity shown by the stagonolide (Yuzikhin *et al.* 2007). At 1 mg/mL only stagonolide H inhibited root growth in 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, 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 pinodes* (Evidente *et al.* 1993a, 1993b) and *A. ochraceus* (Fuchser 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).

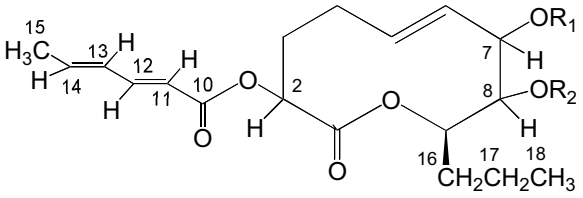
Except stagonolide H, neither other stagonolides nor putaminoxin display host selectivity. However, the host of the pathogens tend to show higher sensitivity to the respective toxins than non-hosts (Evidente *et al.* 1995, 1998b; Yuzikhin *et al.* 2007; Evidente *et al.* 2008c, 2008d). Herbarumin I and stagonolide demonstrated high potency as root growth inhibitors at low concentrations (1×10^{-5}M) on *Amaranthus hypochodriacus* 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.

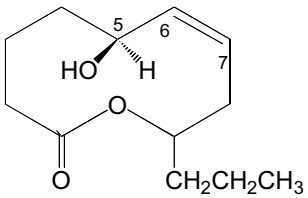
The main phytotoxic metabolite produced by *S. cirsii* in liquid cultures was stagonolide, a new nonenolide. It appears to be a non specific phytotoxin with a low antimicrobial activity. Eight new stagonolides, named stagonolides B-I and modiolide A, were isolated from solid cultures of *S. cirsii* and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

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG SELECTED PHYTOTOXINS

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. pinodes* (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



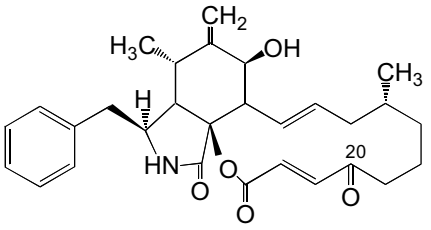


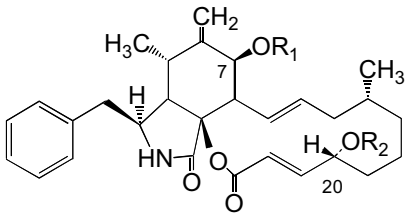
18 $R_1=R_2=H$

20 $R_1=R_2=Ac$

21 $R_1+R_2=-C(Me)_2-$

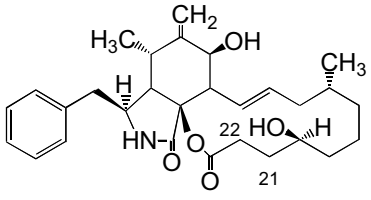
19





22

23 $R_1=Ac$, $R_2=H$
24 $R_1=R_2=Ac$



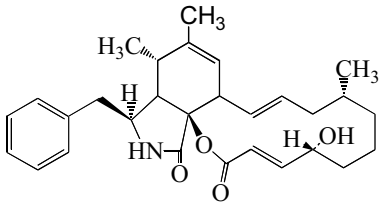


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Fig. 6 Structure of pinolidoxin, its 7,8-*O,O'*-diacetyl and 7,8-*O,O'*-isopropyliden derivatives (**18**, **20** and **21**), putaminoxin (**19**), cytochalasin A (**22**), and 7-*O*-acetyl-, and 7,20-*O,O'*-diacetyl- and 21,22-dihydro-cytochalasin B (**23**-**25**), and cytochalsin T (**26**).

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-dihydrocytochalasin 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**) (Berestetskyi *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 unalterated perihydroisoindolyl 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-

