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On the metabolites produced by *Colletotrichum gloeosporioides* a fungus proposed for the *Ambrosia artemisiifolia* biocontrol; spectroscopic data and absolute configuration assignment of colletochlorin A

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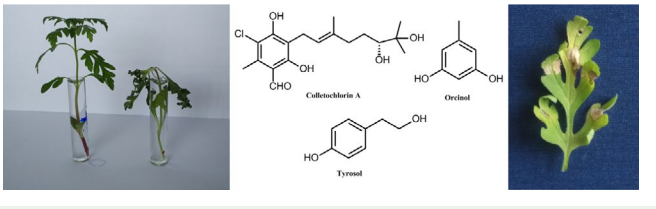
ABSTRACT

Ambrosia artemisiifolia L. is responsible for serious allergies induced on humans. Different approaches for its control were proposed during the COST Action FA1203 "Sustainable management of *Ambrosia artemisiifolia* in Europe" (SMARTER). Fungal secondary metabolites often show potential herbicidal activity. Three phytotoxins were purified from the fungal culture filtrates of *Colletotrichum gloeosporioides,* isolated from infected leaves of *A. artemisiifolia*. They were identified by spectroscopic and chemical methods as colletochlorin A, orcinol and tyrosol (**1**, **2** and **3**). The absolute configuration 6′*R* to colletochlorin A was assigned for the first time applying the advanced Mosher's method. When assayed by leaf-puncture on *A. artemisiifolia* only **1** caused the appearance of large necrosis. The same symptoms were also induced by **1** on ambrosia plantlets associated with plant wilting. On *Lemna minor*, colletochlorin A caused a clear fronds browning, with a total reduction in chlorophyll content.



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1. Introduction

Ambrosia artemisiifolia L., also known as common ragweed, is a widespread invasive weed native to North America. This plant is very competitive and forms large and almost pure populations in natural or uncultivated areas, gardens, railway ballast, yards, roadsides and

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cultivated fields after harvest (Essl et al. 2015). The major concern regarding common ragweed is its highly allergenic pollen that causes sensitisation of the population generating huge medical costs. Moreover, ragweed is becoming a major weed causing severe crop losses and troubles for its control (Gerber et al. 2011).

Chemical herbicides and mechanical control are effective as short-term measures in small infested areas, but are rather insufficient in larger areas and in natural environments, where these methods are unable to prevent flowering, pollen dispersal and seed production, or cannot be used due to environmental issues and safety restrictions. Biological control with the use of microorganisms or their toxic metabolites has been considered a potential alternative approach for managing weeds (Cimmino et al. 2015a) and can be applied to control the common ragweed. At this regard, a pathogenic fungus responsible for necrotic spots of *A. artemisiifolia* was previously isolated from naturally diseased leaves and stems, and identified as *Colletotrichum gloeosporioides* (Berestetskiy, unpublished).

Colletotrichum is a fungal genus comprising a large number of endophytic, saprophytic and plant pathogenic species. It is one of the most economically important plant pathogenic genera, responsible of anthracnose disease of fruits and leaves in a wide range of hosts, with conspicuous crop losses (Sutton 1992; Phoulivong et al. 2010). For this reason, species belonging to this genus have been subjected to extensive studies involving their pathogenesis, morphology, multigene analysis, physiology, host range and disease life cycle. Due to the severe diseases they cause, to the fast symptom appearance, and to their fast growth and easiness to produce conidia *in vitro*, species of this genus had been proposed for biological control of a number of important weeds, such as hemp sesbania (*Sesbania exaltata)* with *Colletotrichum truncatum* (Boyette et al. 1993), or velvetleaf (*Abutilon theophrasti*) by *Colletotrichum coccodes* (Wymore et al. 1988). Two *formae speciales* of *C. gloeosporioides*, namely *malvae* and *cuscutae*, where also commercialised in the past as mycoherbicides for the control of *Malva pusilla* (BioMal) and *Cuscuta* sp. (Lubao), respectively, demonstrating the potential of the genus for weed biocontrol (Committee on Mycoherbicides for Eradicating Illicit Drug Crops 2011).

