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Effect of cultural conditions on the production of radicinin, a specific fungal phytotoxin for buffelgrass (*Cenchrus ciliaris*) biocontrol, by different *Cochlioboulus australiensis* strains

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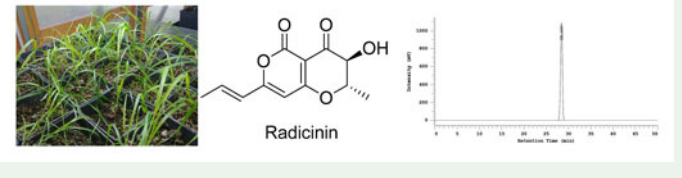
ABSTRACT

Radicinin is a phytotoxic fungal dihydropyranopyran-4,5-dione under evaluation for the development of a target-specific bioherbicide for invasive buffelgrass (*Cenchrus ciliaris*) control. It has already demonstrated high toxicity on host plants, low toxicity to native plants and no negative effects on zebrafish embryos. To continue these studies at the whole-plant level there is a need to obtain much larger quantities of radicinin, either by optimizing its large-scale production by fungal fermentation or through its total stereoselective synthesis. A rapid and sensitive HPLC method for quantification of radicinin in complex mixtures has been developed in order to evaluate its production by different *Cochliobolus australiensis* strains and in different cultural conditions. The analysis proved that radicinin is not produced by all the strains tested and its synthesis is strongly affected by cultural conditions. The HPLC method could be useful in selecting the best fungal source for the production of this promising potential bioherbicide.



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1. Introduction

Buffelgrass (*Pennisetum ciliare* or *Cenchrus ciliaris*) is a perennial grass native to the Old World (Abella et al. 2012) that was intentionally introduced in many semiarid regions

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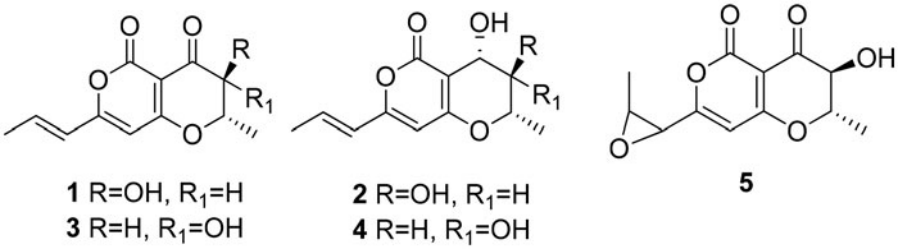

Figure 1. The structures of radicinin (**1**), radicinol (**2**), 3-epiradicinin (**3**), 3-epiradicinol (**4**) and cochliotoxin (**5**).

as a pasture grass (Marshall, Lewis, and Ostendorf 2012). Unfortunately, it has become highly invasive in North America negatively affecting the native vegetation and altering the wildfire regime. The increased fire frequency in the Sonoran Desert of southern Arizona is a real problem for the iconic saguaro cactus and other cacti (Burquez-Montijo et al. 2002; Bowers et al. 2006; Stevens and Falk 2009; Olsson et al., 2012). Few tools are available for buffelgrass control and are essentially based on the use of broad-spectrum herbicides which have a negative environmental and ecological impact (Singh et al. 2006). Phytotoxins produced by weed-pathogenic fungi could be an effective alternative for the design of a natural and safe bioherbicide to manage buffelgrass (Dayan and Duke 2014; Gerwick and Sparks 2014; Cimmino et al. 2015). Thus, the foliar pathogens that commonly occur on buffelgrass in the invaded North American range have been screened for their ability to produce phytotoxic metabolites that could potentially be used as natural herbicides. From the liquid culture of *Pyricularia grisea*, two monosubstituted hex-4-ene-2,3-diols, named pyriculins A and B, were isolated together with (10*S*,11*S*)-(−)-epipyriculol, *trans*-3,4-dihydro-3,4,8-trihydroxy-1(2*H*)-napthalenone, and (4*S*)-(+)-isosclerone (Masi, Meyer, Górecki et al. 2017). From the liquid culture of *Cochliobolus australiensis*, the known radicinin (**1**, Figure 1), radicinol, and their 3-epimers (**2**–**4**, Figure 1) were isolated together with a new phytotoxin which was named cochliotoxin (**5**, Figure 1) (Masi, Meyer, Clement, Cimmino et al. 2017). Successively from the same cultures, two new tetrasubstituted 3-chromanonacrylic acids, named chloromonilinic acids C and D, were also isolated along with the known chloromonilicin and chloromonilinic acid B (Masi, Meyer, Clement, Pescitelli et al. 2017). These fourteen secondary metabolites were evaluated for their potential use as target-specific bioherbicides by testing their phytotoxicity against *C. ciliaris* and six non-target native species (*Digitaria californica*, *Heteropogon contortus*, *Baileya multiradiata, Stanleya pinnata*, *Encelia farinosa* and *Lepidium fremontii*) in leaf puncture bioassays. **1** and (10S,11S)-(−)-epipyriculol showed high toxicity on buffelgrass at 2.5×10^{-3} M but greatly reduced toxicity on native species. At a lower concentration (10^{-3} M), radicinin showed high toxicity on *C. ciliaris* but had no negative effect at all on native species, whereas (10S,11S)-(−)-epipyriculol toxicity did not differ as markedly between buffelgrass and native species. Furthermore, no teratogenic, sublethal or lethal effects were observed for **1** on zebrafish (*Brachydanio rerio*) embryos (Masi et al. 2019). Thus, **1** is now under consideration for development of a target-specific bioherbicide for use against buffelgrass in natural systems where synthetic herbicides cause excessive damage to natives. However, to continue these studies at the whole-plant

