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Influence of Fungal Endophyte Infection on Phenolic Content and Antioxidant Activity in Grasses: Interaction between *Lolium perenne* and Different Strains of *Neotyphodium lolii*

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ABSTRACT: *Lolium perenne* is a major forage and turf grass, which is often naturally infected with a "wild-type" strain (E^{WT}) of the fungal endophyte *Neotyphodium lolii*, establishing a symbiotic relationship. In this study, the impacts of different strains wild type E^{WT}, AR1 (E^{AR1}) and AR37 (E^{AR37}), of *N. lolii* on the phenolic profile, phenolic content, and antioxidant capacity of *L. perenne* were examined. Samples could be ranked according to their phenol content as follows: $E^{AR1} > E^{AR37} \geq E^{-} > E^{WT}$. Radical-scavenging assays showed the same relative ranking of extracts. Flavonoid glycosides and hydroxycinnamic acids were the most abundant polyphenols in *L. perenne* extracts. Chlorogenic acid and its derivatives were the major compounds responsible for the antioxidant activity. Infection with *N. lolii* significantly influenced *L. perenne* phenolic content and antioxidant activity. In conclusion, changes in phenolic composition were merely quantitative. Endophyte infection can have zero, positive, or negative effect on phenol content depending on the endophyte strain.

KEYWORDS: *perennial ryegrass, polyphenols, flavonoids, chlorogenic acid, Poaceae, phenolic profile*

INTRODUCTION

The presence of fungal endophytes in vascular plants, including grasses, is almost ubiquitous.[1] It is estimated that fungal endophytes occur in 20−30% of grass species.[2] The interest in studying endophyte−grass symbiosis is not only due to the economic importance of grasses but also because of the ecophysiological impacts of such relationships. Several biologically active secondary metabolites are produced in endophyte−grass symbiosis. These metabolites play a vital role in improving grass productivity, diversity, and tolerance to biotic and abiotic stresses.[1−4]

Perennial ryegrass, *Lolium perenne* L. (Poaceae), is a major forage and turf grass, because of its quality feed for livestock and adaptability to different environmental and stress conditions. It is a native to Europe, North Africa, and temperate Asia, but has adapted to several temperate areas in America, Australia, and New Zealand.[5] *L. perenne* is often naturally infected with a "wild-type" strain (E^{WT}) of the fungal endophyte, *Neotyphodium lolii*, which establishes a symbiotic relationship.[1] *N. lolii* E^{WT} infection may cause ryegrass staggers, a neuromuscular disease in livestock characterized by staggers, occasional sudden death, and reduced weight gain when animals feed on the infected *L. perenne*.[6] At the same time, *N. lolii* E^{WT} enhances the grass's defense capability against many phytophagous insects and microbial pathogens, further improving its environmental adaptability and leading to greater productivity and persistence.[7,8]

N. lolii E^{WT} infection alters the metabolic profiles of *L. perenne* dramatically.[2] This alteration is characterized by the production of a range of chemically unrelated alkaloids such as ergovaline, lolitrem B, and peramine that have never been detected in endophyte-free (E^{-}) *L. perenne*. Ergovaline and lolitrem B have been linked to livestock health problems;[9] however, alkaloids are reported to enhance *L. perenne* fitness.[10] As a strategy to combat endophyte-associated livestock health problems, while maintaining superior productivity and persistence of the grass, several "novel" *N. lolii* strains have been tested and commercially released in Australia. Strains AR1 (E^{AR1}) and AR37 (E^{AR37}) have enhanced the grass productivity and persistence to levels similar to those reported by E^{WT} with minimal or no health problems to livestock.[11]

Phenolic compounds, also known as polyphenols or biophenols, are the largest group of secondary plant metabolites. However, their functions in plants are not completely understood. Phenolic compounds are the principal antioxidant agents in plants.[12] In addition, they exhibit a wide array of biological activities (e.g., antimicrobial,[13] allelopathic,[14] UV-β protector,[15] and pest deterrent[16]). Yet, little is known about the effect of fungal endophyte infection on plant phenolic quantity, identity, and activity.[2−4,17,18] Quantitative and qualitative changes have been reported. *Neotyphodium typhinum*-infected *Poa ampla* produced isoorientin, tricin, and tricin glycosides contrary to E^{-} plants.[18] Six different flavonoids and gentisic acid were detected in *Lolium multiflorum* E^{+} and not in the E^{-}.[17] Not only was *N. lolii* E^{WT} infection found to enhance the level of chlorogenic acid in *L. perenne*, but it had also increased the concentrations of polyphenol biosynthesis precursors, quinic and shikimic acids.[2,4] Such an enhancement is not specific to the E^{WT} strain, because E^{AR1} and E^{AR37} strains can also enhance quinic, shikimic, and chlorogenic acids in *L. perenne* to even greater levels than those reported in E^{WT}.[2,4]

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Despite the fact that phenolic compounds play a key role in extending the adaptability of the host to a wide range of environmentally different areas and in enhancing the host defense mechanisms,[1] the impacts of endophyte infection on the phenolic content have not been systematically investigated. How *N. lolii* alters the phenol levels and the antioxidant activity in *L. perenne* is not fully understood. In this paper, we have investigated the effects of *N. lolii* on the phenol content and antioxidant activity of *L. perenne*. Specifically, we determined the phenolic content and the antioxidant activity in *L. perenne* E^- and how the strains E^{WT}, E^{AR1}, and E^{AR37} affected them.