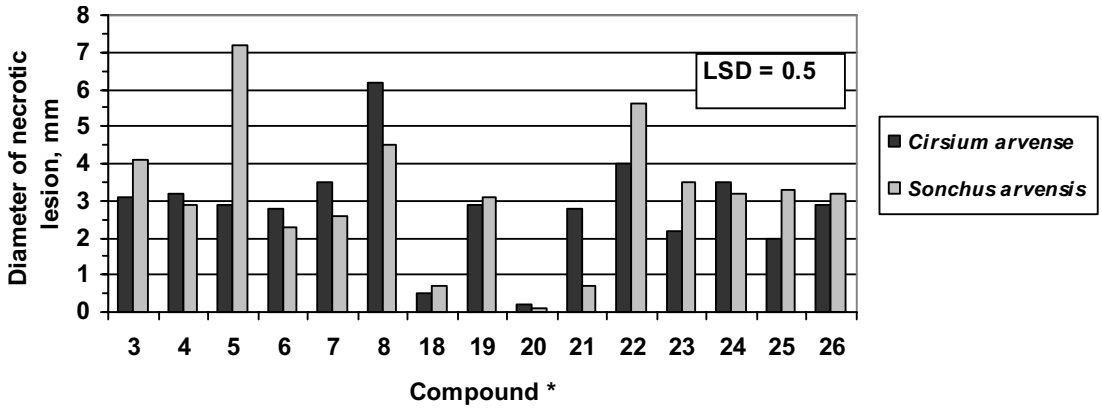


Fig. 7 Effect of different toxins on *C. arvense* and *S. arvensis* using a leaf disc-puncture assay. ***3**, cytochalasin B; **4,** cytochalasin F; **5**, deoxaphomin; **6**, cytochalasin Z2; **7**, cytochalasin Z3; **8**, stagonolide; **18**, pinolidoxin; **19**, putaminoxin; **20**, 7,8-*O*,*O*'-diacetylpinolidoxin; **21**, 7,8-*O*,*O'*-isopropylidene-pinolidoxin; **22**, cytochalasin A; **23**, 7-*O*-acetyl-cytochalasin B; **24**, 7,20-*O*,*O'*-diacetyl-cytochalasin B; **25**, 21,22-dihydro-cytochalasin B; **26**, cytochalasin T. **3-7** and **21-26** are cytochalasins, **8** and **18-21** are nonenolides.

ported from previous studies (Bottalico *et al.* 1990; Capasso *et al*. 1991; Vurro *et al*. 1997; Evidente *et al*. 2002). It is concluded that cytochalasins affect leaf tissues of *S. arvensis* similarly as it does to other sensitive plants (Berestetskiy *et al.* 2008).

Effect of selected toxins on photometric properties

Five toxins were selected to study the effect on relative chlorophyll content in *C. arvense* leaves by measuring the light absorption at 632.8 nm. The first necrosis on leaf disks appeared 6-8 hours post toxin application. Comparing to control, significant changes in the light absorption were caused by cytochalasin A 2 hours post treatment. The ability of *C. arvense* leaves to absorb light was significantly decreased by stagonolide, putaminoxin and both cytochalasins A and B 4 hours post treatment. Negative effect of both nonenolides on the light absorption and relative chlorophyll content was about 2 times higher than that caused by the cytochalasins. The effect of deoxaphomin on relative chlorophyll content was weak. Changes in light absorption and development of lesions on *C. arvense* leaves caused by the toxins did not correlate. Some early effect of cytochalasins on the chlorophyll content can be explained by their well-known inhibition of the light-dependent movement of chloroplasts in leaf cells (Takagi 2003). Cytochalasin E might inhibit photosynthesis because it decreased chlorophyll fluorescence (Kshirsagar *et al.* 2001). Pronounced effect of the nonenolides on chlorophyll content and, hence photosynthesis, was reported for the first time.

Both cytochalasin B and stagonolide caused significant decrease in light absorption at 450 nm 24 hours post treatment of *C. arvense* leaves. This impact is most likely connected to the reduction of the β-carotene and/or chlorophyll b content in leaf tissues because both pigments have a peak of resonant absorption near this wavelength (Britton 1983). The increased level of light absorption at 530 and 550 nm could be caused by either toxins, although stagonolide showed significantly stronger effect at 550 nm. Cytochrome c absorbs the light at 530-550 nm while cytochromes b or f have an absorption peak at 560 nm. Cytochromes are soluble proteins with a heme prosthetic group involved in electron transport. Possibly, these toxins increased the concentration of cytochromes in *C. arvense* leaf tissues.

The reduction of light absorption in the 630–690 nm range was observed for *C. arvense* leaves treated with stagonolide only. Absorption peaks within this region are characteristic of chlorophyll intermediates, protochlorophyllide and chlorophyllide. Tentoxin (a phytotoxin produced by several *Alternaria* spp.) and some synthetic herbicides affected chlorophyll synthesis similarly (Duke *et al.* 1991). Stagonolide also increased light absorption of *C. arvense* 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.