Due to the pathogenic characteristics of these fungi, the studies on the production, identification and chemical and biological characterisation of their secondary metabolites received great attention. However, due to the size of the genus, counting hundreds of species and *formae speciales*, this enormous source of bioactive metabolites has been only partially explored (García-Paión and Collado 2003). Recently, extensive work carried out on *Colletotrichum higginsianum* led to the isolation from the mycelium of two new diterpenoid α-pyrones, named higginsianins A and B, showing antiproliferative activity against a panel of six cancer cell lines (Cimmino et al. 2015b). Furthermore, two new tetrasubstituted pyran-2-one and dihydrobenzofuran, named colletochlorins E and F, which showed phytotoxic activity on *Sonchus arvensis*, *Lemna minor* and *Phelipanche ramosa*, were isolated from the culture filtrates of the same fungus (Masi et al. 2017).

In preliminary observations, the ability of a strain of *C. gloeosporioides* to produce *in vitro* phytotoxic metabolites was ascertained (Zonno et al. 2015, 2016).

In the present study, thanks to the bioactivity-guided fractionation of the cultural organic extract, three metabolites were isolated and identified as colletochlorin A, orcinol and tyrosol. The manuscript reports their isolation, the evaluation of their phytotoxic activity and discusses the potential use in weed biological control. Furthermore, the complete NMR

spectroscopic characterisation and the absolute configuration assignment of colletochlorin A is also reported.

2. Results and discussion

The organic extract obtained from *C. gloeosporioides* culture filtrates was purified as reported in the Experimental section. Three pure metabolites, namely colletochlorin A, orcinol and tyrosol (**1–3**, Figure 1) were isolated and identified by comparison of their physic and spectroscopic (^{1}H NMR and MS) data with those previously reported in the literature (Kimura and Tamura 1973; Kosuge et al. 1973, 1974; Mori and Sato 1982; Capasso et al. 1992; Monde et al. 1998; Cimmino et al. 2017a).

The ^{1}H NMR spectrum of **1** was very similar to that of both the natural and synthetic colletochlorin A (Kosuge et al. 1974; Mori and Sato 1982). The chemical shifts of all colletochlorin A carbons were assigned for the first time based on the couplings observed in the HSQC and HMBC spectra (Berger and Braun 2004) as reported in Table 1. In particular, the coupling observed in the HSQC spectrum allowed to assign the signals resonating at δ 194.5, 121.6, 79.2, 35.9, 29.7, 26.9, 24.4, 22.4, 17.6 and 15.3 to the protonated carbons HCO, C-2′, C-6′, C-5′, C-4′, Me-9′, Me-8′, C-1′, Me-(C-3′), Me-(C-6), respectively, while those observed in the HMBC spectrum allowed to assign the quaternary carbons. Thus, the signals at 162.2, 157.4, 139.7, 136.8, 115.7, 115.4, 114.6 and 75.2 were assigned to C-2, C-4, C-6, C-3′, C-1, C-3, C-5 and C-7′, respectively. Furthermore, its ESI MS spectrum showed the sodium clusters at *m/z* 381 and 379 and the protonated forms at *m/z* 359 and 357 consistent with the presence of a chlorine atom.

Compound **1** was isolated for the first time in 1973 from culture filtrate of *C. nicotianae,* a pathogenic fungus that induce tobacco anthracnose (Kosuge et al. 1973) and recently from the culture filtrate of *C. higginsianum* (Masi et al. 2017). Its total synthesis was realised on

