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Radicinols and Radicinin Phytotoxins Produced by *Alternaria radicina* on Carrots

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The phytotoxin *epi*-radicinol, a diastereomer of radicinol, was isolated from large cultures of *Alternaria radicina* grown on carrot slices and identified by GC-MS, LC-MS, ^{1}H NMR, and ^{13}C NMR. Four strains of *A. radicina* isolated from rotted carrot produced *epi*-radicinol as the major metabolite (up to 39414 μg/g) together with radicinol (up to 2423 μg/g), and, to a lesser extent, radicinin when cultured on carrot slices, whereas on rice they mainly produced radicinin (2486−53800 μg/g). Radicinin and *epi*-radicinol reduced root elongation of germinating carrot seeds at concentrations of 10−20 μg/mL. Carrot samples naturally infected by *A. radicina* contained detectable quantities of *epi*-radicinol also in combination with lower levels of radicinin or radicinol. Accumulation of radicinols and radicinin in stored carrots, either naturally contaminated or artificially inoculated with *A. radicina*, was stimulated by successive temperature rises from 1 to 10 °C and from 10 to 20 °C, reaching maximum levels of 60 μg/g *epi*-radicinol and 26 μg/g radicinin. This is the first report on the production of radicinols by *A. radicina* and its natural occurrence in carrots in association with radicinin.

KEYWORDS: ***Alternaria radicina*; phytotoxins; *epi*-radicinol; radicinol; radicinin; carrot**

INTRODUCTION

Alternaria radicina Meier, Drechs., and Eddy is a seedborne fungal pathogen responsible for black rot disease of carrots (*Daucus carota* L.). The fungus is primarily a root pathogen and causes dry, black, necrotic lesions on the carrot crown and root both in the field and during storage. In the field, disease symptoms include a black decay of the carrot leaf petiole, crown, and root (*1*−*3*). Isolation from diseased plants showed that *A. radicina* attacks carrots at all stages, causing damping-off and rotting of roots, crowns, seedlings, petioles, and leaves of maturing carrots. Up to 70% of mature carrots with crown and shoulder infections were unmarketable, and isolates of *A. radicina* from seeds or infected seedlings were pathogenic to carrot seedlings and carrot disks (*4*). Seeds infected with *A. radicina* are the main source of disease, but the fungus can also attack the plants if present in the soil (*5*). Radicinin, a compound with phytotoxic, antifungal, antibiotic, insecticidal, and plant growth regulatory activities, has been reported in cultures of *A. radicina* (*6*). Although the pathogenicity of *A. radicina* against carrot has been previously described (*1*, *3*−*5*, *7*), no data are available on the possible involvement of radicinin as pathogenicity or virulence factors of *A. radicina* versus this vegetable.

In this investigation we have isolated and characterized *epi*-radicinol from cultures of *A. radicina* isolated from carrot roots. The accumulation of *epi*-radicinol, radicinin, and radicinol in carrots naturally and artificially inoculated with *A. radicina* and stored at different temperatures was studied. The natural occurrence of *epi*-radicinol, in association with radicinol or radicinin, in carrots with black spot symptoms was also investigated together with the phytotoxicity of these toxins toward germinating carrot seeds and the relevant production by strains of *A. radicina*.

MATERIALS AND METHODS

Isolation and Morphological Identification of *A. radicina* Strains. Six carrot samples with black spot symptoms were obtained from Plant Research International (The Netherlands) and used for the isolation of strains of *A. radicina* and for toxin analysis. Tissue fragments were excised from diseased carrots, plated on water agar supplemented with 10 mg of streptomycin sulfate per liter, and incubated for 5−7 days at 25 °C. Fungal colonies were then transferred to plates of potato−sucrose agar (PSA) and incubated for 7 days at room temperature under fluorescent lamps (12-h photoperiod). Single-spore fungal cultures were then obtained on PSA plates to test the color and morphology of the colonies. Conidial morphology was observed using potato−carrot agar (PCA) or a sterile carnation leaf agar (*8*). The identification of fungal colonies was made according to the synoptic keys of Ellis (*9*, *10*). To preserve the cultures, mycelia and conidia from wild strains grown on carnation leaf agar were transferred aseptically in 1 mL of sterile 18% glycerol−water and frozen at −75 °C. The isolates of *A. radicina* were

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deposited in the culture collection (ITEM, http://www.ispa.cnr.it/Collection) of the Institute of Sciences of Food Production, Bari, Italy. The remaining carrot tissues were analyzed for *epi*-radicinol, radicinin, and radicinol as described.