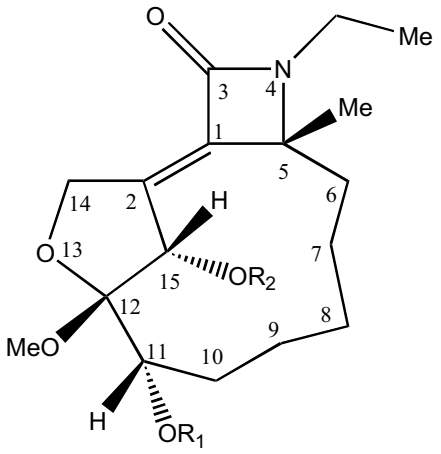
The results of photometric assays performed with different equipment provided similar trends. In fact, the nonenolides, in particular stagonolide and putaminoxin, appear to affect the light absorption at multiple wavelengths. Stagonolide seems to inhibit photosynthesis of *C. arvense* leaves, possibly 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 imparting this activity (Berestetskiy *et al.* 2008).

Effect of selected toxins on conductometric properties

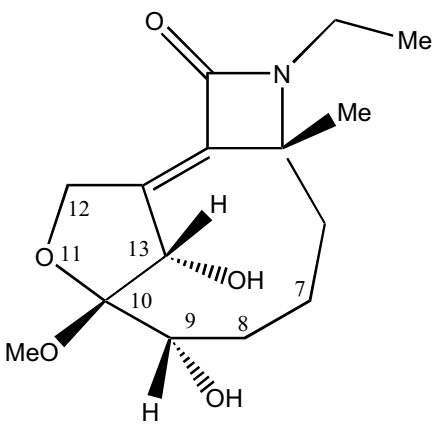
Electrical resistance in *C. arvense* leaves was measured after treatment of phytotoxins *in vivo* using the protocol introduced by Lisker (1991). Under electrical tension, cell ions tend to accumulated along electrodes, thus interfering with the current. For boiled leaf tissues, initial increase in 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).

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 Ca^{2+} 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



27 R_1=R_2=H
28 R_1=H, R_2=Ac
29 R_1=R_2=Ac
30 R_1=H, R_2=*S*-MTPA
31 R_1=H, R_2=*R*-MTPA



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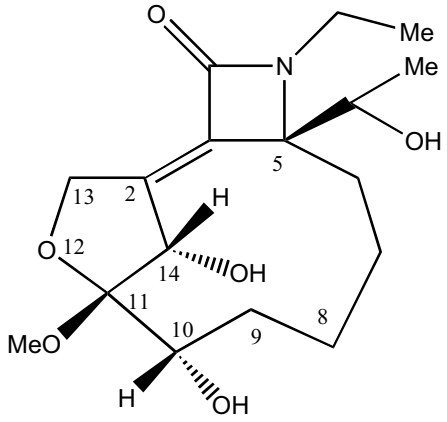

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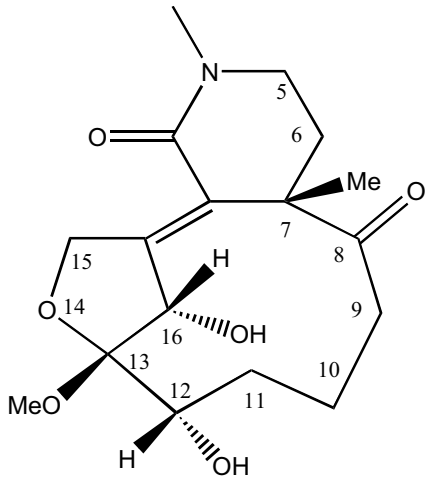

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Fig. 8 Structures of phyllostictines A-D (**27** and **32**-**34**).

demonstrated the highest herbicidal effect on *S. arvensis* leaves. The phytotoxic nonenolides were stronger inhibitors of photosynthesis on *C. arvense* leaves than cytochalasins 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.

PHYLLOSTICTA CIRSII TOXINS

New phytotoxic oxazatricycloalkenones

Recently, the fungus *Phyllosticta cirsii* has been evaluated as a possible biocontrol agent of Canada thistle (Beresteskyi *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 phyllostictine 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 phyllostictine A assigned to the toxin. The phyllostictines B-D appeared to be closely related to phyllostictine A and to each other, but differed from phyllostictine A in the size of macrocyclic ring. Phyllostictine C also differed for the substituent at C-5. Phyllostictine 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 phyllostictine 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 ^{1}H-NMR spectra of its 15-*O*-SMTPA and 15-*O*-RMTPA esters (**30** and **31**, **Fig. 8**) and NOESY spectroscopy. The absolute stereochemistry of phylostictines B-D was assigned after comparing their NMR data with those of phyllostictine A (Evidente *et al*. 2008a).