MATERIALS AND METHODS

Plant Samples. Seeds of *L. perenne* cv. Samson either E^- or infected with one of E^{AR1}, E^{AR37}, or E^{WT} strains were supplied by Dr. David Hume (AgResearch Limited, New Zealand) and were sown in plastic pots and raised to seedlings. The presence of endophyte was detected using the tissue-print immunoblot technique following the method of Hahn et al.[19] Seedlings not in accordance with the required endophyte strain were discarded. All pots were maintained in the shade house at Charles Sturt University, Orange, NSW, Australia, and watered uniformly. Leaves were clipped weekly to promote the production of tillers. Grown plants were transplanted into the experimental site, which consisted of five blocks (4 m^2 each); each block was divided into four 1 m^2 plots. In each plot 16 individuals of *L. perenne* E^-, 16 of *L. perenne* E^{AR1}, 16 of *L. perenne* E^{AR37}, and 16 of *L. perenne* E^{WT} were transplanted. Each fungal endophyte strain was distributed randomly. Eight weeks after transplanting, shoots of grasses from each treatment were harvested individually, pooled, thoroughly mixed, and air-dried in a ventilated oven at 35 °C until they achieved constant mass. Dried samples were ground in a motorized pulverizer to pass through a 1 mm sieve. The ground plant material was stored at (− 20 °C) until analysis.

Chemicals and Reagents. Reagents used were Folin−Ciocalteu reagent, 2,2′-diphenyl-1-picrylhydrazyl (DPPH•) radical, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{•+}$), and sodium nitrite (Sigma-Aldrich, Sydney, Australia); HPLC grade methanol and *n*-hexane (Mallinckrodt, Paris, KY, USA); anhydrous acetonitrile (Unichrome, Sydney, Australia); formic acid, hydrochloric acid (35%), Na_2CO_3, and $Na_2MoO_4{\cdot}2H_2O$ (Univar, Sydney, Australia); $AlCl_3$ (Aldrich, Milwaukee, WI, USA); chloroform (Chem-Supply, Adelaide, Australia); and NaOH and absolute ethanol (Biolab Melbourne, Australia). The water used in all analytical work was purified by a Modulab analytical model (Continental Water Systems Corp., Melbourne, Australia) water system.

Phenolic standards used were catechol, gallic acid, caffeic acid, *p*-coumaric acid, rutin, luteolin, chlorogenic acid, ferulic acid, syringic acid, sinapic acid, *trans*-cinnamic acid, benzyl alcohol, 3,4-dihydroxyphenyl acetic acid, *o*-coumaric acid, naringin, protocatechuic acid, homovanillic acid, *p*-anisic acid, hespiridin, salicylic acid, catechin hydrate, naringenin, benzoic acid, 4-hydroxyphenylacetic acid, neohesperidin, homovanillyl alcohol, hesperetin, and quercetin dihydrate (Sigma-Aldrich); tyrosol (Aldrich); hydroxytyrosol (Cayman, Ann Arbor, MI, USA); oleuropein and verbascoside (Extrasynthese, Genay, France); and *p*-hydroxybenzoic acid (Fluka, Buchs, Switzerland).

Most standards were dissolved in 70% aqueous methanol to prepare stock solutions of 1 mg/mL. Exceptions were quercetin dihydrate and luteolin, which were dissolved in absolute methanol, and rutin, which was dissolved in warm 70% aqueous methanol following the method of Obied et al.[20]

Preparation of Crude Phenolic Extracts. Air-dried ground material of *L. perenne* samples (E^-, E^{WT}, E^{AR1}, and E^{AR37}) were extracted following the method of Obied et al.[20] with some modifications as described below. Extractions were performed in triplicate. Two grams of the ground material was extracted individually with 20 mL of aqueous methanol (70% v/v) at room temperature (20−22 °C) for 30 min with uninterrupted stirring. After filtration, the resulting raffinate was re-extracted with 10 mL of aqueous methanol (70% v/v) of the same extraction solvent for 15 min and filtered into the first filtrate. The combined filtrate was defatted twice with 20 mL of *n*-hexane. The defatted extract was then filtered through GF/F filters followed by filtration through a 0.45 μm nylon syringe filter. All resulting crude extracts were stored at −20 °C until analysis.

Spectrophotometric Analyses. Prior to spectrophotometric analyses, crude extracts were diluted (1:1) with aqueous methanol (70% v/v) for the determination of total phenols using Folin−Ciocalteu reagent (diluted extract A) or (1:5) for the determination of *o*-diphenols, phenolic compound classes, and total flavonoids (diluted extract B). All spectrophotometric analyses including UV−vis scanning, determination of total phenols, *o*-diphenols, phenolic classes, and total flavonoids were performed with a Cary 50 UV−vis spectrophotometer, using Cary WinUV version 3 software (Varian, Australia).

UV−Vis Scanning. Spectra of the aqueous methanolic extracts were obtained at 200−500 nm in neutral, acidic, and alkaline solutions by diluting an aliquot (0.2 mL) of the respective diluted extract A to 10 mL with water or with either HCl (2%) or 2 M NaOH, respectively.

Determination of Total Phenols Using Folin−Ciocalteu Reagent. Total phenol contents in extracted samples of *L. perenne* either E^- or infected with E^{AR1}, E^{AR37}, or E^{WT} were determined as described earlier.[20] Briefly, either an aliquot (100 μL) of the diluted extract A or a blank (methanol 70% v/v) was added to a 10- mL volumetric flask containing 6−7 mL of water. Folin−Ciocalteu reagent (500 μL) was added, and after 1 min, 1.5 mL of aqueous Na_2CO_3 solution (20% w/v) was added. The flask was shaken, and the volume was made up to 10 mL with water. The flask was kept for 1 h at ambient temperature after the contents had been mixed thoroughly. Absorbance was read at 760 nm. Results were expressed as milligrams of gallic acid equivalent per gram dry weight of air-dried material (mg GAE/g). Each crude extract from each treatment was analyzed in triplicate.

Determination of Total Flavonoids. The quantification of total flavonoids in extracted samples of *L. perenne* either E^- or infected with E^{AR1}, E^{AR37}, or E^{WT} was determined according to the $AlCl_3$ colorimetric assay following ref 21 with minor modifications. An aliquot (500 μL) of the diluted extract B was added to a 5 mL volumetric flask and mixed with 150 μL of $NaNO_2$ (5% w/v). After 5 min, 150 μL of $AlCl_3$ (10% v/v) was added. After 1 min of mixing, NaOH (1 M; 1 mL) was added, and the total volume was made up to 5 mL with distilled water. The solution was mixed well, and the absorbance was measured against blank solution at 510 nm. Total flavonoids content was expressed as milligrams of quercetin equivalent per gram dry weight of air-dried material (mg QE/g). Each crude extract from each treatment was analyzed in triplicate.