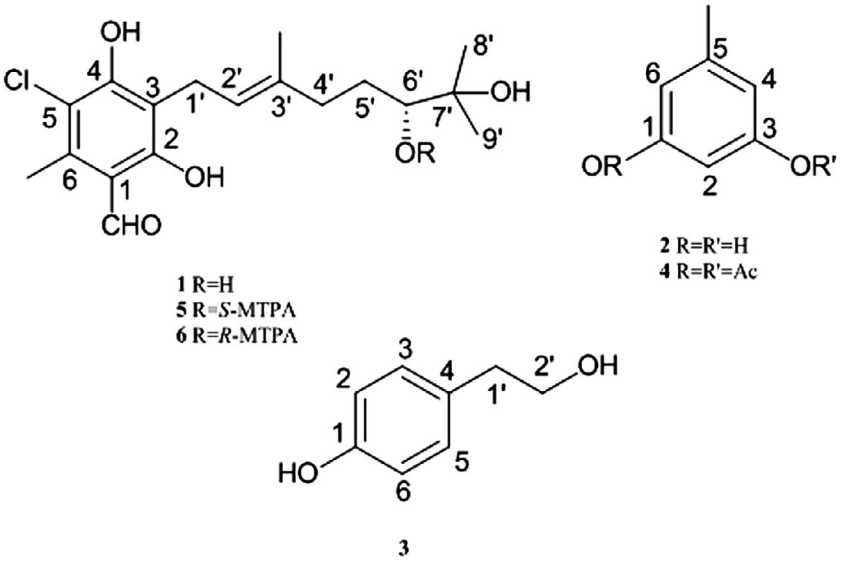

Figure 1. Structures of colletochlorin A (**1**), its 6′-*O*-*S*- and 6′-*O*-*R*-MTPA esters (**5** and **6**), orcinol (**2**), tyrosol (**3**) and 1,3-*O*,*O*′-diacetylorcinol (**4**).

Table 1. ^{1}H and ^{13}C NMR data and HMBC of colletochlorin A (**1**) and ^{1}H NMR data of Its Mosher's derivatives (**5** and **6**).[a,b]

	1			**5**	**6**
Position	δ_C[c]	δ_H (*J* in Hz)[d]	HMBC	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1	115.7 s		Me-6		
2	162.2 s		H_2-1′, HCO		
3	115.4 s		H_2-1′		
4	157.4 s		H_2-1′		
5	114.6 s		Me-6		
6	139.7 s		Me-6		
Me-(C-6)	15.3 q	2.60 (3H) s		2.660 (3H) s	2.656 (3H) s
1′	22.4 t	3.40 (2H) d (7.5)	H-2′	3.543 (2H) d (7.5)	3.541 (2H) d (7.5)
2′	121.6 d	5.27 (1H) t (7.5)	H_2-1′, Me-(C-3′)	5.346 (1H) t (7.5)	5.342 (1H) t (7.5)
3′	136.8 s		H_2-4′, H_2-5′, H_2-1′, Me-(C-3′), H-2′		
4′	29.7 t	1.32–1.73 (2H) m	H_2-5′	2.011 (2H) m	2.008 (2H) m
5′	35.9 t	2.01–2.43 (2H) m	H_2-4′, H-6′, H-2′, Me-(C-3′)	2.347 (2H) m	2.343 (2H) m
6′	79.2 d	3.32 (1H) dd (11.1, 3.1)	H_2-4′, H_2-5′, Me-8′, Me-9′	3.724 (1H) dd (11.1, 3.1)	3.724 (1H) dd (11.1, 3.1)
7′	75.2 s		H-6′, Me-8′, Me-9′		
Me-(C-3′)	17.6 q	1.80 (3H) s	H_2-4′	2.171 (3H) s	2.170 (3H) s
Me-8′	24.4 q[e]	1.16 (3H) s[e]	Me-9′	1.138 (3H) s	1.194 (3H) s
Me-9′	26.9 q[e]	1.13 (3H) s[e]	Me-8′	1.098 (3H) s	1.135 (3H) s
HCO	194.5 d	10.13 (1H) s		10.317 (1H) s	10.315 (1H) s
OH-2		12.68 (1H) s		12.528 (1H) s	12.523 (1H) s
OMe				3.645 (3H) s	3.644 (3H) s
Ph				7.726–7.368 (5H)	7.713–7.368 (5H)

[a]The chemical shifts are in δ values (ppm) from TMS.
[b]2D ^{1}H, ^{1}H (COSY) and 2D ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.
[c]Multiplicities were assigned by DEPT spectrum.
[d]These data are in according to those previously reported (Kosuge et al. 1974; Mori and Sato 1982).
[e]The attribution of these signals could be inverted.