Biological activity of phyllostictines

Tested at about 6×10^{-3} M on Canada thistle using the leaf-disk assay, phyllostictine 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 *Escherichia 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 10^{-3} M, whereas phyllostictine B had only negligible effect. The integrity of oxazatricycloalkenone 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; Tringali 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 phyllostine A in the static M-1-D medium after 4 weeks (**Table 3**). The phytoxicity of culture filtrate seemed to correlate to production of specific metabolites by the fungus under certain cultural conditions (Zonno *et al.* 2008).

Table 2 Toxicity of culture filtrates produced by *Phyllosticta cirsii* in shake-flask media on Canada thistle leaf discs.[a,b]

Culturing duration	Medium			
(days)	M-1-D	Fries	Malt	Kent-Strobel
4	-	-	+	-
8	–	++	-	-
12	–	+++	+	-
16	–	+++	+	+
20	–	+	+	-

[a] Scale of toxicity: - not toxic; +: necrotic lesion < 1 mm; ++: 2-3 mm; +++: 3–5 mm; ++++: > 5 mm
[b] Zonno *et al.* 2008

Table 3 Production of phyllostictine A (mg L^{-1}) by *Phyllosticta cirsii* under static cultural conditions.[a]

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

[a] 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 C_{18} HD column, isocratic elution with acetonitrile and HPLC grade water (1: 1, v/v) with a flow rate of 1 mL/min. Detection was performed at 263 nm, corresponding to the maximum phyllostictine A absorption. This method proved to be very useful for the identification of the best cultural conditions for toxin production, and may also be used for comparing different fungal strains for phyllostictine A production.

The chromatographic peak of metabolite in samples was identified by the retention time with that of phyllostictine A standard, which was eluted at 13.8 min. For all the samples, the matrix substances absorbed at 263 nm were eluted within the first 6 minutes. This finding, together with the high similarity of the standard, allows to suggest that no peaks of other substances overlap those being investigated.

As shown in **Table 3**, the concentration of phyllostictine A in the static culture filtrates can vary substantially depending on the medium and cultural duration. On the M-1-D medium, the fungus produced phyllostictine A in a linear fashion of increase up to the fourth week and decreased after that. This change pattern coincided with that of phytotoxic impact on leaves. In shaken cultures, the fungus reached the peak of phyllostictine A production within 4 days but the toxin yield did not increase after that, irrespective the medium used. Carbon source and concentration also influence the production of phyllostictine A. For example, saccarose is better than glucose for toxin production and reducing its concentration in the M-1-D medium by 50% from a standard recipe lowered the yield substantially (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^{-3} M was highly effective even 1 hour after application, killing most of the protoplasts completely. At 5×10^{-4} M, the toxicity was time dependant, with around 50% kill 1 h after treatment and 100% after 6 h. At 10^{-5} M, however the toxin was almost completely ineffective. For comparison, fusaric acid showed a time-dependant toxicity at 10^{-3} 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 bright fluorescence during flow cytometric analysis, whereas dead protoplasts were non-fluorescent. An example of citogramm obtained using the flow cytometer to analyze tobacco protoplasts treated with phyllostictine A is given in **Fig. 9**. At 10^{-3} M phyllostictine A acted quickly, killing almost all the protoplasts within an hour. At 5×10^{-4} M the toxin was still fast acting, causing around 80% mortality 1 h after the treatment, although the effect was time dependant and considerable only after 3 h, whereas at 10^{-5} M no toxicity was observed. Fusaric acid was highly toxic at 10^{-3} and 5×10^{-4} M, causing almost 100% mortality after 6 h, whereas at 10^{-5} 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.