Determination of *o*-Diphenols. *o*-Diphenol content in extracted samples of *L. perenne* either E^- or infected with E^{AR1}, E^{AR37}, or E^{WT} was determined following ref 20 with minor modifications. An aliquot (250 μL) of diluted extract B or blank (methanol 70% v/v) was mixed with 250 μL of $Na_2MoO_4{\cdot}2H_2O$ solution (5% w/v in ethanol 50%) in a 5-mL volumetric flask. After mixing, the volume was made up to 5 mL with ethanol 50%. After 15 min, the absorbance was measured at 370 nm. Results were expressed as milligrams of of caffeic acid equivalent per gram dry weight of air-dried material (mg CAE/g). Each crude extract from each treatment was analyzed in triplicate.

Determination of Different Phenolic Compound Classes. Determination of different phenolic compound classes in the extracted samples of *L. perenne* either E^- or infected with E^{AR1}, E^{AR37}, or E^{WT} was performed as described previously[20] with minor modifications. An aliquot (500 μL) of diluted extract B was mixed with 1 mL of aqueous ethanol (95% v/v) containing 0.1% HCl in a 10 mL volumetric flask, and the volume was made up to 10 mL with 2% HCl. Absorbance was measured at 280 nm to determine total phenolic compounds using gallic acid as standard, at 320 nm to determine hydroxycinnamic acid derivatives using caffeic acid as standard, and at 360 nm to estimate flavonols using quercetin as standard. Each crude extract from each treatment was analyzed in triplicate.

Extract Hydrolysis. Acid hydrolysis was conducted to free phenolic aglycones. Two milliliters of concentrated HCl was added to 12 mL of the E^- extract in a 100 mL round-bottom flask. The mixture was refluxed at 90 °C for 60 min. The mixture was allowed to cool before it was extracted four times with 20 mL of ethyl acetate. The combined ethyl acetate extract was dried with anhydrous Na_2SO_4. Ethyl acetate was evaporated under vacuum at 50 °C. The residue was dissolved in 10 mL of absolute methanol, filtered through a 0.45 μm nylon syringe filter, and stored at −20 °C until analysis using HPLC.

HPLC-DAD Online ABTS$^{\bullet+}$. Analysis was performed on a Varian Prostar 240 solvent delivery system equipped with a Varian Prostar 335 diode array detector (DAD) and a Varian Prostar 410 autosampler. The HPLC system was controlled by Star Chromatography workstation version 6.41 (Varian, Australia). A flow rate of 0.8 mL/min and an injection volume of 10 μL were used. The outflow from the DAD was connected to a reaction coil through a T-intersection. ABTS$^{\bullet+}$ (diluted from a stock solution of 3 mM to result in absorbance = 0.70 at 734 nm) was pumped to the reaction coil through the T-intersection by a Perkin-Elmer series 10 isocratic HPLC pump. PEEK reaction coil, 3.4 m × 0.178 mm i.d., was maintained at 37 ± 1 °C in a Varian HPLC column temperature controller. Detection of ABTS$^{\bullet+}$ absorbance was monitored at 414 nm by a Varian 9050 UV−vis detector. Data collected from the Varian 9050 UV−vis detector generated positive peaks by reversing the polarity of the analogue signal.

Sample analysis was performed by gradient elution on a 250 mm × 4.6 mm i.d., 5 μM, Gemini C-18 column (Phenomenex, Sydney, Australia) with a SecurityGuard (Phenomenex) guard cartridge. The mobile phases were freshly prepared, degassed under vacuum using Phenomenex nylon 45 μm membranes, and sonicated in a Sanophon ultrasonic bath (Ultrasonic Industries Pty. Ltd., Sydney, Australia) for 15 min prior to HPLC analysis. Solvent A was a 0.1% formic acid in 10 mM ammonium acetate solution, and solvent B was 0.1% formic acid in a mixture of acetonitrile/water (50 + 50, v/v). A gradient elution for a total run time of 67 min was used as follows: initial condition, 100% solvent A; solvent B increased to 25% over 20 min; then solvent B increased to 30% over 5 min; then solvent B increased to 80% over 40 min; and finally back to initial conditions in 2 min. The system was allowed to equilibrate for 15 min between runs.

HPLC-DAD-MS/MS. Samples were analyzed by an Agilent 1200 series liquid chromatographic equipment (Agilent Technologies, Waldbronn, Germany) by gradient elution on a 150 mm × 4.6 mm i.d., 5 μm, Alltima C-18 column (Alltech, Melbourne, Australia). The flow rate was 0.7 mL/min, and the injection volume was 5 μL. The mobile phase used was a gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile/water 50:50, v/v). The total run time was 70 min. A linear gradient was conducted as follows: from 0% solvent B to 5% solvent B in 7 min; isocratic for 3 min; then to 10% solvent B in 5 min; to 20% B in 5 min; to 30% B in 10 min; to 80% B in 25 min; back to initial composition 100% A in 5 min; then isocratic for 10 min. The DAD was set to record chromatograms at 235, 260, 280, 330, and 420 nm. The effluent from the DAD was directed to a 6410 triple-quadrupole mass analyzer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface. MS analysis was performed in the negative ion mode (m/z 100−1200) under the following conditions: nitrogen gas; gas temperature, 300 °C; gas flow rate, 12 L/min; nebulizer pressure, 45 psi; capillary voltage, 4 kV; cone voltage, 100 V. Data were analyzed using Agilent MassHunter workstation version B.01.04 2008 (Agilent Technologies, Waldbronn, Germany).

