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Phytotoxic activity against *Bromus tectorum* for secondary metabolites of a seed-pathogenic *Fusarium* strain belonging to the *F. tricinctum* species complex

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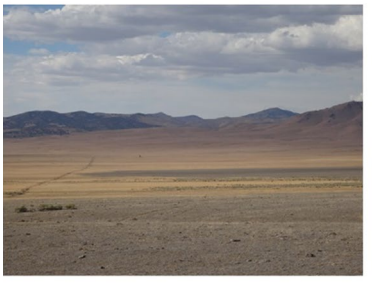
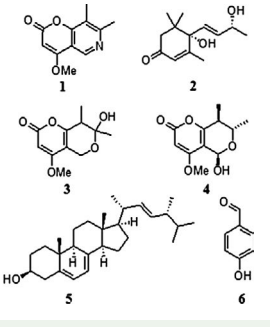
ABSTRACT

The winter annual grass *Bromus tectorum* (cheatgrass) has become highly invasive in semiarid ecosystems of western North America. In these areas, a natural phenomenon, complete cheatgrass stand failure ('die-off'), is apparently caused by a complex interaction among soilborne fungal pathogens. Several *Fusarium* strains belonging to the *Fusarium tricinctum* species complex were isolated from these soils and found to be pathogenic on *B. tectorum* seeds. One of these strains was produced in cheatgrass seed culture to evaluate its ability to produce phytotoxins. Six metabolites were isolated and identified by spectroscopic methods (essentially 1D and 2D NMR and ESIMS) as acuminatopyrone (**1**), blumenol A (**2**), chlamydosporol (**3**), isochlamydosporol (**4**), ergosterol (**5**) and 4-hydroxybenzaldehyde (**6**). Upon testing against *B. tectorum* in a seedling bioassay, (**6**) the coleoptile and radicle length of cheatgrass seedlings were significantly reduced. Compounds **1** and **2** showed moderate activity, while **3**–**5** were not significantly different from the control.





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1. Introduction

Bromus tectorum (cheatgrass, downy brome) is an exotic winter annual grass weed that causes serious losses in intensive agriculture and is also a major problem on semi-arid rangelands in the western U.S.A. (Knapp 1996; Brooks et al. 2004). It has become highly invasive in western North America, forming monocultures across millions of hectares of former shrubland. A frequent but poorly understood phenomenon in these heavily invaded areas is periodic 'die-off' or complete stand failure of this weed. Cheatgrass stand failure is apparently caused by a complex interaction among multiple soilborne fungal pathogens (Meyer et al. 2016). *B. tectorum* seed bank dynamics, seedling emergence and seed production are influenced by five principal soilborne pathogens: *Ustilago bullata* Berk. (head smut pathogen) (Meyer et al. 2010), *Tilletia bromi* (Brockm.) Nannf. (chestnut bunt pathogen) (Castlebury et al. 2005), *Pyrenophora semeniperda* (Brittlebank & Adams) Shoemaker (black fingers of death pathogen) (Baughman & Meyer 2013; Meyer et al. 2015), *Fusarium* Link sp. n. (*Fusarium* seed rot pathogen) (Meyer et al. 2014) and a new species in the Rutstroemiaceae (bleach blonde syndrome pathogen) (Meyer et al. 2016). These fungal pathogens have been studied to understand how they interact with abiotic factors, with the soil microbial community, with their hosts, and with each other to impact stand dynamics in *B. tectorum* and specifically to cause stand failure (Meyer et al. 2016). However, the ability of these pathogens to produce phytotoxins as well as the role of secondary metabolites in fungal community ecology on *B. tectorum* need to be investigated. These studies might help elucidate the 'die-off' phenomenon.