Toxin Production. The *A. radicina* isolates were grown on 50 g of rice kernels moistened with 50 mL of distilled water in 100-mL Erlenmeyer flasks. The substrate was autoclaved for 20 min at 121 °C and then inoculated with potato−dextrose−agar cultures of freshly isolated *A. radicina* strains from decayed carrots. The cultures were incubated at 28 °C for 21 days in darkness. After incubation, the cultures were dried at 40 °C and finely ground. The same isolates of *A. radicina* were also grown on 100 g of carrot slices. Carrot roots were washed under tap water, peeled off, and sliced into disks of ∼1-cm thickness and autoclaved in a 500-mL beaker for 1 min at 121 °C. After autoclaving, carrot slices were transferred to sterile Petri dishes containing a sterile filter paper moistened with 2 mL of sterile water containing 0.5 mg/mL of streptomycin sulfate and chlortetracycline HCl and individually inoculated with potato−dextrose−agar culture plugs of freshly isolated *A. radicina*. The cultures were incubated for 14 days at 28 °C in the darkness, then dried at 40 °C, and finely ground. For the production and isolation of *epi*-radicinol 500 g of carrot was inoculated with *A. radicina*.

Extraction and Isolation of *epi*-Radicinol. The dried carrot culture (19.8 g) of *A. radicina* was blended and extracted three times with 80 mL of a mixture of acetonitrile−methanol−water (45:10:45, v/v/v) at pH 3 (HCl) by shaking for 30 min. After filtration, the filtrates were combined and submitted three times to liquid−liquid extraction with 50 mL of methylene chloride in a separatory funnel. The combined methylene chloride phases were dried over anhydrous sodium sulfate and evaporated to dryness under vacuum at 40 °C. The residue was reconstituted with 10 mL of chloroform and chromatographed on a 22 × 2.2 cm i.d. column packed with silica gel (0.063−0.200 mm particle size, 70−230 mesh) (Merck, Darmstadt, Germany) in chloroform. The column was eluted sequentially with 100 mL of chloroform−0.1% glacial acetic acid (99:1, v/v), 200 mL of chloroform−methanol−0.1% glacial acetic acid (94:6:1, v/v/v), and 100 mL of chloroform−methanol−0.1% acetic acid (88:12:1, v/v/v). Sixteen fractions (F1−F16) of ∼25 mL each were collected and analyzed by reversed phase liquid chromatography with a UV diode array detector (LC-UV/DAD), as described under Toxin Analysis, to obtain a compound having a UV spectrum similar to that of radicinol but with a longer retention time. This compound accumulated in fractions F7, F8, and F9, which were combined, evaporated to dryness under vacuum at 40 °C, reconstituted with 5 mL of chloroform, and chromatographed again on a 30 × 1.4 cm i.d. column with the same packing material. This column was eluted with the same solvent mixtures reported above, and 16 fractions of ∼25 mL each were collected and analyzed by LC-UV/DAD for the radicinol-like compound. Fraction 9 containing the pure radicinol-like compound was evaporated to dryness, reconstituted in a minimum volume of methanol, diluted with water, and freeze-dried to obtain 200 mg of pure compound as a white powder.

Storage Experiment. Ninety-six freshly harvested carrot roots of similar size were selected from a local market in Bari, washed with tap water, air-dried, and used for storage experiments. In particular, 48 carrots were inoculated by immersion for 30 s in a spore suspension of *A. radicina* having a concentration of ∼70000 spores/mL and kept under shaking by a magnetic stirring. The remaining 48 carrots having the natural mycoflora were used as such for comparison. Carrots were maintained in plastic boxes and incubated in a climatic cabinet with controlled conditions (WTB Binder Labortechnik GmbH, Tuttlingen, Germany) for 17 weeks at 1 °C, followed by 4 weeks at 10 °C and 4 weeks at 20 °C. Six carrots were collected from both *A. radicina*-inoculated samples and control samples eight times at regular intervals during storage (after 9, 17, 19, 20, 21, 23, 24, and 25 weeks), blended, and analyzed for *epi*-radicinol, radicinin, and radicinol.

Toxin Analysis. A standard of radicinin was purchased from Sigma; radicinol was kindly provided by Dr. Hiromitsu Nakajima, Faculty of Agriculture, Tottori University, Koyama, Tottori, Japan; and *epi*-radicinol was produced, isolated, and characterized in our laboratory.

Dried and finely ground rice (5 g) or carrot (2 g) cultures were extracted with 20 mL of a mixture of acetonitrile−methanol−water (45:10:45, v/v/v) at pH 3 (HCl) by shaking for 30 min. After filtration on filter paper (Whatman no. 4), the culture extracts were appropriately diluted with water, filtered through a 0.45-μm membrane filter (LCR-Millipore), and analyzed by both LC-UV/DAD and LC-MS. For GC-MS confirmation analyses, filtrates were submitted to liquid−liquid partitioning with methylene chloride that was concentrated prior the injection.