1982, but only in racemic mixture (Mori and Sato 1982). Thus, the absolute configuration (AC) of **1** still remained to be assigned. To reach this goal the AC at C-6′ was determined by applying the modified Mosher's method (Ohtani et al. 1991; Cimmino et al. 2017b). Colletochlorin A was treated with *R*-(-)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) and *S*-(+)-MTPA chlorides, to convert **1** into the corresponding diastereomeric esters at C-6′ (**5** and **6**). The spectroscopic data for the *S*-MPTA and *R*-MTPA esters (**5** and **6**, respectively) were consistent with the structure assigned to **1**. The $\Delta\delta$ (**5–6**) values for all the protons were determined as reported in Figure 2, by subtracting the chemical shift of the protons (Table 1) of the 6′-*O*-*S*-MTPA (**5**) from that of 6′-*O*-*R*-MTPA (**6**) esters. The positive $\Delta\delta$ values were located on the right-hand side and those with negative values on the left-hand side of model A as previously reported (Ohtani et al. 1991; Cimmino et al. 2017b). This model allowed to assign the *R* configuration at C-6′. The assignment of AC to colletochlorin A represents a key part in understanding the mode of action of many secondary bioactive metabolites isolated from microorganisms, plants and marine organisms (Evidente et al. 2011, 2013). In fact the total syntheses of colletochlorin A, its enantiomer, its bromine and fluorine analogues and the corresponding colletorin A and its enantiomer are in progress with the aim to evaluate the structure-activity relationships.

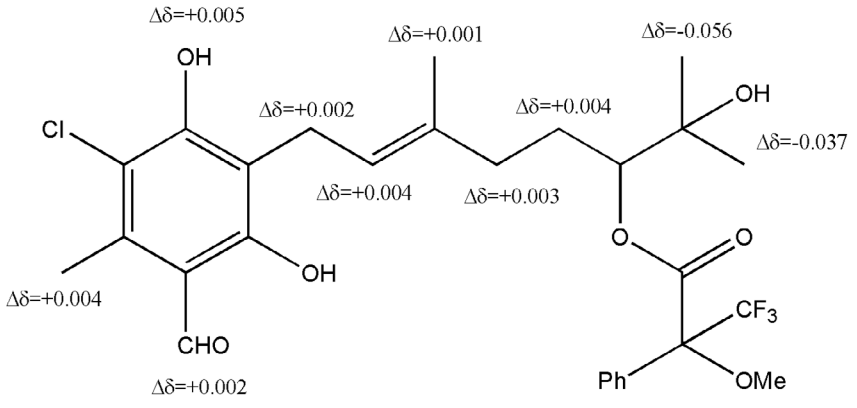

Figure 2. Structures of 6′-*O*-*S*- and 6′-*O*-*R*-MTPA of colletochlorin A (**5** and **6**, respectively), reporting the Δδ values obtained by comparison (**5**–**6**) of each proton system.

Colletochlorin A belong to the class of naturally occurring 2-prenyl orsellinaldehydes bearing a chlorine attached to the aromatic ring (Kosuge et al. 1973; García-Paión and Collado 2003).

The ^{1}H NMR spectrum of **2** was very similar to that reported in literature for orcinol (Monde et al. 1998). Its ESI MS spectrum showed the pseudomolecular $[M + H]^+$ ion at *m/z* 125. To confirm this structure **2** was also converted into the corresponding 1,3-*O,O′*-diacetil derivative (**4**, Figure 1), previously reported as a synthetic compound (Tangestaninejad et al. 2002). Its ^{1}H NMR spectrum differed from that of **2** mainly for the presence of the singlets of the two acetyl groups which appeared overlapped at δ 2.30 Its ESI MS spectrum showed the potassium $[M + K]^+$ and sodium $[M + Na]^+$ clusters at *m/z* 247 and 231, respectively.

The ^{1}H NMR spectrum of **3** was very similar to that reported in literature for tyrosol (Kimura and Tamura 1973; Capasso et al. 1992; Cimmino et al. 2017a). Its ESI MS spectrum showed the dimeric sodiated $[2 M + Na]^+$ form and the sodium cluster $[M + Na]^+$ at *m/z* 295 and 159, respectively.

Orcinol (Turner and Aldridge 1983; Monde et al. 1998) and tyrosol (Kimura and Tamura 1973; Capasso et al. 1992) are well known as bioactive fungal and plant metabolites. Tyrosol was also produced as phytotoxin from fungi pathogenic for some important agrarian crops (Evidente et al. 2010; Andolfi et al. 2012; Cimmino et al. 2017a) and legumes (Andolfi et al. 2013).

