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Nonenolides and cytochalasins with phytotoxic activity against *Cirsium arvense* and *Sonchus arvensis*: A structure–activity relationships study

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Abstract

A structure–activity relationships study was conducted assaying 15 natural analogues and derivatives belonging to two groups of organic compounds, nonenolides and cytochalasins, for their toxicity against the composite perennial weeds *Cirsium arvense* and *Sonchus arvensis* occurring through the temperate region of world. The toxic nonenolides (stagonolide, putaminoxin, pinolidoxin) and cytochalasins (deoxaphomin, cytochalasins A, B, F, T, Z2 and Z3) were isolated from phytopathogenic *Stagonospora*, *Phoma* and *Ascochyta* spp. The pinolidoxin (7,8-*O*,*O*′-diacetyl- and 7,8-*O*,*O*′-isopropylidene-pinolidoxin) and cytochalasins B (21,22-dihydro-, 7-*O*-acetyl- and 7,20-*O*,*O*′-diacetyl-cytochalasin B) derivatives were obtained by chemical modifications of the corresponding toxins. Among the 15 compounds tested, stagonolide and deoxaphomin proved to be the most phytotoxic to *C. arvense* and *S. arvensis* leaves, respectively. The tested phytotoxic nonenolides were stronger inhibitors of photosynthesis in *C. arvense* leaves than cytochalasines A and B. Stagonolide had less effect on membrane permeability in *C. arvense* leaves than cytochalasin B. Significant changes of light absorption by *C. arvense* leaves in visible and infrared spectra were caused by stagonolide. The functional groups and the conformational freedom of the ring, appear to be important structural features for the nonenolides toxicity, whereas and the presence of the hydroxy group at C-7, the functional group at C-20 and the conformational freedom of the macrocyclic ring are important for the cytochalasins toxicity.

Keywords: *Cirsium arvense*; *Sonchus arvensis*; Weeds; Phytotoxins; Nonenolides; Cytochalasins; Structure–activity relationships; Leaf optics

1. Introduction

Perennial weeds are common problem in different crops. They are harmful especially in agricultural systems with low herbicide usage because of their tolerance to traditional mechanical control methods. Among them, *Cirsium arvense* (L.) Scop. and *Sonchus arvensis* L. (both from Asteraceae) commonly called Canada thistle and perennial sowthistle, respectively (Donald, 1990; Lemna and Messersmith, 1990) are particularly important. Few herbicides are recommended for chemical control of perennial weeds in non-organic cropping systems, and they have low selectivity (Lemna and Messersmith, 1990; Kloppenburg and Hall, 1990; Grekul et al., 2005). Obviously, new compounds should be actually developed as herbicides against composite weeds.

Microbial phytotoxins or their synthetic analogues could be used for the development of new herbicidal compounds (Evidente and Abouzeid, 2006; Rimando and Duke, 2006). Many plant pathogens, especially necrotrophic and hemibiotrophic fungi, are able to produce phytotoxins responsible for disease development (Hoppe, 1998), and therefore they could be considered as sources

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of such useful metabolites (Kenfield et al., 1989; Evidente and Motta, 2001).

Numerous surveys were carried out to find pathogens of *C. arvense* (Berestetskiy, 1997; Leth and Andreasen, 2000; Bailey et al., 2000) and, to a lesser extent, of *S. arvensis* (Berestetskiy and Smolyaninova, 1998). Several pathogens, among which *Stagonospora cirsii* Davis and *Ascochyta sonchi* (Sacc.) Grove (syn. *Phoma exigua* Desm. var. *exigua*) were found to be common for both plants and to produce phytotoxic metabolites (Berestetskiy et al., 2005).

A new phytotoxic nonenolide, named stagonolide, was recently isolated from the culture filtrate of *S. cirsii* (Yuzikhin et al., 2007). It proved to be structurally related to other known phytotoxins such as herbarumins from *Phoma herbarum* West. (Rivero-Cruz et al., 2000), pinolidoxins from *Ascochyta pinodes* L.K. Jones (Evidente et al., 1993a,b) and putaminoxins from *Phoma putaminum* Speg. (Evidente et al., 1995, 1997, 1998a). Although some studies on structure–activity relationships of some of the mentioned phytotoxins were carried, the activity of stagonolide was never compared to other nonenolides against *C. arvense* and *S. arvensis*.