For the analysis of carrots from the storage experiment and the six carrot samples with black spot symptoms, filtrates were purified on a C_{18} SPE column prior to LC-UV/DAD analysis to improve the limit of quantification. In particular, carrots were homogenized with a blender (Sterilmixer Lab, International Pbi, Milan, Italy), and a 20 g test portion was extracted with 50 mL of acetonitrile−methanol−water (45:10:45, v/v/v) at pH 3 (HCl) by shaking for 30 min. After filtration through a filter paper, 2.5 mL of filtrate was diluted with 3 mL of distilled water and purified on a C_{18} SPE column, 10 mL capacity containing 500 mg of sorbent (Waters, Milford, MA) previously conditioned with 3 mL of acetonitrile and 3 mL of distilled water. The diluted filtrate was passed through the column at ∼1−2 drops/s, and the eluate was discarded. The toxins were eluted from the column with 2 mL of acetonitrile−glacial acetic acid (99:1, v/v) into a 4-mL vial. The eluate was evaporated to dryness under a nitrogen stream at ∼60 °C. The purified residue was redissolved in 500 μL of methanol, diluted with 500 μL of water, and stored at ∼4 °C until LC analysis. Recovery experiments were performed in triplicate by spiking 20 g of blended carrot samples at two levels (1 and 5 μg/g) of both radicinin and *epi*-radicinol. Three samples were spiked with 200 μL each of the 0.10 μg/mL spiking solution and three samples with 200 μL each of the 0.5 μg/mL spiking solution containing radicinin and *epi*-radicinol and prepared in methanol. Mean recoveries of radicinin and *epi*-radicinol from spiked carrot were 60−63 and 65−75%, respectively. Results of within laboratory repeatability (RSD_r) were 5−6 and 10−15% for radicinin and *epi*-radicinol, respectively.

Analyses by LC-UV/DAD were performed with a liquid chromatograph (Thermo Quest Inc., San Jose, CA) equipped with a quaternary gradient pump delivering a 1 mL/min constant flow rate (Spectra Series gradient pump P4000), a vacuum membrane degasser (SCM 1000), an autosampler injection system with a 50 μL loop (AS 3000), a column oven set at 30 °C, a diode array detector (DAD, UV 6000 LP detector) set at 224, 317, and 345 nm, and a chromatography data system for Windows 2000 (ChromQuest version 2.53). A stainless steel 150 × 4.6 mm i.d., 5 μm, SymmetryShield C_{18} reversed-phase column (Waters) preceded by a guard filter (3 mm, 0.5 μm) was used. The mobile phase consisted of a linear gradient of acetonitrile in water from 10 to 30% in 22 min at a flow rate of 1 mL/min. Radicinin, radicinol, and *epi*-radicinol were identified in sample extracts by comparing retention times and UV spectra of peaks recorded in the chromatogram with those of the relevant standards. Quantification of the toxins was performed according to the external standard method, integrating peak areas at retention times relevant to the corresponding standards. Chromatograms were recorded at 345 and 317 nm for quantification of radicinin and radicinols, respectively.

For GC-MS analyses of selected samples of carrots with black spot symptoms the filtrates were purified on a C_{18} SPE column, but the final eluates were evaporated to dryness and reconstituted with 500 μL of methylene chloride. GC-MS was performed by a 6890 series GC system (Agilent Technologies, Inc., Palo Alto, CA) coupled with a 5973N mass selective detector and equipped with a GC-MSD ChemStation and a 30m × 0.25 mm i.d., 0.25 μm, HP-MS-5 capillary column. GC operating conditions: splitless injection; injector temperature, 250 °C; helium carrier gas with a constant flow of 2.9 mL/min; ionization temperature, 250 °C; column temperature program of 70 °C for 2 min, raised at 25 °C/min to 150 °C, raised at 3 °C/min to 200 °C, raised at 8 °C/min to 280 °C, and held at 280 °C for 10 min; run time, 42 min; mass spectrometer setting, electron impact ionization mode with 70 eV energy; injection volume, 1 μL.

Analyses (LC-MS) were performed by a Hewlett-Packard 1050-Ti chromatographic system and a UV detector set at 317 nm interfaced to an API 165 mass spectrometer equipped with a turbo-ionspray interface (Perkin-Elmer Sciex, Norwalk, CT). A 150 × 4.6 mm i.d., 5 μm, SymmetryShield C_{18} reversed-phase column was used. The mobile phase consisted of an isocratic mixture of acetonitrile−0.1%glacial acetic acid (25:75, v/v) at a flow rate of 1 mL/min. Splitting of the LC flow was performed to allow just 200 μL/min to enter the turbo-ionspray interface. The mass spectrometric conditions (positive ionization) were