Considerable work has been done in this field for *P. semeniperda*, which has been proposed as a potential biocontrol agent for *B. tectorum* (Meyer et al. 2013). This fungus produced large quantities of cytotoxic cytochalasin B when grown in wheat and cheatgrass seed culture, along with smaller quantities of the related compounds cytochalasins A, F and deoxaphomin (Masi et al. 2014a). Three new phytotoxic sesquiterpenoids named pyrenophoric acid and pyrenophoric acids B and C were also isolated, together with the known abscisic acid, and characterised as three new penta-2,4-dienoic acids by spectroscopic methods (NMR and HR ESIMS) (Masi et al. 2014b, 2014c). The phytotoxicity of these metabolites was evaluated in cheatgrass seedling bioassays. All these compounds induced growth suppression of both radicles and coleoptiles, but only abscisic acid had an effect on germination per se. When the same fungus was grown on potato dextrose broth medium, a new γ-lactam named spirostaphylotrichin W was isolated, together with the well-known spirostaphylotrichins A, C, D, R, V and triticone E. These compounds had little effect on cheatgrass seedlings but some of them caused necrosis in leaf puncture bioassays, suggesting that their production by *P. semeniperda* may be a legacy of its descent from a group of primarily foliar pathogens (Masi et al. 2014d).

Fusarium is a large genus of filamentous fungi which are ubiquitous in soils worldwide including many important pathogens of cultivated plants, particularly vegetables and winter cereal crops (Nelson et al. 1981). The occurrence of *Fusarium* species in natural ecosystems is also frequently reported, but its role in the microbial ecology of these ecosystems is much less well documented (Walsh et al. 2010). Many species of *Fusarium* are able to produce bioactive secondary metabolites which belong to different classes of natural compounds (Chelkowski 2014). Recently, some endophytic strains were studied for their ability to produce bioactive metabolites and some new compounds were isolated for the first time (Bashyal &

Leslie Gunatilaka 2010; Dame et al. 2016; Liang et al. 2016; Shen et al. 2016). Several *Fusarium* strains pathogenic on *B. tectorum* seeds have been isolated from *B. tectorum* die-off soils (Meyer et al. 2014). Molecular–genetic characterisation using both EF1 (translation elongation factor) and RPB1/RPB2 (RNA polymerase II largest and second-largest subunit) sequence data led to their classification as one or more undescribed members of the *Fusarium tricinctum* species complex (FTSC) (O'Donnell et al. 2013; Franke et al. 2014). To evaluate the ability to produce phytotoxic metabolites, one of these strains was grown for the first time on *B. tectorum* seed solid culture. This manuscript reports on the isolation and identification of secondary metabolites produced by *Fusarium* strain Eden-C5 of the FTSC in this cultural condition and on the evaluation of the phytotoxic activity of these metabolites against *B. tectorum*.

2. Results and discussion

The strain Eden-C5 produced in *B. tectorum* seed solid culture was extracted as reported in detail in the Experimental section. The organic extract, showing phytotoxic activity, was purified using different steps of column chromatography and TLC yielding six pure compounds (**1**–**6**, Figure 1).

The preliminary ^{1}H and ^{13}C NMR investigation of the first compound showed signal systems which were very similar to those reported in literature for acuminatopyrone (**1**, Figure 1) a compound previously isolated from *F. tricinctum* of the FTSC (Visconti et al. 1994). This result was also confirmed by ESI-TOF MS, recorded in positive modality, which showed the pseudomolecular ion $[M + H]^+$ at *m/z* 206, and by comparison of other spectroscopic data of **1** (UV and IR) with those reported in literature (Visconti et al. 1994). Compound **1** was also isolated from *Fusarium acuminatum* of the FTSC (Grove & Hitchcock 1991). Recently, it was also isolated from the fermentation broth of the wood-decay fungus *Xylaria allantoidea* BCC 23163 (Isaka et al. 2014). However, no biological activity has been reported.