Preliminary results showed that some *A. sonchi* strains produce cytochalasins (Evidente, unpublished) besides ascosonchine, a previously described phytotoxic enol tautomer of 4-pyridylpyruvic acid (Evidente et al., 2004). Many other cytochalasins are reported to be produced by 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). However, their activity was not tested against perennial weeds.

In this paper, we show the results of a structure–activity relationships study, with respect to the phytotoxic activity of several nonenolides and cytochalasins and their derivatives against *C. arvense* and *S. arvensis*.

2. Results and discussion

2.1. Phytotoxic activity of different fungal toxins on leaves of Canada thistle and perennial sowthistle

Among the 15 compounds tested by leaf disc-puncture bioassay, stagonolide (**1**, Fig. 1) showed the highest toxicity (necrosis diameter >6 mm) to leaves of *C. arvense*. Other nonenolides, i.e.: putaminoxin (**2**, Fig. 1) and 7,8-*O*,*O*′-isopropylidene-pinolidoxin (**5**, Fig. 1), were significantly less toxic (necrosis diameter ~3 mm). Among cytochalasins, only cytochalasin A (**7**, Fig. 2) was to be highly toxic (necrosis diameter 4 mm) for the weed (Fig. 3).

Deoxaphomin (**6**, Fig. 2) was the most toxic compound (necrosis diameter ~7 mm) for punctured leaf discs of *S. arvensis*. Stagonolide (**1**), cytochalasin A and cytochalasin B (**7** and **8**, Fig. 2) had high phytotoxicity (necrosis diameter ~4.5, 5.5 and 4 mm, respectively). Other cytochalasins were moderately toxic (necrosis diameter between 2 and 3.5 mm) (Fig. 3).

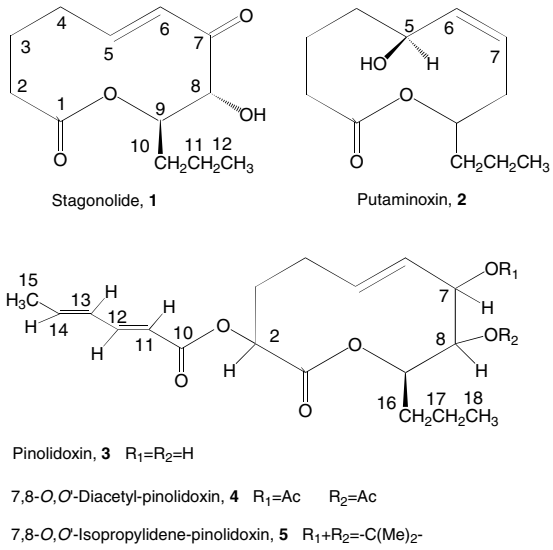
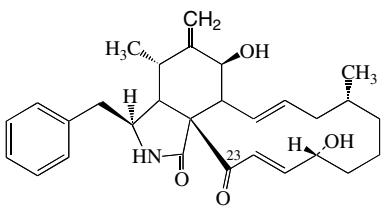


Fig. 1. Structures of stagonolide, putaminoxin, pinolidoxin (**1**–**3**) and two pinolidoxin derivatives (**4** and **5**).

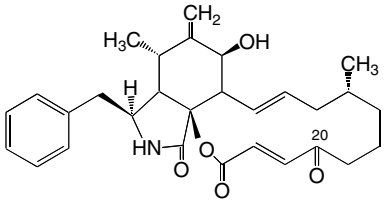
Pinolidoxin and 7,8-*O*,*O*′-diacetylpinolidoxin (**3** and **4**, Fig. 1) were almost completely inactive (necrosis diameter <1 mm) to leaves of both weeds (Fig. 3).