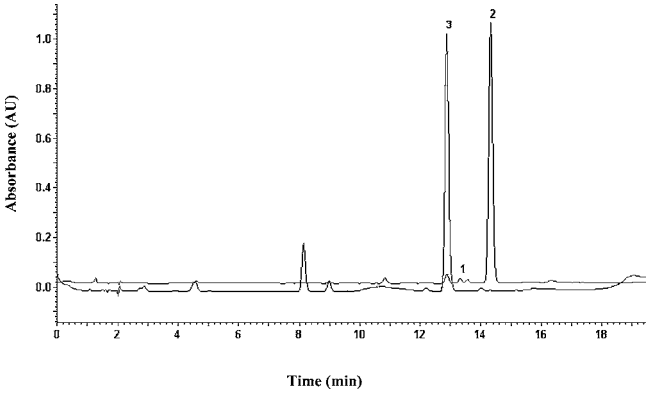

Figure 1. LC-UV/DAD chromatograms (λ = 317 nm) relevant to extracts of *A. radicina* strain ITEM 4342 grown on rice (lower trace) and carrot slices (upper trace) containing radicinin (**3**), radicinol (**1**), and *epi*-radicinol (**2**).

as follows: nebulizer gas (air) 1.5 L/min; curtain gas (nitrogen) temperature, 300 °C; mass range, 70.0−800.0 amu; scan time, 2 s; needle voltage, 5000 V; ring voltage, 200 V; and orifice voltage, 25 V.

NMR Analysis. Proton (^{1}H NMR) and carbon nuclear magnetic resonances (^{13}C NMR) were recorded on a DRX500 Advance Bruker instrument equipped with probes for inverse detection and with *z* gradient for gradient-accelerated spectroscopy. Standard Bruker automation programs were used for 2D NMR experiments. Correlation spectroscopy 2D (COSY) experiments were performed using COSY DFTP (double-quantum-filtered phase-sensitive COSY) and COSY GS (gradient-accelerated COSY) sequences. NOESY-EXY spectra were acquired using mixing times of 0.6−0.9 s. Inverse detected normal and long-range ^{1}H and ^{13}C heterocorrelated 2D NMR spectra were obtained by using the gradient-sensitivity enhanced pulse sequences INVIEAGSSI and INV4GPLRND, respectively. $CDCl_3$ was used as the solvent in all of the NMR experiments. Residual ^{1}H and ^{13}C peaks of the solvent (δ_H 7.26, δ_C 77.0) were used as internal standards to calculate chemical shifts with reference to tetramethylsilane.

Phytotoxicity Assay. The phytotoxic activity of *epi*-radicinol and radicinin was tested on germinating carrot seeds following the method described by Visconti et al. (*11*). Seeds were kept overnight under running water and then sterilized with 1% NaOCl for 10 min. After several rinses with sterile distilled water, seeds were left to germinate on water-impregnated filter paper at 25 °C in the darkness for 48 h. Ten germinating seedlings (root-length range = 2−5 mm) were selected per test and put in a Petri dish (diameter = 5 cm) containing the same size filter paper. Two milliliters of sterile distilled water containing 1% methanol (control) or a methanolic solution of *epi*-radicinol or radicinin was added. Radicinin and *epi*-radicinol were tested at 10 and 20 μg/mL, respectively. Root elongation measurements were made after 2 days of exposure to fluorescent lamps (12-h photoperiod, 25 °C). Two replicates per test were performed.

RESULTS

Identification of *epi*-Radicinol. LC-UV/DAD analyses of *A. radicina* culture extracts showed different metabolite profiles depending on the culture substrate. As shown in **Figure 1**, radicinin was the main toxin produced by *A. radicina* grown on rice, whereas another compound, having a longer retention time, was the main metabolite produced on carrots. This compound had a UV spectrum similar to that of radicinol, with UV absorbance maxima at 224 and 317 nm as measured with a UV diode array detector. To identify this compound, the carrot culture extracts of *A. radicina* were analyzed by GC-MS and compared with a standard solution of radicinin and radicinol. GC-MS analysis showed for radicinol and radicinin retention times of 19.85 and 24.6 min, respectively, whereas the radicinol-like compound eluted with a retention time of 20.38 min. The mass spectrum (EI mode) of the radicinol-like compound was

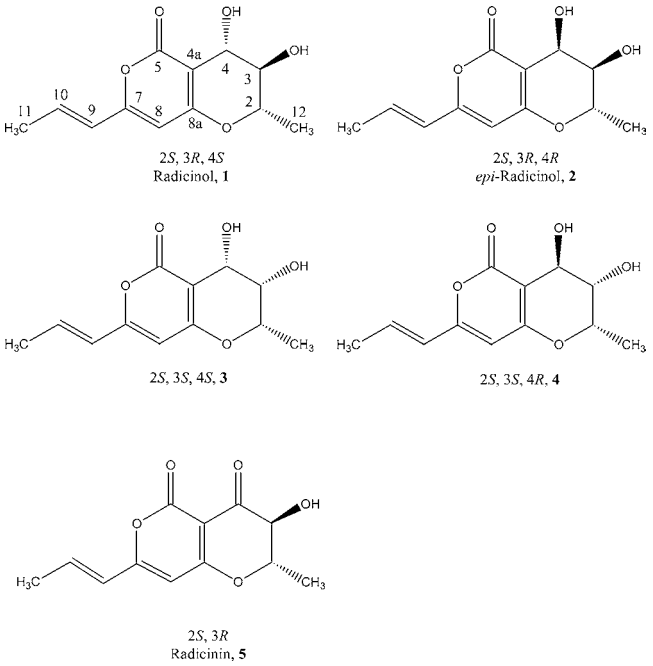

Figure 2. Structures of radicinol (**1**), *epi*-radicinol (**2**), radicinol isomers 2*S*,3*S*,4*S* (**3**) and 2*S*,3*S*,4*R* (**4**), and radicinin (**5**).