Assayed on punctured leaves, colletochlorin A caused the fast appearance of large necrosis on *A. artemisiifolia* leaves (Figure 3(A)). Tyrosol was weakly active, causing modest necrosis at the assayed concentration. Orcinol was completely inactive.

Assayed by uptake on ambrosia plantlets, colletochlorin A caused the wilting of the plants already 24 h after the transfer to water, with the appearance of large necrosis on the leaves margins, meaning an accumulation of the toxin due to the transpiration (Figure 3(B)). Conversely, the stem appeared only slightly damaged. In the same assay, orcinol was weakly active, causing only a slight withering, whereas tyrosol was inactive.

In the assay on *Lemna minor*, colletochlorin A caused a clear fronds browning, with a total reduction in chlorophyll content of around 70% (data not shown), whereas the other two compounds were completely inactive at the concentration tested.

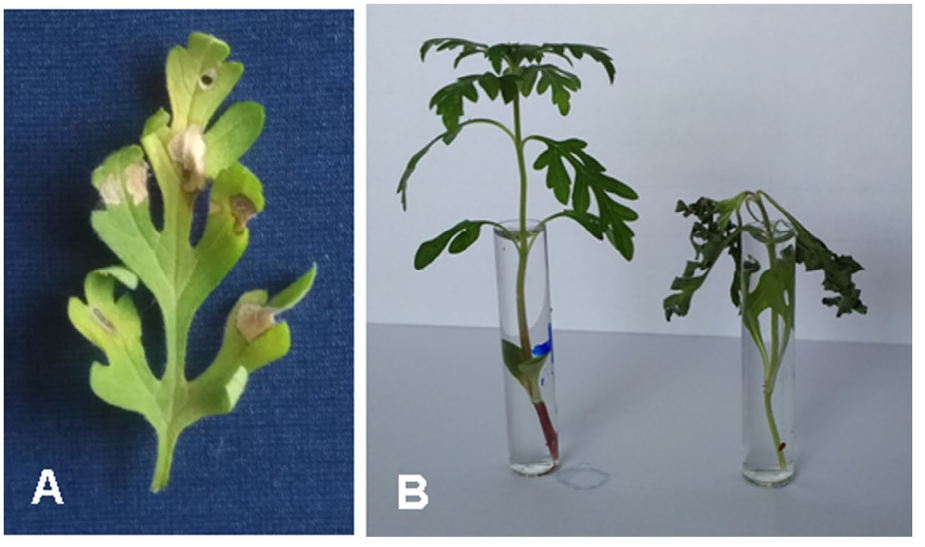

Figure 3. A: necrosis caused by colletochlorin A (**1**) on *A. artemisiifolia* leaves by leaf-puncture assay; B: wilting of ambrosia plantlet after treatment with colletochlorin A (**1**) (right) compared with the control (left).

In the antibiosis assay, colletochlorin A was active only against the gram + bacterium, *Bacillus subtilis*, inhibiting its growth as clearly shown by the inhibition halo around the diskette (data not shown), whereas was inactive against *Escherichia coli*. The other two compounds were inactive against both bacteria. No toxic effects were visible in the *Artemia salina* assay.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured in a MeOH solution on a Jasco P-1010 digital polarimeter (Tokyo, Japan); ^{1}H and ^{13}C NMR spectra were recorded at 400/100 or 500/125 MHz in $CDCl_3$ on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA), spectrometers, respectively, using the same solvent as internal standard. ESI MS was performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity, Milan, Italy). Analytical and preparative TLC was performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) and on reversed phase (Merck Kieselgel 60 RP-18, F_{254}, 0.20 mm) plates (Merck, Darmstadt, Germany). The spots were visualised by exposure to UV radiation (253), or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed using silica gel (Merck Kieselgel 60, 0.063–0.200 mm) (Merck).

3.2. Fungal strain

The fungus was isolated from diseased leaves of *A. artemisiifolia* and identified as *C. gloeosporioides* (Berestetskiy 2016). A monoconidial isolate was deposited in the culture

collection of both the All-Russian Research Institute of Plant Protection, Pushkin, Saint-Petersburg, Russia (MIF 13.14) and the Institute of Sciences of Food Production, Bari, Italy (ITEM 17400). The isolate was routinely grown and maintained in plates and slants containing potato-dextrose agar (PDA, Sigma-Aldrich, Chemie Gmbh, Buchs, Switzerland).