The results demonstrated a different behaviour of the two plants (*C. arvense* and *S. arvensis*) in response to the compounds assayed (Fig. 3). The natural nonenolides were more toxic than cytochalasins to *C. arvense*. The most toxic nonenolides, stagonolide and putaminoxin (**1** and **2**), differ by the location of nucleophilic (hydroxy and double bond) groups on the same fragment between C-5 and C-8 of the macrocyclic ring. Pinolidoxin (**3**), and its two derivatives (**4** and **5**), having a marked modifications in respect to **1** and **2** in both the functional groups and the conformational freedom of the nonenolide ring, showed a strong decrease or practically the total loss of toxicity. These results are in fully agreement with data on a structure–activity relationships study previously performed by assaying putaminoxin and pinolidoxin together to their natural and synthetic analogs on several weedy and crop plants (Evidente et al., 1998b).

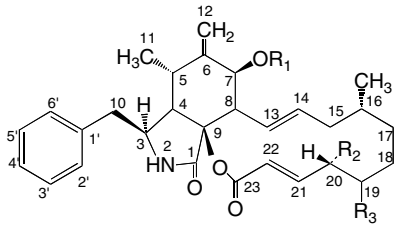
The cytochalasins tested were more toxic than nonenolides to *S. arvensis*. Among them, deoxaphomin, cytochalasins A and B (**6**–**8**) were the most toxic compounds, which possess a [13]carbocyclic or a [14]lactonic macrocyclic ring, respectively, jointed with an unalterated perihydroisoindolyl residue. In this latter moiety, the presence of the secondary hydroxyl on C-7, which lacks in **12**–**14** (Fig. 2) or is acetylated in **10** and **11** (Fig. 2), appears to be an important feature to impart toxicity. Furthermore, the significant decrease of toxicity observed testing the 21,22-dihydroderivative of cytochalasin B and cytochalsins Z3 (**9** and **15**, Fig. 2) also indicates the importance of the functionalization on C-20 and the conformational freedom of the macrocyclic ring. These results are in accordance with those previously described in the struc-



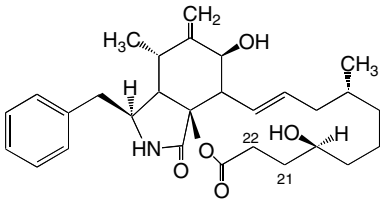
Deoxaphomin, **6**



Cytochalasin A, **7**



Cytochalasin B, **8** R_1=R_3=H, R_2=OH

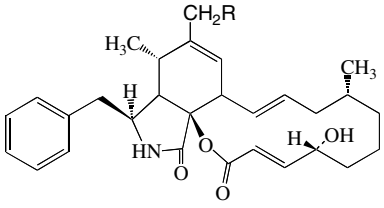


21,22-Dihydro-cytochalasin B, **9**

7-*O*-Acetyl-cytochalasin B, **10** R_1=Ac, R_2=OH, R_3=H

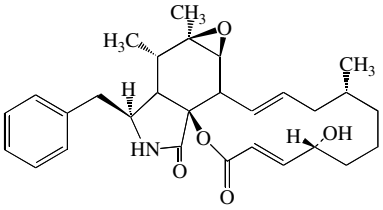
7,20-*O,O'*-Di-acetyl-cytochalsin B, **11** R_1=Ac, R_2=OAc, R_3=H

Cytochalasin Z3, **15** R_1=R_2=H, R_3=OH



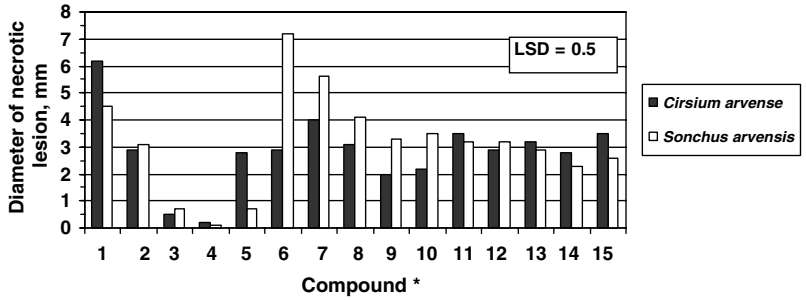
Cytochalasin T, **12** R=H

Cytochalasin Z2, **14** R=OH



Cytochalasin F, **13**

Fig. 2. Structures of deoxaphomin and cytochalasins A, B, F, T, Z2 and Z3 (**6**–**8** and **12**–**15**) and three cytochalasins B derivatives (**9**–**11**).