similar to that of radicinol and showed its characteristic fragmentation with prominent ions at *m*/*z* (%) $[M]^+$ 238 (6), 181 (100), 111 (25), and 69 (37). LC-MS analyses of carrot culture extracts of *A. radicina* showed a similar mass spectrum for both radicinol and the radicinol-like compound. In the positive ionization mode the mass spectrum of the two radicinol isomers showed two major ions at *m*/*z* 239 $[M + H]^+$ (base peak) and *m*/*z* 221 $[M + H - H_2O]^+$. The analysis by LC-MS, positive ionization mode, of radicinin showed prominent ions at *m*/*z* 237 $[M + H]^+$ (base peak), 259 $[M + Na]^+$, 219 $[M + H - H_2O]^+$, and 495 $[M + M + Na]^+$, clearly indicating the 2 amu difference with respect to radicinol isomers due to the carbonyl group, instead of a hydroxy group, at the C-4 position.

To ascertain the stereochemistry of the radicinol isomer, the compound (200 mg) was isolated from a carrot culture of *A. radicina* (ITEM 4342) and submitted to extensive NMR studies. Considering that radicinol (3,4-dihydroxy-2-methyl-7-propenyl-3,4-dihydro-2*H*-pyrano[4,3-*b*]pyran-5-one) is characterized by the presence of three chiral centers in the dihydropyran ring, our compound should be one of the eight possible stereoisomers of radicinol. However, only three stereoisomers have been reported in the literature as fungal metabolites, namely, radicinol (**1**; 2*S*,3*R*,4*S*), *epi*-radicinol (**2**; 2*S*,3*R*,4*R*), and the radicinol isomer 2*S*,3*S*,4*S* (**3**) (*12*−*14*). All of them have the same *S* configuration at the C2 of the dihydropyran ring found in the radicinin, which is considered to be an oxidized precursor of radicinol isomers (*12*). Therefore, the natural radicinol isomers seem to be restricted to the possible configurations at the C3 and C4 shown in **Figure 2**. ^{1}H NMR data of the radicinol isomer obtained in our laboratory and those of the three isomers of radicinol, reported as fungal metabolites, are reported in **Table 1**. The comparison of the ^{1}H NMR data reported in **Table 1** allowed the structure of the isomer under investigation to be defined as *epi*-radicinol. ^{13}C NMR data of *epi*-radicinol, not reported in the literature, are shown in **Table 2** together with ^{13}C data for the other known radicinol isomers. It should be noted that slight differences observed in the ^{1}H spectrum of *epi*-radicinol with respect to literature data may be due to a reduced resolution available in older NMR spectrometers and do not support an alternative stereoisomeric assignment. The

Table 1. ^{1}H NMR Data for *epi*-Radicinol (**2**) Obtained in the Present Work and Assigned also on the Basis of COSY and NOESY Spectra Compared with Literature Data of *epi*-Radicinol (**2e**), Radicinol (**1**), and Radicinol Isomer 2*S*,3*S*,4*S* (**3**)

	2	**2e**[a]	**1**[a]	**3**[b]
CHCH$_3$(2)	4.27 dq (8.5, 6.7)	4.30 dq (8.5, 6.5)	4.23 dq (8, 6.5)	4.37 dq (1.1, 6.3)
CHOH(3)	3.63 dd (8.5, 4.3)	3.60 dd (8.8, 4.0)	3.76 dd (8, 6.5)	3.88 br
CHOH(3)	3.02 bs		4.75 bs	
CHOH(4)	3.87 bs			
CHOH(4)	4.76 d (4.3)	4.78 d (4)	4.70 d (6.5)	4.60 d (2.8)
CH(8)	5.76 s	5.80 s	5.85 s	5.79 s
CH(9)	5.96 dq (15.3, 1.8)	5.99 dq (1.5, 16)	6.02 dq (16, 2)	5.97 dq (15.7, 1.5)
CH(10)	6.71 dq (15.3, 6.7)	6.75 dq (7, 16)	6.72 dq (16, 7)	6.72 dq (15.7, 6.8)
CH$_3$(11)	1.90 dd (6.7, 1.8)	1.90 dd (7, 1.5)	1.92 dd (7, 2)	1.91 dd (6.8, 1.5)
CHCH$_3$(12)	1.46 d (6.7)	1.45 d (6.5)	1.52 d (6.5)	1.50 d (6.3)

[a] Data from Nukina and Marumo (*12*). [b] Data from Nakajima et al. (*13*).