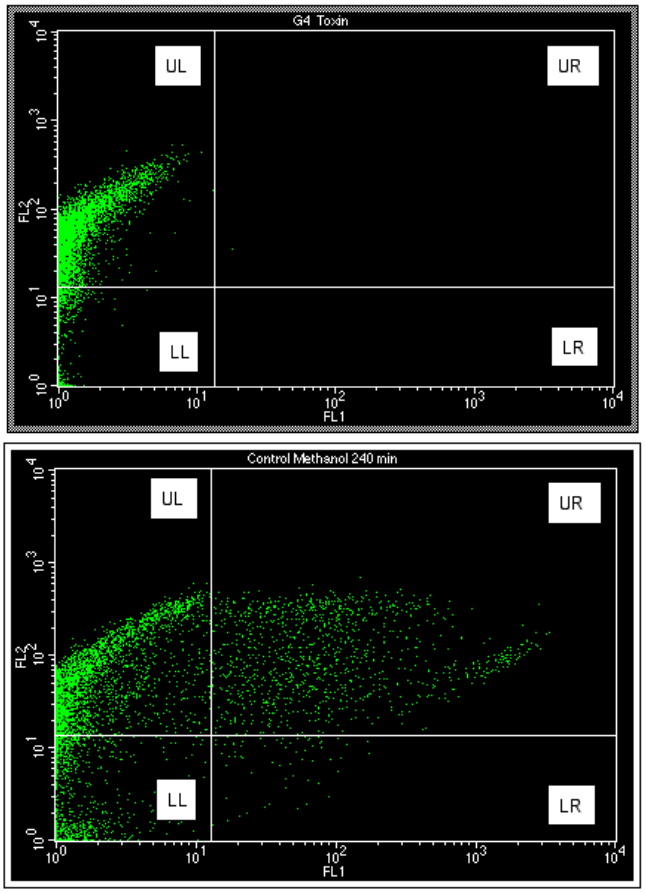


Fig. 9 Citogramm (Dot-plot) of tobacco protoplast vitality exposed to phyllostictine A at 10^{-3} M (upper) and to methanol (1%) as control (lower) for 240 min. Quadrants: UR + LR = Live protoplasts UL + LL = Debris and dead cells.

Phyllostoxin and phyllostin

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. Phyllostin 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 synthesis (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**.

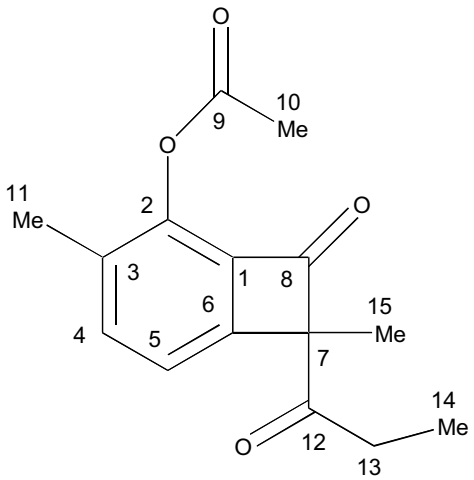
Bond lengths and angles in phyllostin are in the normal range (Rendle and Trotter 1975; Schweizer and Dunitz 1982; Ayer *et al.* 1993). The molecule contains two 6-membered rings. In the 2-oxo-1,4-dioxane ring (ring A) which assumes a distorted half chair conformation with C3/C2/O1/C8a atoms nearly coplanar 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.*



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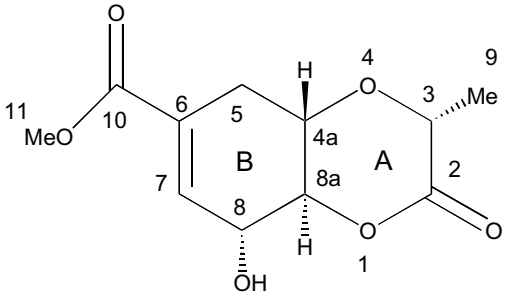

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Fig. 10 Structure of phyllostoxin and phyllostin (**35** and **36**).