*1, stagonolide; **2**, putaminoxin; **3**, pinolidoxin; **4**, 7,8-*O,O*'-diacetylpinolidoxin; **5**, 7,8-*O,O'*-isopropylidene-pinolidoxin; **6**, deoxaphomin; **7**, cytochalasin A; **8**, cytochalasin B; **9**, 21,22-dihydro-cytochalasin B; **10**, 7-*O*-acetyl-cytochalasin B; **11**, 7,20-*O,O'*-diacetyl-cytochalasin B; **12**, cytochalasin T; **13,** cytochalasin F; **14**, cytochalasin Z2; **15**, cytochalasin Z3. **1-5** are nonenolides, **6-15** are cytochalasins.

Fig. 3. Effect of different toxins on *C. arvense* and *S. arvensis* using a leaf disc-puncture assay. The results are reported as diameter of necrosis.

ture–activity relationships studies carried out by testing the phytotoxicity of several cytochalasins and their derivatives on different crop plants (Bottalico et al., 1990; Capasso et al., 1991; Vurro et al., 1997; Evidente et al., 2002). So, cytochalasins affect leaf tissues of *S. arvensis* similarly to other sensitive plants.

2.2. Effect of selected toxins on photometric properties of C. arvense leaves

Five toxins were selected to study their effect on the relative chlorophyll content in *C. arvense* leaves by measuring the light absorption at the wavelength of 632.8 nm. The

first necrosis on leaf discs appeared 6–8 h post toxin application. Absorption of light by *C. arvense* leaves (Table 1) was significantly decreased by stagonolide, putaminoxin and both cytochalasins A and B (**1**, **2**, **7** and **8**) 4 h after treatment. The effect of deoxaphomin (**6**) on relative chlorophyll content was negligible. Changes in the light absorption and lesion development in *C. arvense* leaves were not correlated. Some early effects of cytochalasins on the chlorophyll content can be explained by their well-known effect on inhibition of the light-dependent movement of chloroplasts in the leaf cells (Takagi, 2003).

Twenty four hours after the treatment both cytochalasin B and stagonolide (**8** and **1**) caused significant decrease of the light absorption at the wave 450 nm (Table 2). This observation is most likely connected with the reduction of the content of β-carotene or/and chlorophyll *b* in leaf tissue of *C. arvense*, because both pigments have a peak of resonant absorption near this wavelength (Britton, 1983).

The increased light absorption at 530 and 550 nm was caused by both toxins (Table 2). However, stagonolide (**1**) had significantly stronger effect at the wavelength 550 nm than cytochalasin B (**8**). As cytochromes have a peak of absorption in the range 530–550 nm, the toxins probably increased the concentration of these proteins in leaf tissue of *C. arvense* and, hence, did not affect electron transport.

The reduction of light absorption in the wavelength region of 630–690 nm by *C. arvense* leaves was observed after the treatment of leaf discs by stagonolide (**1**) only (Table 2). The peaks of light absorption in this region are characteristic for chlorophyll intermediates, protochlorophyllide and chlorophyllide. These results are in agreement with those previously observed with some natural and synthetic herbicides (Duke et al., 1991).

Stagonolide (**1**) was also found to significantly increase light absorption by *C. arvense* leaves in near infrared spectra (Table 2). At the wavelength higher than 700 nm leaves of healthy plants are usually transparent. Therefore, increasing of absorption in the near infrared region can be related with level of leaf damage caused by phytotoxins (Kräutler, 2002; Merzlyak et al., 2002).

The results of photometric assays performed with two different equipments were in accordance each other. In fact, the nonenolides appear to affect more the light absorption

Table 1
Effect of toxins on light absorption by leaves of *C. arvense* at 632.8 nm

Toxins	Absorption (%)			Change after 4 h (%)
	Before treatment	After 2 h post-treatment	After 4 h post-treatment	
Stagonolide	68.1	63.4	53.5[a]	−21
Putaminoxin	62.0	53.4	47.8[a]	−24
Deoxaphomin	60.6	60.9	56.6	−7
Cytochalasin A	66.3	57.8[a]	58.8[a]	−11
Cytochalasin B	66.3	60.8	54.4[a]	−18
Control	63.8	61.6	61.8	−3

[a] Values are significantly ($p < 0.05$) differed from values before treatment.