Table 2. ^{13}C NMR Data for *epi*-Radicinol (**2**) Obtained in the Present Work (Assigned also on the Basis of Normal and Long-Range ^{1}H and ^{13}C Heterocorrelated 2D NMR) Compared with Literature Data for Radicinol (**1**) and Radicinol Isomer 2*S*,3*S*,4*S* (**3**)

	2	**1**[a]	**3**[b]		**2**	**1**[a]	**3**[b]
C(2)	73.7	76.8	72.4	C(8)	98.7	99.1	99.3
C(3)	69.0	72.5	69.1	C(8a)	165.3	164.5	166.6
C(4)	60.6	68.0	63.2	C(9)	122.5	122.7	122.6
C(4a)	99.3	100.6	99.5	C(10)	135.5	135.7	135.2
C(5)	164.2	165.4	165.4	C(11)	18.0	18.4	18.4
C(7)	158.7	158.9	158.5	C(12)	16.7	17.0	15.8

[a] Data from Nukina and Marumo (*12*). [b] Data from Nakajima et al. (*13*).

^{1}H coupling constants obtained for the C2, C3, C4 linkage of the dihydropyran ring were in agreement with those expected for *epi*-radicinol from a detailed study on correlation of coupling costants and dihedral angles for the most favored conformation of the four stereoisomers **1**−**4** searched by MOPAC/PM3 calculation (*13*). Moreover, the assignment of the 2*S*,3*R*,4*R* configuration, typical for *epi*-radicinol, was supported by the presence of NOESY cross-peaks in the NOESY-EXY spectrum, which correlate the methyl group at C2 not only with H-2 but also with H-3 and H-4, indicating that the methyl group at C2 and the H-3 and H-4 are on the same side of the dihydropyran ring. Consistently, H-4 showed NOESY cross-peaks with H-3 and the two OH groups but not with H-2. EXY cross-peaks were also observed between the two OH groups at C3 and C4. ^{13}C NMR data of *epi*-radicinol (**Table 2**) were very similar to those reported for the other known radicinol isomers. As expected, the observed major differences in chemical shift were related to the C2,C3,C4 linkage of the dihydropyran ring.

Toxigenicity of *A. radicina* Strains on Different Culture Media. Rice and carrot culture extracts as well as the purified extracts of carrots from the storage experiment and the six carrot samples with black spot symptoms were analyzed by LC-UV/DAD. A good separation of radicinin, radicinol, and *epi*-radicinol was obtained with a reversed phase column eluted with a mobile phase consisting of a linear gradient of acetonitrile in water. Radicinin, radicinol, and *epi*-radicinol had retention times of 12.80, 13.30, and 14.30 min, respectively (**Figure 1**). Confirmation of radicinin, radicinol, and *epi*-radicinol in sample extracts was performed by comparing both retention times and UV spectra with authentic standards. Radicinin, radicinol, and *epi*-radicinol from culture extracts showed a similarity index of 0.99 when the relevant UV spectra, obtained by the DAD, were compared with those of the authentic toxin standards. A similarity index of 0.98 or higher is accepted to correctly identify a compound.

Table 3. Levels of Radicinin and Radicinols (Micrograms per Gram) in Rice and Carrot Slice Cultures of *A. radicina* Isolated from Diseased Carrot Roots

	rice			carrot slices		
strain	radicinin	radicinol	*epi*-radicinol	radicinin	radicinol	*epi*-radicinol
ITEM 4218	53 800	nd[a]	299	nd	1854	3494
ITEM 4223	11 903	993	nd	nd	2423	3241
ITEM 4342	17 841	nd	nd	1018	532	39414
ITEM 4481	2486	828	nd	nd	33	180

[a] Not detected, limit of quantification (signal-to-noise ratio of 6:1) = 0.2 μg/g.

Table 4. Natural Occurrence of *epi*-Radicinol, Radicinol, and Radicinin (Micrograms per Gram) in Carrots Affected by *A. radicina* Black Rot

carrot cv.	*epi*-radicinol	radicinol	radicinin
Royal Chantenay Rola (lot 133087)	8.78	0.70	nd[a]
Royal Chantenay Rola (lot 142568)	2.35	0.09	nd
Nanco	0.86	nd	4.87
Tempo	0.46	nd	0.09
Kira	0.25	nd	nd
Aston	0.20	nd	nd

[a] Not detected, <0.01 μg/g.