Compound **2** (Figure 1) showed ^{1}H and ^{13}C NMR and optical rotation data very similar to those reported in literature for the pentasubstituted cyclohexenone blumenol A (Masuoka et al. 2003). This identification was supported by the data of its ESI-TOF MS which showed the sodium dimeric form $[2 M + Na]^+$ and the sodium cluster $[M + Na]^+$ at *m/z* 471 and 247, respectively. Furthermore, the pseudomolecular ion, by loss of H_2O, generated the ion $[M + H + H_2O]^+$ at *m/z* 207. The other spectroscopic data (UV and IR) of **2** were very similar to those

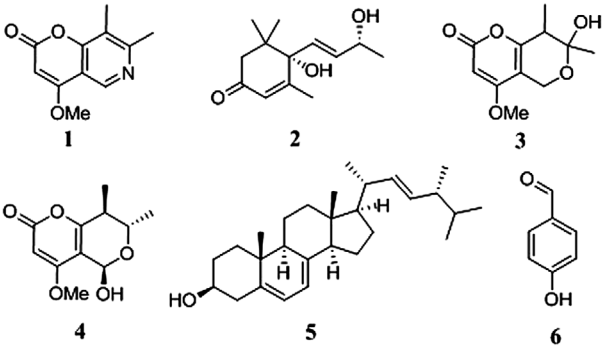

Figure 1. Structures of metabolites **1**–**6**.

reported in literature for blumenol A when it was first isolated from the plant *Podocarpus blumei* (Galbraith & Horn 1972). Its absolute stereochemistry, as well as that of some analogues, was determined by chiroptical properties (Weiss et al. 1973). Our CD data were in accord with the literature, confirming the (+)-(6*S*,9*R*) configuration (Weiss et al. 1973). Compound **2** was also isolated from other plants such as *Rauwolfia vomitoria* (Pousset & Poisson 1969), *Croton sparsiflorus* (Satish & Bhakuni 1972) and *Vinca rosea* (Bhakuni et al. 1974). This is the first report of the production of blumenol A (**2**) by a *Fusarium* sp., but it has been isolated from other fungi such as the endophyte *Colletotrichum gloeosporioides* GT-7 (Wei et al. 2016) and from the carnivorous fungus *Arthrobotrys entomophaga* (Wu et al. 2013). Many natural products are known to be produced by both plants and fungi (e.g. Strobel et al. 1996, Rehman et al. 2008). Plant hormones such as abscisic acid and (1*R*,2*R*)-(−)-jasmonic acid are also produced by different species of fungi (Andolfi et al. 2014; Masi et al. 2014c). Compound **2** showed weak cytotoxic activity when tested against human solid tumour cells (Liu et al. 1999), and phytotoxicity indicated by inhibition of the growth of cress (*Lepidium sativum* L.) roots and shoots (Kato-Noguchi et al. 2012).

Compound **3** (Figure 1) was isolated as an inseparable epimeric mixture in a ratio 1:1 (as deduced from ^{1}H NMR) and identified as chlamydosporol (**3**, Figure 1) by comparing its spectroscopic data (^{1}H NMR, UV, IR and ESI-TOF MS) with those reported in literature (Abbas et al. 1992). Compound **3** has been isolated from different *Fusarium* species (Savard et al. 1990; Abbas et al. 1992; Solfrizzo et al. 1994) and is considered a mycotoxin because it causes food refusal and weight loss in rats, cytotoxic effects to cultured mouse and human fibroblast cells and mortality to chick embryos (10 to 70%) over a concentration range from 0.5 mg to 4 mg per egg (Abbas et al. 1992).

Compound **4** was identified as the known metabolite isochlamydosporol by comparing its ^{1}H and ^{13}C NMR spectra, as well as its UV and IR data, with those reported in literature for the same metabolite produced by some species of *Fusarium* (Solfrizzo et al. 1994) and by the ascomycetous fungus *Tolypocladium inflatum* (SCK6-CP14) (Lin et al. 2011)**.** Its ESI-TOF MS, recorded in positive modality, consistently showed the pseudomolecular ion $[M + H]^{+}$ at *m/z* 227. No biological activities were reported for this compound.