3.3. Production, extraction and purification of phytotoxic metabolites

The strain of *C. gloeosporioides* used in this study was grown on a minimal-defined liquid medium named M1-D (Pinkerton and Strobel 1976). Small plugs of mycelium obtained by colonies actively growing on PDA were used to seed 1 L Roux bottles containing 200 mL of the sterile medium above mentioned. Bottles were kept in still condition at 25 °C in the dark in an incubator for 4 weeks, then filtered to remove the mycelium and lyophilised. The lyophilised material, obtained by 11 L of culture filtrates was dissolved under stirring in 600 mL ultrapure water and extracted with EtOAc (3 × 600 mL). The organic extracts were then combined, dehydrated (Na_2SO_4), filtered and evaporated under reduced pressure, yielding a dark oily residue (285 mg). The organic residue was purified by silica gel column chromatography eluted with $CHCl_3$-*i*-ProOH (95:5) obtaining 10 groups of homogeneous fractions. The residue of the fifth fraction (14.3 mg) was further purified by preparative TLC on silica gel eluted with *n*-hexane-Me_2CO (6:4) yielding colletochlorin A (**1**, 10.4 mg, 0.94 mg/L) as a homogenous compound. The residue of the sixth fraction (16 mg) was further purified by preparative TLC on silica gel eluted with *n*-hexane-Me_2CO (6:4) yielding orcinol (**2**, 9.4 mg, 0.85 mg/L) as a homogenous compound. The residue of the eighth fraction (25.7 mg) was further purified by preparative TLC on silica gel, eluted with $CHCl_3$-*i*-ProOH (95:5) and by reverse phase TLC, eluted with Me_2CO-H_2O (8:2) yielding tyrosol (**3**, 12.3 mg, 1.11 mg/L) as white solid.

Colletochlorin A (**1**): amorphous solid; $[\alpha]_D^{25}$ + 6.24 (*c* 0.20) [In Ref. Kosuge et al. 1973: $[\alpha]_D^{25}$ + 11.6 (*c* 10.0)]; ^{1}H and ^{13}C NMR (400/100 MHz, in $CDCl_3$), are reported in Table 1; ESIMS (+) *m/z*: 381 $[M + 2 + Na]^+$, 379 $[M + Na]^+$, 359 $[M + 2 + H]^+$, 357 $[M + H]^+$. These data are in agreement with those previously reported (Kosuge et al. 1973**,** 1974; Mori and Sato 1982; Masi et al. 2017).

Orcinol (**2**): amorphous solid; ^{1}H NMR (500 MHz, in $CDCl_3$), *δ*: 6.77 (br s, H-4 and H-6), 6.36 (br s, H-2), 2.33 (s, Me-5). ESIMS (+) *m/z*: 125 $[M + H]^+$. These data are in agreement with those previously reported (Monde et al. 1998).

Tyrosol (**3**): white solid; ^{1}H NMR (500 MHz, in $CDCl_3$), *δ*: 7.20 (d, *J* = 8.0 Hz, H-3 and H-5), 6.80 (d, *J* = 8.0 Hz, H-2 and H-6), 4.90 (s, OH), 3.80 (t, *J* = 6.4 Hz, H_2-2′), 2.80 (t, *J* = 6.4 Hz, H_2-1′). ESI/MS (+), *m/z* 295 $[2\ M + Na]^+$, 159 $[M + Na]^+$. These data are in agreement with those previously reported (Kimura and Tamura 1973; Capasso et al. 1992; Cimmino et al. 2017a).

1,3-*O,O*′-Diacetylorcinol (**4**). Orcinol (**2**, 1.4 mg), dissolved in pyridine (20 µL) was acetylated with acetic anhydride (20 µL) at room temperature. After 24 h the reaction was stopped by addition of MeOH and the azeotrope formed by addition of C_6H_6 was dried under reduced pressure. The residue (1.2 mg) was purified by preparative TLC using $CHCl_3$ as eluent to yield 1,3-*O,O*′-diacetylorcinol (**4**, 1.2 mg). Compound **4** had: ^{1}H NMR (500 MHz, in $CDCl_3$), *δ*: 6.82 (br s, H-4 and H-6), 6.73 (br s, H-2), 2.30 (s, Me-5), 2.19 (s, OAc-1 and OAc-2). ESIMS (+), *m/z*: 247 $[M + K]^+$ and 231 $[M + Na]^+$.