The toxigenicity of the four strains of *A. radicina* isolated from rotted carrot tissues with black spot symptoms was first ascertained on rice, a substrate commonly used to test the toxigenicity of *Alternaria* spp. (*15*−*17*), and then on carrot slices to verify the toxigenicity of the fungus on the host plant **(Table 3)**. All four strains on rice produced high amounts of radicinin together with minor levels of radicinol (two strains) or *epi*-radicinol (one strain). When grown on carrot slices, the four strains of *A. radicina* showed a different metabolic profile characterized by the production of *epi*-radicinol (up to 39.4 mg/g) and radicinol (up to 2.4 mg/g) by all four strains, whereas only one strain produced radicinin. The finding of *epi*-radicinol as the main toxin produced by *A. radicina* on carrot substrate (**Table 3** and **Figure 1)** was also confirmed by the analysis of carrot samples naturally infected with *A. radicina* and bearing clear rot (black spot) symptoms. As shown in **Table 4**, all tested samples were found to be contaminated with *epi*-radicinol at levels ranging from 0.2 to 8.8 μg/g, whereas two samples also contained radicinin (0.1 and 4.9 μg/g) and two radicinol isomers (0.1 and 0.7 μg/g). The identity of *epi*-radicinol, radicinol, and radicinin in selected culture extracts and in two purified extracts of carrot samples with black spot symptoms was confirmed by GC-MS. This is the first report on the production of *epi*-radicinol and radicinol by *A. radicina* and their natural occurrence in carrots in association with radicinin.

Formation of Toxins during Storage. Accumulation of radicinols and radicinin in carrots, naturally contaminated or artificially inoculated with *A. radicina* during storage for different periods at different temperatures, is graphically shown in **Figure 3**. No toxin accumulation was observed in naturally infected carrots stored for 17 weeks at 1 °C, whereas low levels of radicinin (2.2 μg/g) and *epi*-radicinol (0.9 μg/g) were found after 17 weeks of storage at 1 °C in carrots artificially infected with *A. radicina*. The increase of storage temperature to 10 °C