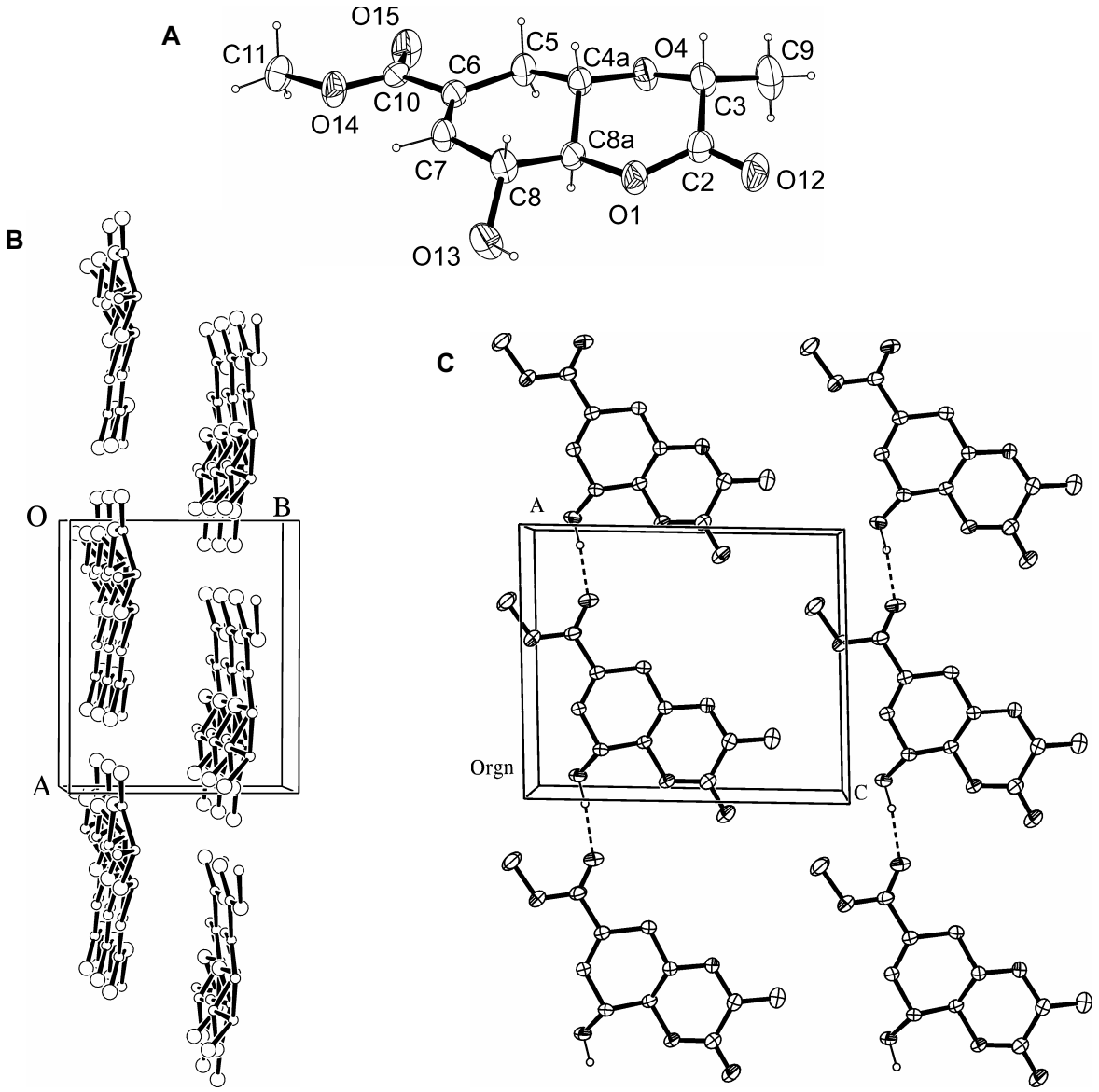

Fig. 11 (**A**) ORTEP view of phyllostin (**36**) showing atomic labelling. *Displacement ellipsoids* are drawn at 30% probability level. (**B**) Crytal packing viewed along *c* axis, showing the *flat shape* of molecule and the stacking of layers in the [0 1 0] direction. Atoms are drawn as *sphere* of arbitrary radius, all H atoms are omitted for clarity. (**C**) Partial packing viewed along *b* axis, showing chains of molecules through O-H···O hydrogen bond (*dashed lines*). Only hydroxyl H atom is reported, all other H atoms are omitted for clarity.

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. Phyllostctine A showed antibiotic activity against Gram+ bacteria and a noticeable zootoxic activity at a high concentration. Phyllostoxin tuned out to be highly phytotoxic in the same bioassay, while phyllostin was ineffective.

TOXINS FROM *PHOMOPSIS CIRSII*

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 *Aldus* spp., a noxious weed for forestall 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. v*itians* (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 potential herbicide activity in field trials for control of some troublesome weeds in forest (Doworth and Glover 1991).

CONCLUSIONS

Phytopathogenic fungi belonging to the genera *Ascochyta*, *Stagonospora*, *Phyllosticta* and *Phomopsis* have been proposed for biocontrol of *C. arvense* 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. arvense*, 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. arvense* 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 oxazatricicloalchenones, which were named as phyllostictines A-D. Phyllostictine A was most phytotoxic against *C. arvense*, but its effect would depend on the size and functionality of the macrocyclic ring. Due to a lack of zootoxic and antimicrobial activity, phyllostictine 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. arvense* 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.

ACKNOWLEDGEMENTS

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