Table 1. Analytical characteristics of the calibration curve[a] for radicinin (**1**) quantification.

Rt (min)	Range (μg)	R^2	Number of data points	Detection limit (μg)
28.437	0.001 - 5	0.9998	21	0.001

[a]Calculated in the form y = a + bx, where y is the chromatographic peak area and x is the μg of radicinin. The calibration curve for this analysis run was: y = 1E + 07x + 247112.

level and under greenhouse and field conditions there is a need to obtain much larger quantities of radicinin, either by optimizing its large-scale production by fungal fermentation or through total stereoselective synthesis. A rapid and sensitive high-pressure liquid chromatography method for quantification of radicinin in complex mixtures has been developed in order to select the best fungal source and the optimal growth conditions. This manuscript reports on this method and its application to evaluate the radicinin production by different *C. australiensis* strains and in different cultural conditions.

2. Results and discussion

To quantify radicinin (**1**, Figure 1) in complex mixtures an HPLC method was optimized as reported in the Experimental section. Construction of the HPLC calibration curve (Table 1) for quantitative **1** determination was performed with absolute amounts of radicinin standard dissolved in MeOH in the range between 250 and 1 μg/mL, in triplicate for each concentration. The retention times were highly reproducible, varying less than 0.500 min. A linear regression curve (absolute amount against chromatographic peak area) for **1** was obtained based on weighted values calculated for 7 concentrations of the standard in the above range. The quantitative determination of the metabolite was calculated by interpolating the mean area of the chromatographic peak using the equation from the calibration curve (Table 1). The chromatographic profile of **1** at a concentration of 250 μg/mL is reported in Figure 2. The limit of detection (LOD) was extrapolated from the calibration graphics according to the guidelines provided by IUPAC (Armbruster and Pry 2008) while the validation of the HPLC method (in terms of limit of quantitation (LOQ), intra-and inter-assay precision, and accuracy) was achieved following the rules reported in the "Guidance for Industry-Bioanalytical Method Validation" of the Food and Drug Administration (FDA, USA) (Epifano et al. 2018).

This method has been successfully applied to quantify **1** in the organic extracts of six strains of *C. australiensis* as reported in the Experimental section. The strains, the culture conditions and the yield of extraction are reported in Table 2. The presence of **1** in all the extracts was first ascertained by TLC analysis and then confirmed by HPLC also by co-injection with the standard. The peak of **1** in the chromatograms was almost coincident to the 28.437 min retention time of the standard and the radicinin content in the fungal organic extracts is reported in Table 2. The quantity of radicinin detected varied among the strains grown on PDB. In particular, **1** was not detected in the organic extracts of LJ3B1 and LJ3C1 strains and very low amounts were produced by 2MG2F, LJ3B2 and SNM4C1 strains, while the maximum production of **1** (26.20 ± 0.32 mg/L) was obtained for the strain LJ4B. Thus, this strain was also grown in two other liquid media and on wheat seed solid culture to evaluate their effect on