Table 2
Effect of toxins on light absorption by leaves of *C. arvense* in the range of 450–950 nm

Wavelength (nm)	Absorption (%)			Comparison of means with Student's coefficient		
	Control	Cytochalasin B	Stagonolide	Control vs. cytochalasin B	Control vs. stagonolide	Cytochalasin B vs. stagonolide
450	62.6	58.6	58.5	4.40[a]	2.77[a]	0.07
470	77.1	76.8	76.8	0.31	0.10	0.02
490	74.1	74.9	71.1	0.36	1.98[a]	1.93
510	59.9	63.3	62.2	1.81	0.99	0.74
530	44.5	48.7	53.4	2.04[a]	3.70[a]	1.94
550	44.4	49.4	54.4	2.38[a]	4.85[a]	2.02[a]
590	65.2	66.4	63.9	0.84	0.87	1.61
610	68.4	69.8	66.1	0.70	1.37	1.71
630	70.9	72.4	66.7	0.84	2.14[a]	2.76[a]
650	78.5	78.2	70.5	0.15	2.15[a]	1.98[a]
670	85.6	85.7	76.1	0.12	3.65[a]	3.62[a]
690	62.6	64.8	55.0	0.98	3.46[a]	3.49[a]
710	19.1	21.9	26.0	0.97	3.02[a]	1.47
730	1.3	2.9	16.8	1.07	5.91[a]	4.79[a]
750	0.5	2.6	16.2	1.64	7.12[a]	5.44[a]
770	2.2	3.9	18.0	0.91	6.93[a]	5.39[a]
790	3.1	4.0	16.8	0.43	5.07[a]	4.31[a]
810	4.3	5.4	17.5	0.49	5.63[a]	2.60[a]
830	4.2	6.0	17.0	0.82	5.47[a]	4.27[a]
850	3.3	4.2	14.3	0.49	4.96[a]	3.95[a]
870	1.4	3.25	10.8	1.12	4.70[a]	3.25[a]
890	1.0	2.2	4.2	0.97	1.68	0.91
910	0.19	1.2	3.1	1.32	1.98	1.13
930	0.02	0.9	3.4	1.49	2.41[a]	1.61

[a] Values marked with asterisk are significantly ($p < 0.05$) differed from each other.

at different wavelengths than cytochalasins (cytochalasins A and B, **7** and **8**) and, consequently, even the relative content of pigments. Probably the same structural features above discussed for each group of compounds are important to impart this activity.

2.3. Effect of selected toxins on conductometric properties of *C. arvense* leaves

In vivo measurement of the electrical resistivity in *C. arvense* leaf tissues showed its growth (up to 100 Ω) during course of the electrical current in the intact discs (Fig. 5). Under electrical tension cell ions were accumulated at electrodes and interfered the current. In boiled discs initial increasing of resistivity was changed by its falling to the minimal values (about 10 Ω) 150 s after the first measurement. Leaf discs treated with cytochalasin B (**8**) did not express considerable changes of resistivity during the measurement time. The resistivity dynamics of discs treated with stagonolide (**1**) was linear and similar to control but with a lower angle (Fig. 4). The results allow to assume that stagonolide (**1**) practically did not affect the permeability of cellular membranes, while cytochalasin B (**8**) caused electrolyte leakage in cells of leaf tissues of *C. arvense*.

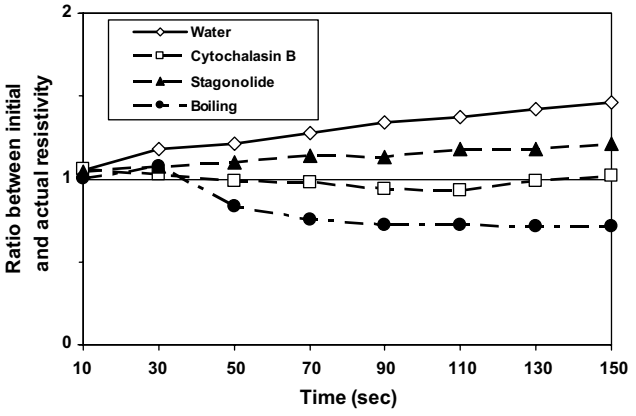


Fig. 4. Effect of cytochalasin B and stagonolide on *in vivo* resistivity of *C. arvense* leaves.