DPPH$^\bullet$ Radical-Scavenging Assay. DPPH$^\bullet$ scavenging activities of samples were evaluated following ref 22 with minor modifications. Diluted extracts (1:5; 50 μL) were added to an aqueous methanolic solution (82%) of DPPH$^\bullet$ (32 mg/L; 3 mL) in plastic macrocuvettes (1 cm). The macrocuvettes were covered, shaken well, and maintained in darkness for 60 min; the absorbance was measured at 517 nm. The percentage scavenging of DPPH$^\bullet$ was calculated according to the formula

$$\%\ \text{scavenging} = 100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$$

A five-point calibration curve was prepared using Trolox (50−500 μg/mL). Trolox equivalent antioxidant capacity (TEAC) was determined as the amount of Trolox equivalent to the amount of test substance that resulted in equal scavenging of DPPH$^\bullet$.

ABTS$^{\bullet+}$ Radical-Scavenging Assay. The stock solution of ABTS$^{\bullet+}$ was prepared as described previously[23] by mixing ABTS$^{\bullet+}$ reagent (7.0 mM) with potassium persulfate (2.45 mM). The solution was kept in darkness overnight. The working solution of ABTS$^{\bullet+}$ was prepared by diluting an aliquot of ABTS$^{\bullet+}$ stock solution to a final absorbance of 0.70 ± 0.02 at 734 nm. Diluted extracts (1:5; 50 μL) were added to 3 mL of ABTS$^{\bullet+}$ working solution in plastic macrocuvettes (1 cm). The macrocuvettes were covered, shaken well, and incubated at room temperature for 30 min; the absorbance was measured at 734 nm. The percentage scavenging of ABTS$^{\bullet+}$ was calculated according to the formula

$$\%\ \text{scavenging} = 100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$$

Data Analysis. Data are expressed as means of triplicates ± standard deviation. Statistical comparisons were made using one-way ANOVA and posthoc Duncan test. The Pearson correlation coefficient has been used to describe the correlation between variables. Data analyses were performed by Microsoft Excel and PASW Statistics package version 17.0 (SPSS Inc., Chicago, IL, USA). Results were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

UV−Vis Spectra. The UV−vis spectra of diluted extracts (1:100) provided preliminary data on the nature of the compounds present in *L. perenne* and enabled the choice of appropriate wavelengths for HPLC monitoring. UV−vis spectra of *L. perenne* extracts, either E^- or infected with E^{WT}, E^{AR1}, or E^{AR37}, were almost identical. Although the UV−vis spectrum is a very gross qualitative test, the high level of similarity among various extracts suggested that differences are more likely to be quantitative rather than qualitative. Figure 1A represents the UV−vis spectrum of E^- sample. Apart from the "chlorophyll *a*" band at 665 nm, the extract showed a major band at 330 nm with shoulders at 245, 265, and 300 nm. In acidic solution, a low-intensity broad shoulder was obvious around 420 nm. In alkaline solution, three major bands at 220, 265, and 380 nm and a wide shoulder at 475 nm were evident. The band at 330 nm had undergone a large bathochromic shift, 50 nm, which could be due to the presence of flavones and/or hydroxycinnamic acid derivatives.[24] Both chlorophylls and carotenes absorb in the 400−500 nm range, yet the large observed bathochromic shift, 55 nm, suggests a phenolic nature. Most probably this band is due to a degradation product as examination of the major peaks by HPLC-DAD did not show any compounds absorbing beyond 400 nm.

Phenolic Content and Antioxidant Capacity. A few studies have reported the phenolic content in grasses (e.g., *Lolium*[2,4,17]), but none has systematically described the content of phenolics and their antioxidant activity in *L. perenne*. In the present study, we report for the first time the phenolic content of *L. perenne* shoots. Methanolic extracts of *L. perenne* either E^- or infected with E^{WT}, E^{AR1}, or E^{AR37} contained substantial amounts of polyphenols (Table 1). Total phenols, *o*-diphenols, total flavonoids, flavonols, and hydroxycinnamic acids in *L. perenne* were variably affected by endophyte infection (Table 1). Total phenol content ranged between 13.6 and 16.1 (Folin−Ciocalteu) mg GAE/g dry weight and between 14.5 and 16.7 (absorbance at 280 nm) mg GAE/g dry weight. These values are close to those reported for *Festuca arundinacea* (Schreb) (tall fescue),[3] (current valid name being *Lolium arundinaceum* (Schreb)). Whereas the concentrations of *o*-