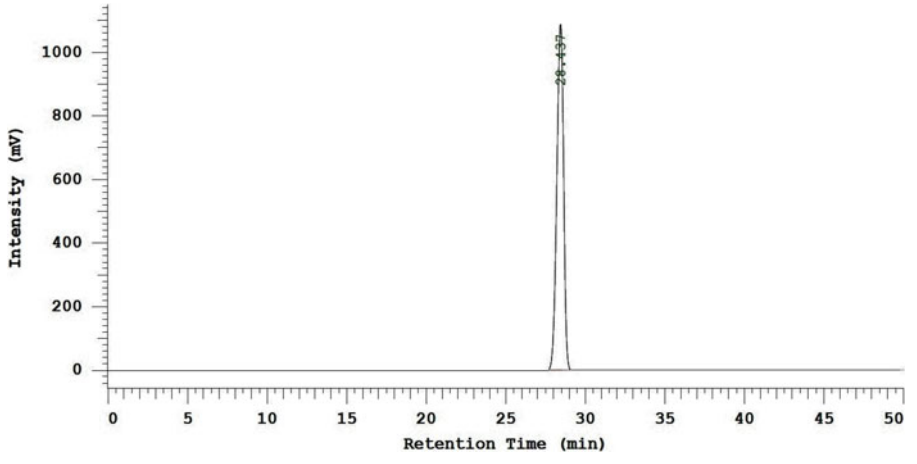

Figure 2. Chromatographic profile of radicinin used as reference at a concentration of 250 µg/mL.

Table 2. Radicinin production in different cultures condition for six *C. australiensis* strains.

Strain	Cultural mediums[a]	Organic extracts[b] (mg)	Radicinin detected (µg)	% of radicinin in the extract	radicinin (mg/L or mg/Kg)
LJ3B1	PDB	8.28	0	0	0
2MG2F	PDB	5.87	12.49 ± 0.50	0.21	0.12 ± 0.01
LJ3B2	PDB	7.04	62.57 ± 0.88	0.89	0.63 ± 0.02
LJ3C1	PDB	8.38	0	0	0
SNM4C1	PDB	4.90	10.01 ± 0.13	0.20	0.10 ± 0.01
LJ4B	PDB	25.93	2620.29 ± 2.24	10.11	26.20 ± 0.32
LJ4B	M1D	12.10	2184.88 ± 3.68	18.06	21.85 ± 0.24
LJ4B	SSS	23.37	80.14 ± 1.38	0.34	0.80 ± 0.02
LJ4B	WSC18	66.0	111.31 ± 1.54	0.17	1.11 ± 0.03
LJ4B	WSC25	108.0	1006.64 ± 2.01	0.93	10.07 ± 0.11

[a]PDB (Potato Dextrose Broth), M1D (M1D Medium), SSS (Soy Sauce Sucrose), WSC18 (wheat seeds solid culture at 18 days), WSCC25 (wheat seeds solid culture at 25 days); [b]EtOAc organic extracts corresponding to 100 mL of liquid cultures or 100 g of dried solid cultures.

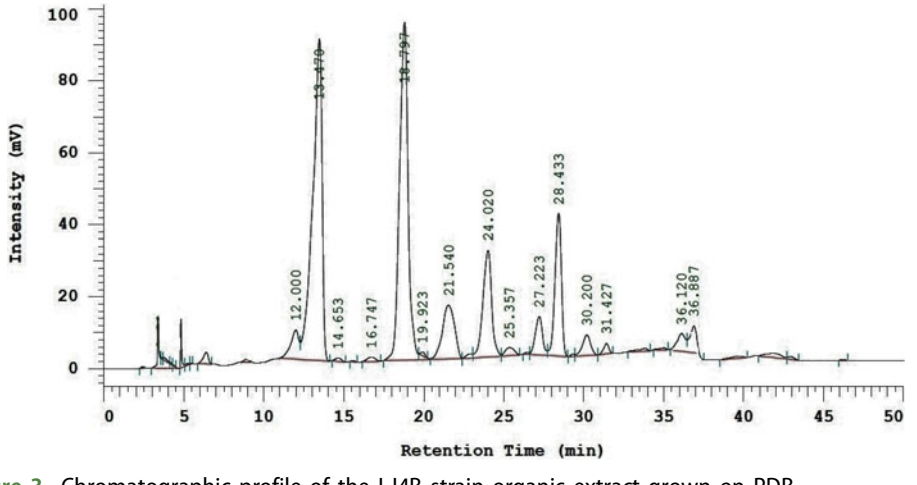

Figure 3. Chromatographic profile of the LJ4B strain organic extract grown on PDB.