Using the above-cited techniques, compounds **5** and **6** (Figure 1) were identified as the well known ergosterol (Ying et al. 2013) and 4-hydroxybenzaldehyde (Ren-Yi et al. 2015), respectively. Compound **5** is a component of yeast and other fungal cell membranes, serving many of the same functions that cholesterol serves in animal cells (Weete et al. 2010). In fact, given its protective role against mechanical and oxidative stress, ergosterol may have evolved as a fungal alternative to cholesterol in response to the selective pressure induced by drying/wetting cycles occurring in fungal habitats (Dupont et al. 2012). Its function in the secretome of *Fusarium* strain Eden-C5 is unknown, as ergosterol is not commonly secreted.

Compound **6** (4-hydroxybenzaldehyde) is a natural product produced by several classes of fungi as well as some insects and plants (Venkatasubbaiah et al. 1991). Its phytotoxicity was reported for the first time when it was isolated from *Botryosphaeria obtusa* inducing black rot of apple fruit and frogeye leaf spot (Venkatasubbaiah et al. 1991). This was later confirmed when the compound was isolated from *Phaeoacremonium aleophilum* and *Fomitiporia mediterranea*, two fungi associated with grapevine trunk diseases (Andolfi et al. 2011). Recently it was also isolated from *Diaporthe gulyae*, a fungus proposed as a mycoherbicide for saffron thistle (*Carthamus lanatus*) biocontrol (Andolfi et al. 2015), and its inhibitory

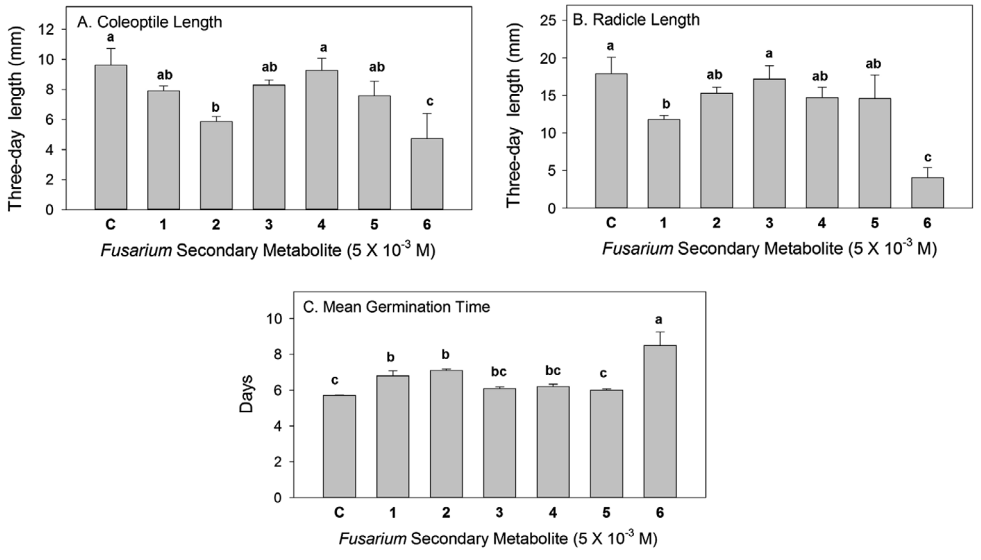

Figure 2. Results of *B. tectorum* seedling bioassays with acuminatopyrone (**1**), blumenol A (**2**), chlamydosporol (**3**), isochlamydosporol (**4**), ergosterol (**5**) and 4-hydroxybenzaldehyde (**6**) produced by the seed-pathogenic *Fusarium* strain Eden-C5 tested at a concentration of 5×10^{-3} M in 2% DMSO for: (A) mean three-day coleoptile length, (B) mean three-day radicle length, and (C) mean germination time. Control seeds treated with 2% DMSO designated as C. Error bars represent standard error of the mean ($n = 3$). For each response variable, bars headed by the same letter are not significantly different at $P < 0.05$ according to a means separation test from analysis of variance.

effect on seed germination was determined on wheat (*Triticum aestivum*) and radish (*Raphanus sativus*) (Ren-Yi et al. 2015).