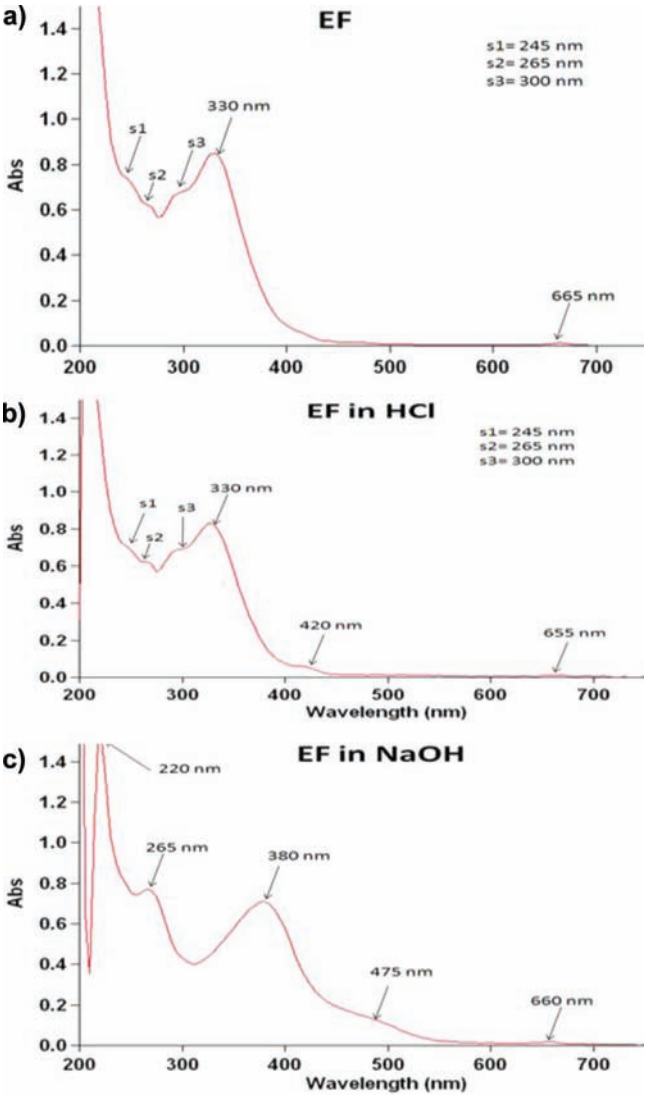

Figure 1. Representative UV−vis spectra of *L. perenne* E^- in hydroalcoholic (A), acid (HCl, 2%) (B), and alkali (NaOH, 2 M) (C) solutions.

diphenols, flavonols, and hydroxycinnamic acids were in the range of 0.011−0.018% DW, total flavonoids were 10-fold greater at 0.107−0.155% DW. Although this high concentration of total flavonoids, as measured using the $AlCl_3$ method, is exceptional, some earlier studies reported a similar trend in some Chinese herbs.[25] Flavonoids are apparently the most abundant class of polyphenols in our samples (Table 1). No previous studies on grasses, in general, tried to quantitate different polyphenol classes. Hence, we cannot determine whether the abundance of flavonoids is characteristic for *Lolium* or a common feature in grasses. Whereas the relative difference between the highest total phenol content, E^{AR1}, and the lowest total phenol content, E^{WT}, was 15%, the relative difference in total flavonoids was around 30%.

Samples could be ranked according to their phenol content as follows: $E^{AR1} > E^{AR37} \geq E^- > E^{WT}$. Consistently, the E^{AR1} sample showed the highest phenol content in all employed assays, whereas the E^{WT} sample showed the lowest content. No significant difference occurred between E^- and E^{AR37} samples in all assays ($p < 0.05$) apart from their flavonol content. The phenol content of E^{WT} was significantly less than that of E^- apart from total phenols (at 280 nm absorbance) and flavonol content. Concurrently, the phenol content of E^{AR1} was significantly greater than that of E^- except for *o*-diphenols and total flavonoid content. On the basis of the results of cluster analysis of phenol content (Figure 2), E^- and E^{AR37} have been grouped in one cluster, whereas E^{AR1} and E^{WT} have been grouped separately.

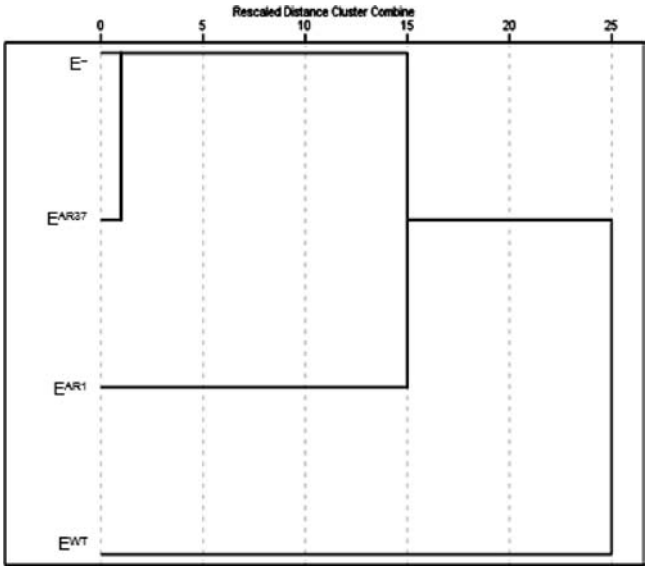

Figure 2. Cluster analysis plotted as dendrogram using average linkage (between groups).

Antioxidants can play a pivotal role in enhancing plant stress tolerance.[26] Endophytes can generate reactive oxygen species that stimulate antioxidant production in their host plants.[27] The antioxidants in turn are responsible for protecting the host from oxidative stress.[27] Antioxidant activity is a multidimensional phenomenon that requires more than one assay for accurate evaluation.[22] The antioxidant capacity of methanolic extracts of *L. perenne* was determined using two stable free radical scavenging model systems, namely, $DPPH^•$ and $ABTS^{•+}$ (Table 2). Both assays showed the same relative ranking of

Table 1. Phenolic Content of *L. perenne* E^- or Infected with Fungal Endophyte *N. lolii* Strain E^{WT}, E^{AR1}, or E^{AR37}[a]

extract	total phenols (FC)[b]	total phenols (280 nm)[b]	*o*-diphenols[c]	total flavonoids[d]	flavonols[d]	HCA[c]
E^-	14.6 ± 0.5 a	15.3 ± 0.7 a	16.8 ± 1.3 a	128.8 ± 6.6 a	10.8 ± 0.4 a	11.8 ± 0.4 a
E^{WT}	13.6 ± 0.7 b	14.5 ± 0.5 a	14.7 ± 0.9 b	107.4 ± 5.9 b	10.5 ± 0.5 a	10.9 ± 0.6 b
E^{AR1}	16.1 ± 0.5 c	16.7 ± 0.5 b	18.4 ± 1.4 a	155.6 ± 10.0 a	12.5 ± 0.3 b	13.0 ± 0.3 c
E^{AR37}	15.4 ± 0.6 ac	15.2 ± 0.9 a	17.6 ± 0.8 a	140.2 ± 10.9 a	11.7 ± 0.7 b	12.2 ± 0.6 a

[a]Different letters in the same column indicate significantly different ($p > 0.05$) mean ± SD. HCA, hydroxycinnamic acids; FC, Folin−Ciocalteu. [b]mg GAE/g DW. [c]mg CAE/g DW. [d]mg QE/g DW.