radicinin production. The amount of **1** in the organic extract of SSS (soy sauce sucrose medium) culture was very low while that of the M1D culture was comparable with that of PDB culture with a yield of 21.85 ± 0.24 mg/L. It is interesting to note that the percentage of **1** in the M1D organic extract was higher than PDB (18.06% compared to 10.01% observed for the PDB production). Regarding the solid cultures on wheat seeds, different amounts of radicinin were produced at 18 and 25 days indicating that time is also an important factor for the production of **1**. The other main metabolites (**2**–**5**) contained in the organic extract of LJ4B on PDB culture (its HPLC chromatographic profile is reported in Figure 3) were recognized by their retention time and co-injection with the corresponding standard. The analysis of each standard has been repeated three times and all the retention times were highly reproducible, varying of less than 0.500 min.

From the results obtained in this study *C. australiensis* may not be the best natural source of radicinin. However, the HPLC method developed in this study could also be used to evaluate the production of **1** by other fungi. In fact, **1** has been isolated from the organic extract of several fungi belonging to different genera such as *Stemphylium radicinum* (Clarke and Nord 1953), *Cochliobolus lunatus* (Nukina and Marumo 1977), *Alternaria chrysanthemi* (Robeson, Gray, and Strobel 1982), *Alternaria helianthi* (Tal et al. 1985), *Phoma andina* (Noordeloos et al. 1993), *Alternaria petroselini* (Pryor and Gilbertson 2002), *Bipolaris coicis* (Nakajima et al. 1997), *Alternaria radicina* (Solfrizzo et al. 2004), and *Curvularia* sp. (Zhang et al. 2011). Screening of additional strains of *C. australiensis* could also result in discovery of a higher-yielding strain.

3. Experimental

3.1. General experimental procedures

Analytical TLC was performed on silica gel (Kieselgel 60 F_{254}, 0.25) plates (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (253 nm), 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.

3.2. Fungi and metabolites

The six strains of *Cochliobolus australiensis* used in this study (namely, LJ3B1, 2MG2F, LJ3B2, LJ3C1, SNM4C1 and LJ4B) were obtained from leaf spot lesions on buffelgrass tissue collections made near La Joya (LJ strains) and Magdalena (MG strains), Hidalgo County, in south Texas, USA, and in Saguaro National Monument (SNM strain), Pima County, Arizona, USA, in September 2014 and identified by Sanger sequencing of the ITS region as previously described (Masi, Meyer, Clement, Cimmino et al. 2017). These strains are stored in the collection of US Forest Service Rocky Mountain Research Station, Shrub Sciences Laboratory, Provo, USA and routinely maintained on Potato Dextrose Agar (PDA) (BD Difco™, Franklin Lakes, NJ, USA) plates and also as sporulated cultures on desiccated buffelgrass leaf tissue previously inoculated with each strain. Pure compounds **1**–**5** (Figure 1) were made available as reference material as previously described (Masi, Meyer, Clement, Cimmino et al. 2017).

3.3. Fungal production

The six strains of *Cochliobolus* were grown in Potato Dextrose Broth (PDB) (BD Difco™, Franklin Lakes, NJ, USA) culture at room temperature (22 °C) by inoculating 100 mL of sterile broth in 500 mL Erlenmeyer flasks with fragments of mycelial mat produced on PDA and incubating in shaker culture for 14 days. The mycelium was then removed from the medium by centrifugation and filtering, and the resulting filtrates were lyophilized and frozen at −20 °C until extraction and analysis. The strain LJ4B was also grown in liquid media on M1D (BD Difco™, Franklin Lakes, NJ, USA) and Soy Sauce Sucrose (SSS) (BD Difco™, Franklin Lakes, NJ, USA), following the same procedure used for the PDB culture, and solid culture on wheat seeds. For this latter culture *C. australiensis* conidia were produced on sterile buffelgrass leaf segments plated onto water agar. Approximately 4 mg of conidia suspended in sterile H_2O were added to 200 g of soaked, autoclaved wheat seeds, and the mixture was placed in a sterile 1 L Erlenmeyer flask with an aluminum foil cap at 22 °C. The flask was hand-shaken periodically during the 18 and 25 days incubation periods to prevent caking together of the grains. The culture was spread in pans and air-dried for at least several weeks prior to extraction.