Compounds **1–6** were bioassayed on *B. tectorum* seedlings, as reported in detail in the Experimental section. The bioassay results include mean germination time (days) as well as radicle and coleoptile length three days post-germination following treatment with **1–6** (Figure 2). Compound **6** (4-hydroxybenzaldehyde) proved to be the most phytotoxic of the six compounds. It increased mean germination time from 5.7 to 8.5 days relative to the control, reduced coleoptile length by half, and decreased radicle length by a factor of four. Acuminatopyrone (**1**) and blumenol A (**2**) showed moderate activity. Both compounds slightly but significantly increased mean germination time relative to the control. In addition, **1** significantly reduced radicle elongation while **2** significantly reduced coleoptile elongation. The other three compounds (**3–5**) were not significantly different from the control for any of the response variables.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured in $CHCl_3$ on a Jasco P-1010 digital polarimeter (Tokyo, Japan); IR spectra were recorded as glassy film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer (Madison, WI, USA); UV spectra were measured in MeCN or MeOH on a Jasco V-530 spectrophotometer (Tokyo, Japan); CD spectra were measured in MeCN on

a Jasco J-815 spectropolarimeter (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) or Varian INOVA (Palo Alto, CA, USA) spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra. ESI-TOF spectra were recorded on an Agilent 6230 TOF LC/MS instrument (Santa Clara, CA, USA). Analytical and preparative TLC were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively) plates (Merck, Darmstadt, Germany). The spots were visualised by exposure to UV radiation (254, or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH.

3.2. Fungus

Fusarium strain Eden-C5 was isolated from a killed *B. tectorum* seed that had been planted into cheatgrass die-off soil from Eden Valley, Nevada USA (Meyer et al. 2014). Pure cultures were maintained on potato dextrose agar (PDA, Fluka, Sigma-Aldrich Chemic GmbH, Buchs, Switzerland) and stored at 4 °C in the strain collection of S. Meyer at the USFS RMRS Shrub Sciences Laboratory, Provo UT, USA.

3.3. Production, extraction and purification of metabolites

To produce solid culture on *B. tectorum* seeds, 500 mL of actively growing culture in potato dextrose broth (PDB) was thoroughly mixed along with 200 mL of fresh medium with 1 kg soaked, autoclaved *B. tectorum* seeds, and the mixture was spread into sterile aluminum pans with plastic lids and incubated at 22 °C in a sterile hood for four weeks. The culture was then air-dried for at least several weeks prior to extraction. The dried material was minced using a laboratory mill and extracted with 1 L of MeOH–H_2O (1% NaCl) (55:45). The mixture was centrifuged for 1 h at 10,000 rpm. The pellet was extracted again with the same solvent mixture in the same conditions, and the two supernatants were then pooled (pH 5.5), defatted by *n*-hexane (2 × 500 mL) and extracted with CH_2Cl_2 (3 × 500 mL). The CH_2Cl_2 organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure to yield a brown solid residue (908.3 mg) that showed significant phytotoxic activity in a *B. tectorum* seedling bioassay. It was then subjected to bioassay-guided fractionation through column chromatography (750 × 30 mm) on silica gel, eluted with $CHCl_3$-*i*-PrOH (95:5). Thirteen fractions were collected and pooled on the basis of similar TLC profiles. The residue of the second fraction (33.3 mg) was further purified by TLC eluted with $CHCl_3$-*i*-PrOH (95:5) yielding ergosterol (**5**, 6.18 mg, R_f: 0.46 $CHCl_3$-*i*-PrOH, 95:5 and 0.84 EtOAc-*n*-hexane, 6:4) as a white amorphous solid. The residue of the third fraction (74.23 mg) was further purified by column chromatography (350 × 25 mm) on silica gel eluted with $CHCl_3$-*i*-PrOH (95:5) yielding three homogeneous fractions. The residue of the third fraction of this latter column (8.02 mg) was purified by TLC eluted with petroleum ether-Me_2CO (75:25) yielding 4-hydroxybenzaldehyde (**6**, 3.79 mg, R_f: 0.76 EtOAc-*n*-hexane, 6:4 and 0.54 petroleum ether-Me_2CO, 75:25) as a homogeneous amorphous solid. The residue of the fourth fraction of the first column (36.3 mg) was further purified by TLC eluted with *n*-hexane-Me_2CO (65:35) yielding acuminatopyrone (**1**, 2.53 mg, R_f: 0.30 petroleum ether-Me_2CO, 75:25), chlamydosporol (**3**, 1.02 mg, R_f: 0.33 $CHCl_3$-*i*-PrOH, 95:5 and 0.20 EtOAc-*n*-hexane, 6:4) and isochlamydosporol (**4**, 1.06 mg, R_f: 0.26 $CHCl_3$-*i*-PrOH, 95:5 and 0.16 EtOAc-*n*-hexane, 6:4) as amorphous solids. The residues of the ninth and tenth fractions were combined (50.1 mg) and further purified first by TLC eluted