Table 2. Trolox Equivalent Antioxidant Capacity (TEAC) Values for *L. perenne* E^- or Infected with *N. lolii* Strain E^{WT}, E^{AR1}, or E^{AR37} in DPPH$^\bullet$ and ABTS$^{\bullet+}$ Scavenging Assays[a]

	E^-	E^{WT}	E^{AR1}	E^{AR37}
DPPH$^\bullet$	105.6 ± 2.1 a	94.2 ± 3.9 b	107.2 ± 2.0 a	106.0 ± 3.6 a
ABTS$^{\bullet+}$	84.9 ± 2.2 a	79.4 ± 4.1 b	92.5 ± 2.6 c	86.3 ± 4.7 a

[a]Different letters in the same row indicate significantly different mean ± standard deviation ($p < 0.05$). μmol Trolox equiv/g DW.

extracts, which is the same ranking observed for total phenol content: $E^{AR1} > E^{AR37} > E^- > E^{WT}$. In the ABTS$^{\bullet+}$ scavenging assay, E^{AR1} was significantly ($p < 0.05$) greater than all other samples. In both ABTS$^{\bullet+}$ and DPPH$^\bullet$ scavenging assays, E^{WT} was significantly ($p < 0.05$) lower than the other samples. The ABTS$^{\bullet+}$ scavenging assay demonstrated higher sensitivity and ability to discriminate among different samples compared with the DPPH$^\bullet$ scavenging assay (Table 2).

Values from DPPH$^\bullet$ and ABTS$^{\bullet+}$ scavenging assays were strongly correlated ($r = 0.85$). Overall, a strong correlation between phenol contents and antioxidant capacity prevailed. Total phenol content (at 280 nm absorbance) showed the lowest correlation with antioxidant capacity assays ($r = 0.26$ with ABTS$^{\bullet+}$ scavenging assay and $r = 0.61$ with DPPH$^\bullet$ scavenging assay). This can be explained by the low specificity of this assay in which all nonphenolic species absorbing UV close to 280 nm are interfering. Meanwhile, the ABTS$^{\bullet+}$ scavenging assay had a higher correlation with all other phenol content assays ($r = 0.93–0.99$) than the DPPH$^\bullet$ scavenging assay. Pearson's correlation coefficients with total phenols (Folin–Ciocalteu) and total flavonoids were 0.95 and 0.97, respectively, compared with 0.85 and 0.91 for the DPPH$^\bullet$ scavenging assay. For the ABTS$^{\bullet+}$ scavenging assay, the highest correlation was found with hydroxycinnamic acid content ($r = 0.99$), whereas the DPPH$^\bullet$ scavenging assay had the highest correlation with *o*-diphenols ($r = 0.96$).

Belesky et al.[3] have studied the impact of endophyte infection on the phenol content of *Lolium arundinaceum*. Shoots and roots of *L. arundinaceum* infected with *Neotyphodium coenophialum* E^{WT} had nearly 10% more total phenols than their E^- counterparts. Moreover, the response to endophyte infection on *L. arundinaceum* phenol content differed according to genotype, the type of tissue investigated (shoots and roots), site conditions such as shading and defoliation, and abiotic stress factors such as phosphorus deficiency.[3,28] Ponce et al.[23] found that endophyte infection of *L. multiflorum* influences both the quantity and the variety of polyphenols. In contrast, the present study indicates that endophytic infection can have either positive or negative effects on phenol content in *L. perenne* based on the strain of endophyte involved.

Phenolic and ABTS$^{\bullet+}$ Scavenging Profiles of *L. perenne*. Literature reports on the phenolic composition of *L. perenne* are either scarce or incomplete. Rasmussen et al.[2,4] described the metabolomics analysis of *L. perenne* with no detailed description of the composition of phenolic content. In the present paper, we report for the first time the phenolic profile of *L. perenne* (Figure 3). Chromatograms at 260 nm provided more than 20 peaks (Figure 3A). The few early-eluting peaks (peak 1–4) were invisible at 325 nm (Figure 3B). However, the majority of the peaks were visible at 325 nm, supporting our earlier findings that hydroxycinnamic acids and flavonoids are the principal components of *L. perenne* extracts. The ABTS$^{\bullet+}$ scavenging profile of *L. perenne* (Figure 3C) shows a few peaks. The major contributors to *L. perenne* extract

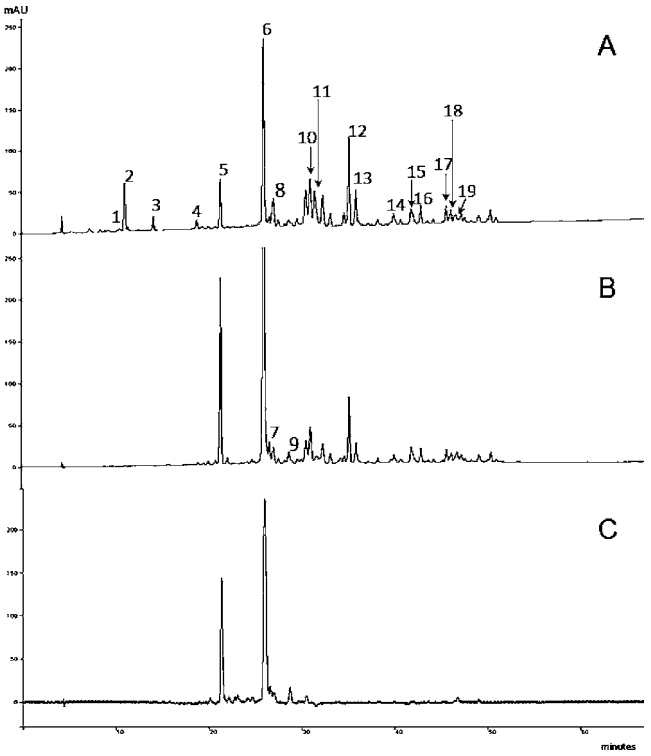

Figure 3. HPLC-DAD-ABTS$^{\bullet+}$ chromatograms of *L. perenne* E^-: (A) chromatogram detected at 260 nm; (B) chromatogram detected at 325 nm; (C) reversed chromatogram for ABTS$^{\bullet+}$ scavenging activity detected at 414 nm.