3.4. Extraction of fungal metabolites

The lyophilized PDB cultures (100 mL) of the six strains studied and the M1D and SSS cultures (100 mL) of LJ4B were dissolved in distilled H_2O (30 mL) and then extracted with EtOAc (3 × 30 mL). The organic extracts were combined and dried with Na_2SO_4, and the solvent was evaporated under reduced pressure, yielding colored solid residues as reported in Table 2. 10 g of dried material of the wheat solid cultures (200 g) of LJ4B at 18 and 25 days were minced using a laboratory mill and extracted with 100 mL of MeOH − H_2O (1:1). The mixtures were centrifuged for 1 h at 7000 rpm. The supernatants were pooled and defatted with *n*-hexane (3 × 50 mL). The resulting aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under reduced pressure yielding two brown solid residues as reported in Table 2. Before accurate quantification described below, the presence of **1**–**5** in all the extracts obtained was ascertained by TLC analysis carried out on silica gel in comparison with a reference of pure samples by using $CHCl_3$–*iso*-PrOH (97:3) and CH_2Cl_2–MeOH (95:5) as solvent systems.

3.5. HPLC apparatus

The HPLC system (HITACHI) consisted of a pump (5160) and a spectrophotometric detector (5410). The HPLC separations were performed using a Merck (Darmstadt, Germany) C-18 reversed-phase column Lichrocart (250 × 4.6 mm i.d.; 5 μm).

3.6. Optimization and calibration of curves

The mobile phase used to elute the samples was MeCN–H_2O at a flow rate of 0.5 mL/min. The analysis was performed using a MeCN−H_2O gradient starting from 10%

MeCN increased linearly to 15% in 6 min, 20% in 16 min, 25% in 22 min, 40% in 40 min, 90% in 45 min and finally re-balanced to the initial rate for 5 min. Detection was performed at 226 nm, corresponding to the maximum UV absorption of radicinin (Nakajima et al. 1997). Samples were injected using a 20-μL loop and monitored for 50 min.

3.7. Quantification of radicinin

An amount for each fungal extract obtained was accurately weighed in order to prepare a 0.5 mg/mL solution in MeOH. An aliquot of the samples (20 μL) was injected for the analysis into the HPLC instrument as previously described, and each sample was assayed in triplicate.

4. Conclusions

Larger quantities of radicinin are needed to continue the studies for its potential application as selective bioherbicide against buffelgrass at the whole-plant level and under field conditions. The method developed in this study could be used to select the best fungal source and the best culture conditions to optimize large-scale radicinin production by fungal fermentation. Although the results obtained evaluating the effects of strain and cultural conditions of *C. australiensis* on the production of radicinin are not encouraging, other fungal sources could be screened prior to initiation of total stereoselective synthesis as an alternative production method.

Disclosure statement

The authors declare that there are not conflict of interest.

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References

Abella SR, Chiquoine LP, Backer DM. 2012. Ecological characteristics of sites invaded by buffelgrass (*Pennisetum ciliare*). *Invasive Plant Sci Manag*. 5(4):443–453.

Armbruster DA, Pry T. 2008. Limit of blank, limit of detection, limit of quantitation. *Clin Biochem Rev*. 29:49–52.

Bowers JE, Bean TM, Turner RM. 2006. Two decades of change in distribution of exotic plants at the Desert Laboratory, Tucson, Arizona. *Madroño*. 53:254–265.

Burquez-Montijo AM, Miller ME, Yrizar AM, Tellman B. 2002. Mexican grasslands, Thornscrub, and the transformation of the Sonoran Desert by invasive exotic buffelgrass (*Pennisetum ciliare*). In Tellman B. editor. *Invasive exotic species in the Sonoran region*, Tucson: The University of Arizona Press, The Arizona-Sonora Desert Museum. p 126–146.

Cimmino A, Masi M, Evidente M, Superchi S, Evidente A. 2015. Fungal phytotoxins with potential herbicidal activity: chemical and biological characterization. *Nat Prod Rep*. 32(12): 1629–1653.

Clarke DD, Nord FF. 1953. Radicinin: a new pigment from *Stemphylium radicinum*. *Arch Biochem Biophys*. 45(2):469–470.

Dayan FE, Duke SO. 2014. Natural compounds as next-generation herbicides. *Plant Physiol*. 166(3):1090–1105.

Epifano F, Preziuso F, Taddeo V, Fiorito S, Genovese S. 2018. An example of a novel efficient plant extraction technique: electromagnetic induction heating. *Molecules*. 23(11):3048.

Gerwick BC, Sparks TC. 2014. Natural products for pest control: an analysis of their role, value and future. *Pest Manag Sci*. 70(8):1169–1185.