with EtOAc-*n*-hexane (6:4) and then by TLC on reverse phase eluted with MeCN-H_2O (1:1) yielding blumenol A (**2**, 2.72 mg, R_f: 0.27 $CHCl_3$-*i*-PrOH, 9:1) as a homogeneous oil.

3.4. Identification of the fungal metabolites

The spectroscopic and physic data of compounds **1–6** are provided as supplementary material.

3.5. Bromus tectorum *Seedling Bioassay*

Acuminatopyrone (**1**), blumenol A (**2**), chlamydosporol (**3**), isochlamydosporol (**4**), ergosterol (**5**) and 4-hydroxybenzaldehyde (**6**) were first dissolved in DMSO, and then brought up to the assay concentration of 5×10^{-3} M with distilled water (the final concentration of DMSO was 2%). For each compound, 250 µL of the solution was pipetted into each of three 3.5 cm Petri dishes onto the surface of one filter paper. Seeds were incubated in 2% DMSO in the control treatment. Four host seeds from a 2010 bulk collection collected in a die-off-prone area in Skull Valley, Utah (Baughman & Meyer 2013) were arranged onto the surface of each filter paper in a pattern that made it possible to track individual seeds. Petri dishes were sealed with parafilm to retard moisture loss and incubated at 20 °C with a 12:12 h photoperiod. Germination was scored each day, and germination day was tracked individually for each seed. Three days after germination for each seed, the coleoptile and radicle length of each seedling were measured and recorded using electronic calipers. All seeds germinated within 14 days. Seeds that produced a radicle but no coleoptile were scored with a coleoptile length of zero.

3.6. Statistical Analysis

Analysis of variance (ANOVA) using a mixed model procedure (SAS Proc MIXED; Statistical Analysis Institute, Boca Raton, FL, USA) for a randomised block design was used to compare differences among the six compounds and the control in terms of mean seed germination time and coleoptile and radicle length three days post-germination. A Petri dish was considered as a block replicate for a total of three blocks. Response variables were log-transformed to meet the assumptions of ANOVA prior to analysis. The LSMeans option in Proc MIXED was used to generate means separations for each of the three response variables.

4. Conclusions

These results demonstrate that phytotoxic metabolites produced by *Fusarium* strain Eden-C5 when grown in solid culture on *B. tectorum* seeds could play some part in pathogenesis on seeds of this species in the field. However, the biological functions of these compounds and their role in interactions both with the host and with other soilborne fungal pathogens involved in the cheatgrass 'die-off' phenomenon remain largely unknown. *Fusarium* species produce numerous toxins that are involved in pathogenesis (O'Donnell et al. 2013). The strain we examined produced several toxins previously known from the FTSC as discussed above, helping to confirm its taxonomic placement in this group, but we did not detect mycotoxins previously reported from members of the FTSC, e.g. enniatins. This could be due to cultural

conditions or to an inherent inability to produce these compounds. Genomic characterisation of our strain, currently in progress, will enable us to determine whether it has the gene cluster responsible for enniatin biosynthesis. Fungal pathogens that can cause *B. tectorum* emergence failure have potential for biocontrol of this invasive weed. It is important to know whether *Fusarium* strains selected for biocontrol development are capable of producing mycotoxins that could impact human or animal health.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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