antiradical activity are peaks 5, neochlorogenic acid, and peak 6, chlorogenic acid (Table 3). The ABTS$^{\bullet+}$ scavenging activity can provide valuable insights into the phenolic chemistry. Studying the structure–activity relationship of 31 phenolic standards (data not shown), we found that ABTS$^{\bullet+}$ scavenging activity (a peak in the ABTS$^{\bullet+}$ scavenging profile) requires a catechol moiety either as an *o*-hydroxy or as an *o*-methoxy group. Monohydroxyphenols were not ABTS$^{\bullet+}$ scavengers. These findings are in accordance with previous results.[29]

UV–vis and MS spectra were compared with available phenolic standards and literature data to identify the major peaks in the phenolic profile of *L. perenne* (Figure 3; Table 3). Major peaks can be grouped on the basis of their chemical class into three groups: Group 1 comprises the early-eluting peaks 1–4, most likely simple monohydroxyphenol derivatives on the basis of their UV–vis spectra (Table 3) and lack of ABTS$^{\bullet+}$ scavenging activities (Figure 3C). These compounds are more hydrophilic than chlorogenic acid, earlier eluting, suggesting a highly polar nature. Group 2 includes peaks 5–7, 14, and 19, which are hydroxycinnamic acid derivatives. Chlorogenic (peak 6) and ferulic acids (peak 14) were identified by comparison with reference standards. Neochlorogenic (peak 5), cryptochlorogenic (peak 7), and diferulic acids (peak 19) were determined by matching their UV–vis and MS spectra, ABTS$^{\bullet+}$

Table 3. Compounds Detected in *L. perenne* E$^-$ or Infected with *N. lolii* Strain E^{WT}, E^{AR1}, or E^{AR37}

peak	compound	RT	λ_{max}	FR[a]	MW	identification	ref
1	unknown	9.6	258	−	134		
2	unknown	10.2	268	−	342		
3	unknown	13.6	258	−	192		
4	unknown	18.5	278	−	204		
5	neochlorogenic acid	21.3	305s, **325**	+	354	UV, MS, RRT	30
6	chlorogenic acid	25.8	302s, **326**	+	354	standard	
7	cryptochlorogenic acid	26.5	307s, **327**	+	354	UV, MS, RRT	30
8	luteolin triglycoside	27.0	265 and 352	+	756	UV, MS	
9	isorhamnetin triglycoside	28.6	252, 267s, and 352	+	786	UV, MS	
10	flavonoid diglycoside	30.9	254, 268s, and 347	−	640	UV, MS	
11	flavonoid triglycoside	31.4	254, 268s, and 353	−	712	UV, MS	
12	flavonoid derivative	35.0	265 and 353	−	696	UV, MS	31
13	flavonoid triglycoside	35.8	265 and 355	−	726	UV, MS	
14	ferulic acid	38.2	317	+	194	standard	
15	rutin	41.7	256 and 356	+	610	standard	
16	flavonoid dihexoside	42.8	265 and 345	−	624	UV, MS	
17	kaempferol-3 *O*- deoxyhexosylhexoside	45.5	265 and 350	−	594	UV, MS	31
18	kaempferol-3 *O*- deoxyhexosylhexoside	46.0	265 and 350	−	594	UV, MS	31
19	diferulic acid	46.6	245 and 323	+	386	UV, MS	32

[a]FR, ABTS$^{\bullet+}$ scavenging activity.

scavenging activities, and relative retention times with literature data (Table 3). Group 3 is made up of other peaks that showed two UV absorption bands: band I (345−355 nm) and band II (250−270 nm). The occurrence of two UV absorption bands is characteristic of flavones and 3-*O*-substituted flavonols.[33] By comparison with a reference standard, the compound represented by peak 15 was identified as rutin. Flavonoids mono-, di-, and triglycosides were abundant in *L. perenne* (Table 3). Acid hydrolysis of extracts produced a variety of flavonols and flavones, including luteolin, apigenin, quercetin, kaempferol, isorhamnetin, and traces of tricin. The absence of NMR data prevented us from elucidating the structures of flavonoid glycosides (Table 3). Whereas the Mexico-grown *L. arundinaceum* demonstrated a flavonoid pattern similar to that of our samples with quercetin, luteolin, apigenin, and tricin glycosides,[34] the chief flavonoid of the New Zealand-grown *L. arundinaceum* was isorhamnetin.[35] On the contrary, Ponce et al.[17] have isolated 13 flavonoids from *L. multiflorum* including 8 new ones.[17] Although Ramussen et al.[2] reported the presence of anthocyanins in the Canada-grown *L. perenne*, our samples were devoid of any anthocyanins. This can be attributed to either geographical or seasonal variations as leaf anthocyanins are known to peak in autumn.