Marshall VM, Lewis MM, Ostendorf B. 2012. Buffelgrass (*Cenchrus ciliaris*) as an invader and threat to biodiversity in arid environments: a review. *J Arid Environ*. 78:1–12.

Masi M, Meyer S, Górecki M, Mandoli A, Di Bari L, Pescitelli G, Cimmino A, Cristofaro A, Clement S, Evidente A. 2017. Pyriculins A and B, two monosubstituted hex-4-ene-2,3-diols and other phytotoxic metabolites produced by *Pyricularia grisea* isolated from buffelgrass (*Cenchrus ciliaris*). *Chirality*. 29(11):726–736.

Masi M, Meyer S, Clement S, Cimmino A, Cristofaro M, Evidente A. 2017. Cochliotoxin, a dihydropyranopyran-4,5-dione, and its analogues produced by *Cochliobolus australiensis* display phytotoxic activity against buffelgrass (*Cenchrus ciliaris*). *J Nat Prod*. 80(5):1241–1247.

Masi M, Meyer S, Clement S, Pescitelli G, Cimmino A, Cristofaro M, Evidente A. 2017. Chloromonilinic acids C and D, two phytotoxic tetrasubstituted 3-chromanonacrylic acids isolated from *Cochliobolus australiensis* with potential herbicidal activity against buffelgrass (*Cenchrus ciliaris*). *J Nat Prod*. 80(10):2771–2777.

Masi M, Freda F, Sangermano F, Calabrò V, Cimmino A, Cristofaro M, Meyer S, Evidente A. 2019. Development of a target-specific bioherbicide for invasive buffelgrass (*Cenchrus ciliaris*) control. *Molecules*. 24:1806.

Nakajima H, Ishida T, Otsuka Y, Hamasaki T, Ichinoe M. 1997. Phytotoxins and related metabolites produced by *Bipolaris coicis*, the pathogen of job's tears. *Phytochemistry*. 45(1):41–45.

Noordeloos ME, De Gruyter J, Van Eijk GW, Roeijmans HJ. 1993. Production of dendritic crystals in pure cultures of *Phoma* and *Ascochyta* and its value as a taxonomic character relative to morphology, pathology and cultural characteristics. *Mycol Res*. 97(11):1343–1350.

Nukina M, Marumo S. 1977. Radicinol, a new metabolite of *Cochliobolus lunata*, and absolute stereochemistry of radicinin. *Tetrahedron Lett*. 37:3271–3272.

Olsson AD, Betancourt JL, Crimmins MA, Marsh SE. 2012. Constancy of local spread rates for buffelgrass (*Pennisetum ciliare* L.) in the Arizona Upland of the Sonoran Desert. *J Arid Environ*. 87: 136–143.

Pryor BM, Gilbertson RL. 2002. Relationships and taxonomic status of *Alternaria radicina*, *A. carotiincultae*, and *A. petroselini* based upon morphological, biochemical, and molecular characteristics. *Mycologia*. 94(1):49–61.

Robeson DJ, Gray GR, Strobel GA. 1982. Production of the phytotoxins radicinin and radicinol by *Alternaria chrysanthemi*. *Phytochemistry*. 21(9):2359–2362.

Singh HP, Batish DR, Kohli RK. 2006. Handbook of sustainable weed management, ed. by Singh HP, Batish DR and Kohli RK. The Haworth Press Inc, New York, 2006.

Solfrizzo M, Vitti C, De Girolamo A, Visconti A, Logrieco A, Fanizzi FP. 2004. Radicinols and radicinin phytotoxins produced by *Alternaria radicina* on carrots. *J Agric Food Chem*. 52(11): 3655–3660.

Stevens J, Falk DA. 2009. Can buffelgrass invasions be controlled in the American Southwest? Using invasion ecology theory to understand buffelgrass success and develop comprehensive restoration and management. *Ecol Restor*. 27(4):417–427.

Tal B, Robeson DJ, Burke BA, Aasen AJ. 1985. Phytotoxins from *Alternaria helianthi*: Radicinin, and the structures of deoxyradicinol and radianthin. *Phytochemistry*. 24(4):729–731.

Zhang YL, Kong LC, Jiang DH, Yin CP, Cai QM, Chen Q, Zheng JY. 2011. Phytotoxic and antifungal metabolites from *Curvularia* sp. FH01 isolated from the gut of *Atractomorpha sinensis*. *Bioresour Technol*. 102(3):3575–3577.