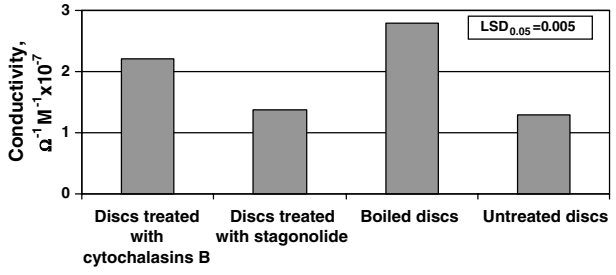


Fig. 5. Effect of cytochalasin B and stagonolide on electrolyte leakage assay. Bars mean electrical conductivity of water extracts obtained from treated leaf discs of *C. arvense*.

This observation was supported by another experiment. It was shown that conductivity of water extracts obtained from leaf discs treated with stagonolide (**1**) was similar to control treatment and was two times lower than conductivity of the extracts from discs treated with cytochalasin B (Fig. 5).

These results did not surprise considering the well known effects of cytochalasins, e.g. cytochalasin B (**8**), in certain plants. This cytochalasin inhibited cytoplasmatic streaming, organelle movement, cell division, pollen germination, cell wall metabolisms and auxin transport (Natori and Yahara, 1991).

3. Conclusions

Among 15 phytotoxic compounds tested stagonolide (**1**) was the most phytotoxic to *C. arvense* leaves and deoxaphomin (**6**) demonstrated the highest herbicidal effect to *S. arvensis* leaves. The tested phytotoxic nonenolides were stronger inhibitors of photosynthesis in *C. arvense* leaves than cytochalasins A and B (**7** and **8**). Although the photometric observations allowed to suppose that both stagonolide (**1**) and cytochalasin B (**8**) did not inhibit electron transport, stagonolide (**1**) had a much lower effect on membrane permeability in *C. arvense* leaves than cytochalasin B (**8**). All above indicates different modes of action of phytotoxic nonenolides and cytochalasins. Stagonolide (**1**) seems to inhibit selectively the photosynthesis in *C. arvense*. Study of its mode of action looks perspective because most of the known herbicides affecting photosynthesis inhibits electron transport in photosystem II and, further, causes membrane damage (Fedtke and Duke, 2004; Wakabayashi and Böger, 2004; Duke et al., 2005).

Analysis of structure–activity relationships showed the importance of functional group and the conformational freedom of the ring for the toxicity of nonenolides. The presence of the hydroxy group at C-7 and the functional group at C-20 and the conformational freedom of the macrocyclic ring appear to be important structural features of toxic cytochalasins.

The equipment (Lisker, 1991) used for *in vivo* photometric and conductometric assays can be useful to study mode of action of natural herbicides.

4. Experimental

4.1. Cultures of the fungi

For stagonolide production a monoconidial strain of *S. cirsii* (C-163, Culture collection of All-Russian Research Institute of Plant Protection, Saint-Petersburg, Russia) was used. The fungus was grown in liquid culture as recently reported (Yuzikhin et al., 2007). A strain of *P. putaminum* isolated from diseased *Erigeron annuus* (L.) Pers. leaves was grown in liquid culture as previously reported (Evi-

dente et al., 1995) to produce putaminoxins. A strain of *A. pinodes* (ITEM 1094, the fungal collection of Institute of Sciences of Food Production, Bari, Italy), isolated from infected pea pods, was used as pinolidoxin source. The toxin was isolated from wheat kernels culture as previously reported (Evidente et al., 1993a). The strain of *P. exigua* var. *heteromorpha* (ITEM 330) was isolated in 1985 from necrotic spots of oleander (*Nerium oleander* L.) leaves and used in this study to produce cytochalasins A, B, F, T, Z2 and Z3, 7-*O*-acetyl cytochalasin B and deoxaphomin. The fungus was grown on autoclaved wheat kernels as previously reported (Evidente et al., 2003).