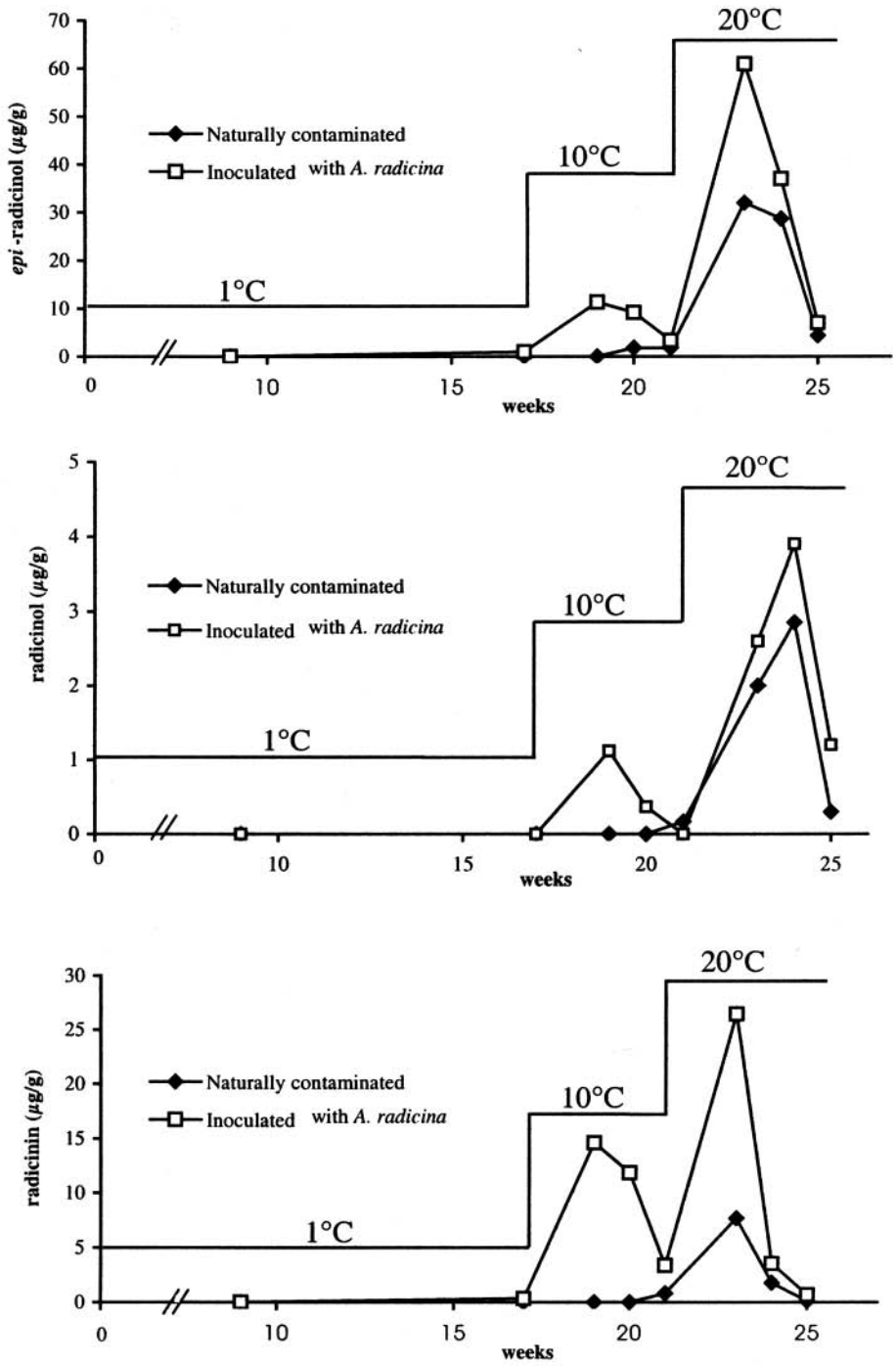

Figure 3. Accumulation of radicinols and radicinin in carrots, naturally contaminated or artificially inoculated with *A. radicina*, during storage for different periods at different temperatures.

at week 17 led to the formation of low levels of the three toxins (0.8 μg/g radicinin, 0.2 μg/g radicinol, and 1.8 μg/g *epi*-radicinol) in the naturally infected carrots after 21 weeks of storage (17 weeks at 1 °C + 4 weeks at 10 °C). Under the same conditions the artificially infected carrots showed a higher accumulation of toxins that reached maximum levels (14.6 μg/g radicinin, 11.3 μg/g *epi*-radicinol, and 1.1 μg/g radicinol) at week 19 and decreased in two successive weeks. The increase of storage temperature to 20 °C stimulated toxin accumulation in both naturally and artificially infected carrots. High levels of *epi*-radicinol (60.9 μg/g) and radicinin (26.5 μg/g) were observed together with radicinol (2.6 μg/g) in artificially inoculated carrots after 23 weeks storage (17 weeks at 1 °C + 4 weeks at 10 °C + and 2 weeks at 20 °C). Again, a prolonged storage of two additional weeks at constant temperature produced a consistent reduction of the three examined toxins as shown in **Figure 3**. A similar toxin profile was observed in naturally infected carrots but with lower levels of toxins probably caused by the lower amount of *A. radicina* inoculum present on these carrots as compared to artificially inoculated carrots.

Phytotoxic Activity of *epi*-Radicinol and Radicinin. Radicinin and *epi*-radicinol showed a phytotoxic effect on carrot seedlings producing root growth inhibition. In particular, 20 μg/mL *epi*-radicinol resulted in 43% root growth inhibition, whereas radicinin produced 25% root growth inhibition at a concentration of 10 μg/mL. Within the six samples reported in **Table 4**, higher levels of radicinin or *epi*-radicinol were found in Nanco and

Royal Chantenay Rola cultivars, which are quite susceptible to *A. radicina* (K. Langerak, personal communication).

DISCUSSION

Radicinin has been reported to be produced by *A. radicina*, *Alternaria chrysanthemi*, *Alternaria helianthi*, *Bipolaris coicis*, *Cochliobolus lunata*, and *Phoma andina*, radicinol by *A. chrysanthemi* and *C. lunata*, and *epi*-radicinol by *A. chrysanthemi* (*12*−*14*, *18*−*22*). The coproduction of these toxins by the same fungus has been reported only for *A. chrysanthemi* (*14*). The data reported in this work demonstrate that *A. radicina* isolated from carrot produces these three toxins in vitro and that the production of each toxin is influenced by the culture medium. In particular, *epi*-radicinol was produced in a larger amount when *A. radicina* was grown on carrot and was found more frequently in naturally contaminated carrots having black spot symptoms. These toxins accumulated in carrot during storage, and their levels were related to the amount of *A. radicina* inoculum and storage temperature and duration. Enzymes of carrot tissues or microflora could be responsible for the reduction of toxin levels occurring after long periods of storage at 10 or 20 °C.

Our findings show that radicinin and *epi*-radicinol are phytotoxic against carrot seedlings and can be found in naturally contaminated carrot roots as a result of infection by *A. radicina*. Previously, culture filtrates of *A. radicina* isolated from carrot, containing stemphylone (syn. of radicinin) plus another radicinol-like unidentified compound were previously reported to cause carrot wilting and leaf burning and to inhibit root elongation of germinated carrot seeds (*23*). Radicinin phytotoxicity was previously reported against Job's tears (*Coix lachrymal-jobi*), causing necrotic lesions on the leaves at 0.3 μg/leaf (*13*). Radicinin and radicinol were reported to cause interveinal necrosis of cuttings of Canada thistle and were detected in necrotic lesions of chrysanthemum leaves artificially infected with *A. chrysanthemi* (*20*). These results together with the finding of higher levels of these phytotoxins in carrot cultivars more susceptible to *A. radicina* suggest that radicinin and radicinols could play a role in the pathogenicity of *A. radicina* against carrot.

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