6′-*O*-(*S*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of colletochlorin A (**5**). (*R*)-(-)-MPTA-Cl (20 µL) was added to **1** (1.9 mg) dissolved in dry pyridine (40 µL). The mixture was kept at room temperature for 1 h and then the reaction was stopped adding

MeOH and the azeotrope formed by addition of C_6H_6 was dried under reduced pressure. The residue (2.1 mg) was purified by preparative TLC, eluted with $CHCl_3$-*i*-PrOH (98:2), yielding **5** (1.0 mg) as a homogeneous oil. It had: ^{1}H NMR spectrum see Table 1; ESIMS (+) *m/z* 471 $[M + 2 + H]^+$ and 469 $[M + H]^+$.

6′-*O*-(*R*)-α-Methoxy-α-trifluoromethyl-α-phenylacetate (MTPA) ester of colletochlorin A (**6**). (*S*)-(+)-MPTA-Cl (20 µL) was added to **1** (1.9 mg) dissolved in dry pyridine (40 µL). The reaction was carried out under the same conditions used for preparing **5** from **1**. The purification of the crude residue (2.0 mg) by preparative TLC eluted with $CHCl_3$-*i*-PrOH (98:2), to give **6** (0.9 mg) as homogeneous oil. It had: ^{1}H NMR spectrum see Table 1; ESIMS (+) *m/z* 471 $[M + 2 + H]^+$ and 469 $[M + H]^+$.

3.4. Biological assays

The three metabolites were first dissolved in MeOH and then diluted with distilled water to the desired concentration.

3.4.1. Leaf-puncture assay

The toxins were tested at 2 µg/µL on leaves of *A. artemisiifolia*. Droplets (20 µL) of the solution containing the compounds were applied to young detached leaves previously punctured with a needle. Five replications were used for each compound. Leaves were kept in a moistened chamber under continuous fluorescent lights at 25 °C. The eventual appearance of symptoms was observed three days after droplet application. Control treatments were carried out by applying droplets containing only water with the MeOH.

3.4.2. Cut plant assay

Young ambrosia plants (around 1 month old) grown in growth chamber were cut and the stem (having 5–6 leaves) immersed in the solutions (10^{-3} M) for 24 h. Plants were then immersed in water for 48 h before observing symptom appearance.

3.4.3. Lemna minor assay

The compounds were tested on *Lemna minor* using a protocol previously described (Vesonder et al. 1992) at a concentration of 2 µg/µL using 100 µL per each pot. Chlorophyll content was determined as reported and expressed as percentage reduction in comparison to the untreated fronds (Vurro et al. 2006).

3.4.4. Antibiosis assay

A disc assay on both one Gram+(*Bacillus subtilis*) and one Gram- (*Escherichia coli*) bacterium was carried out by following the procedure already described (Bottalico et al. 1990). Metabolites were assayed up to 150 µg per diskette.

3.4.5. Zootoxic assay

This assay was performed on *Artemia salina* (brine shrimps) by following the procedure already described by Bottalico et al. (1990). A concentration of 70 µg/well was used for each compound. A negative control was also assayed (solvent in water).

4. Conclusions

Three metabolites were isolated for the first time from the culture filtrates of *C. gloeosporiodes* and identified as the already known colletochlorin A, orcinol and tyrosol. Among them colletochlorin A was significantly phytotoxic against the host plant *A. artmisiifolia* showing also activity against *L. minor* associated to only inhibitory activity against gram + bacteria. Colletochlorin A was only recently reported, from some of the authors, for its modest phytotoxic activity against *Sonchus arvevsis* (Masi et al. 2017). Considering the lack of zootoxicity and the activity against gram- bacteria, colletochlorin A appears to be a potential bioherbicide against *A. artemisiifolia*. Thus, the development of its total enatioselective synthesis, now in progress, represents an alternative for its large-scale preparation in respect to the fermentative production.

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Disclosure statement

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