4.2. Chemical methods

Stagonolide, putaminoxin and pinolidoxin (**1**, **2** and **3**) were obtained by purification of the organic extract of *S. cirsii*, *P. putaminum* and *A. pinodes* cultures, respectively, as previously described (Evidente et al., 1993a, 1995; Yuzikhin et al., 2007). Deoxaphomin and cytochalasins A, B, F, T, Z2 and Z3, and 7-*O*-acetyl-cytochalasin B (**6**, **7**, **8**, **13**, **12**, **14**, and **15**, and **10**) were obtained by the purification of the organic extract of *P. exigua* var. *heteromorpha* solid culture as previously reported (Evidente et al., 2003). 7,8-*O*,*O*′-Diacetyl- and 7,8-*O*,*O*′-isoprophylidene-pinolidoxin (**4** and **5**) were prepared from **3** according to the chemical derivatization previously reported (Evidente et al., 1993a) as well as 21,22-dihydro- and 7,20-*O*,*O*′-diacetyl-cytochalasin B (**9** and **11**), were prepared by chemical modification of **8** as previously reported (Bottalico et al., 1990).

4.3. Leaf disc-puncture bioassay

Discs (1 cm diameter) were cut out from well expanded leaves of *C. arvense* and *S. arvensis* grown in greenhouse. They were placed on moistened filter paper in transparent plastic boxes and wounded with sharp needle in the centre. Samples of 15 toxins were dissolved in EtOH (nonenolides) or DMSO (cytochalasins) and brought up to final concentration of 5×10^{-3} M with bidistilled H_2O. Concentration of the solvents was 20% (v/v), in which they were non-toxic to leaves of both weeds. A drop of test solution (10 μl) was placed in the leaf disc centre. The treated discs were incubated under alternate artificial light and temperature: 8 h in darkness at 20 °C and 16 h under light at 24 °C. After 48 h of the incubation the leaf disc necrotic area was measured.

4.4. Photometric assays

Light absorption of leaves treated with phytotoxins was recorded *in vivo* with a photometer LAFOT and a spectrophotometer SPEFOT. Both instruments were developed in Saint-Petersburg Agrophysical Institute (Saint-Petersburg, Russia) (Lisker, 1991). LAFOT works at the wave of 632.8 nm. At this wavelength the absorption level closely correlates with the chlorophyll content in plants (Lisker and Dmitriev, 1998, 1999). SPEFOT reads the optical parameters of plant tissue in the spectrum from 450 to 1100 nm and can register relative quantitative changes of a number of plant pigments including chlorophylls and carotenes. The absorption values were expressed as percentage of the initial radiation. Ten leaf discs per treatment were analysed with LAFOT 0 (control), 2 and 4 h after toxin application. Photometric assay with SPEFOT at the wavelength range of 450–950 nm was conducted 24 h after treatment of discs with stagonolide and cytochalasin B.

4.5. Electrolyte leakage assays

Conductivity meter Tercon-04 (Agrophysical Institute, Saint-Petersburg, Russia) was used for *in vivo* evaluation of the electrical resistivity of leaf tissues of *C. arvense* 24 h post-treatment with stagonolide, cytochalasin B, and water (control). The discs boiled in water for several minutes were used as a positive control. For the assay, a treated leaf disc (10 replicate discs per treatment) placed between two copper electrodes was exposed to current at the electric tension of 1 V. Its electric resistivity was measured with determined intervals since 5–150 s after beginning of the current. The resulted data were expressed as a ratio between the first measurement (5 s post-beginning of the current) and following measurements of the resistivity.

Data obtained with the above-mentioned technique were compared with observations based on routine electrolyte leakage assay. Treated leaf discs were washed with distilled water, cut into small pieces and soaked for 30 min in water, and electrical conductivity of resulted water extracts was measured by a conductivity meter.

4.6. Statistical analysis

Means were compared using *t*-Student or by Fisher's LSD coefficients.

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