Due to the paucity of information on the phenolic composition of *L. perenne*, we compared our findings with published data on other species of *Lolium*[3,17,35] and other grasses from the family Poaceae.[18,36] We conducted a chemical screening of *L. perenne* samples using available commercial standards and compounds known to be present in grasses.[31] We confirmed the absence of the following unbound simple phenols: catechol, tyrosol, hydroxytyrosol; unbound benzoic acids benzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dihdroxyphenylacetic acid, gallic acid, protocatechuic acid, syringic acid, salicylic acid; and unbound cinnamic acid derivatives *trans*-cinnamic acid, sinapic acid, *o*-coumaric acid, *p*-coumaric acid, caffeic acid, sinapic acid, methyl cinnamate, and verbascoside. Apigenin, catechin, hesperetin, kaempferol, luteolin, naringenin, quercetin, and tricin were not detected in *L. perenne* extracts. In general, flavonoid aglycones are rarely found in plants.[24]

Chlorogenic acid (peak 6) was the most commonly occurring peak in all tested samples, matching with the findings of Rasmussen et al.[2,4] Chlorogenic acid was determined as a major component of *L. arundinaceum*[37] but not in *L. multiflorum*.[17] Isomers of chlorogenic acid (5-*O*-caffeoylquinic acid), 3-*O*-caffeoylquinic acid (neochlorogenic acid), and 4-*O*-caffeoylquinic acid (cryptochlorogenic acid) are reported herein for the first time in *L. perenne*. Ferulic acid (peak 14), rutin (peak 15), and diferulic acid (peak 19) occur as minor components. Flavone and flavonol glycosides constituted the other peaks in *L. perenne* chromatograms (Table 3 and Figure 3).

Effect of Endophyte Infection on Phenolic Profiles of *L. perenne* Samples. Phenolic profiles in plants change according to environmental, geographical, seasonal, and varietal factors. Agronomic practices and pathogenic infestations also affect phenolic profiles. Searching for qualitative differences in the phenolic composition among E$^-$ and different endophyte strain-infected shoots, we studied the phenolic profiles by LC-DAD-ABTS$^{\bullet+}$ and LC-MS/MS techniques. Chromatograms were collected at 220, 260, 280, and 325 nm. On the basis of the peak intensity and number of peaks, chromatograms at 260 nm were selected to compare the phenolic profiles of different samples. Figure 4 shows that all extracts had similar phenolic profiles (peak identity), whereas differences were mainly quantitative (peak intensity). Hence, *N. lolii* infection affects only the level of polyphenol expression rather than changing the number or type of polyphenols in *L. perenne*. Our results contradict with the earlier findings of Ju et al.[18] and Ponce et al.,[17] who reported significant qualitative along with quantitative changes in the phenolic composition of *P. ampla* and *L. multiflorum* infected with their respective *Neotyphodium* spp. Knowing that not only were different plants used by Ju et al. and Ponce et al. but different species of *Neotyphodium* were used as well, we conclude that the influence of endophyte infection on polyphenol composition of grasses is case specific

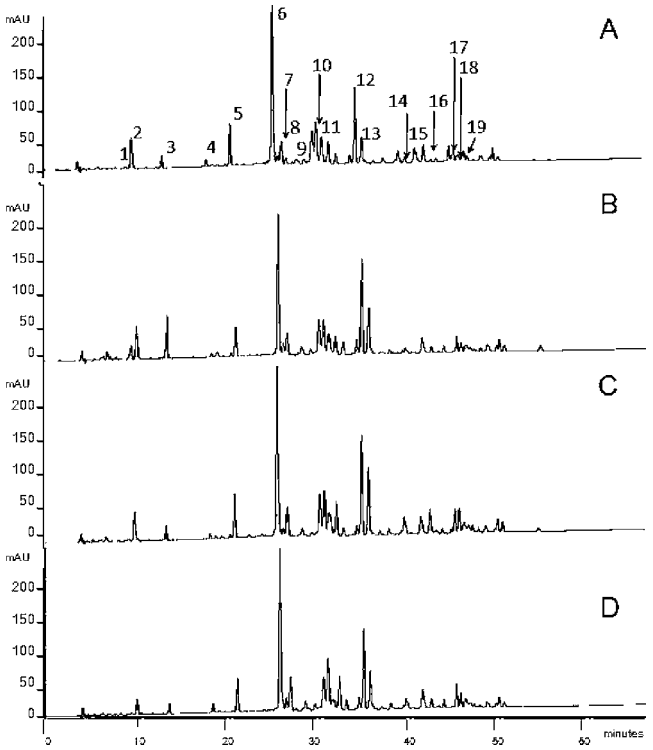

Figure 4. HPLC chromatograms at 260 nm of the phenolic profiles of *L. perenne* E^- (A) or infected with *N. lolii* strain E^{WT} (B), E^{AR1} (C), or E^{AR37} (D).

and depends on several factors including, but not restricted to, plant type, endophyte species, and endophyte strain.

It was also important to know whether the level of individual polyphenols follows the same pattern of total phenols. To test this hypothesis, we studied the change in the peak area (concentration) of the three major peaks (5, 6, and 12). Whereas peaks 5 and 6 had the same pattern and were following the total phenol trend, peak 12 did not (Figure 5). Thus, within the same plant−endophyte system, individual polyphenols were affected differently. Consequently, the effect of endophyte infection on total phenol content should not be used to extrapolate to the effect on individual compounds.

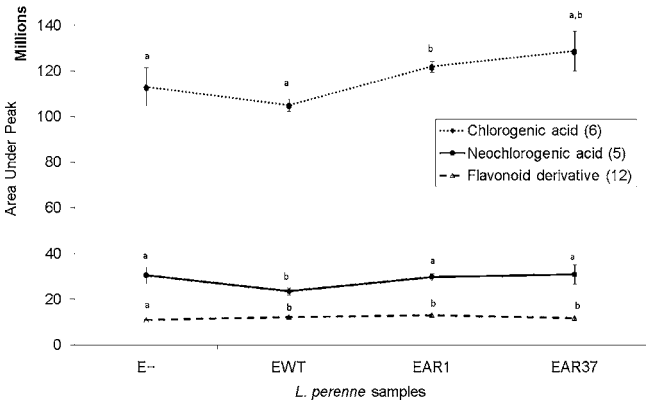

Figure 5. Concentrations of chlorogenic acid (peak 6), neochlorogenic acid (peak 5), and flavonoid in derivative (peak 11) in different *L. perenne* samples. Concentrations are represented as area under the peak units obtained from chromatograms at 325 nm. Data points are the average of three separate measurements, and error bars represent standard deviation of means; values for the same compound that are significantly different from each other at $p < 0.05$ have different letters.

We have reported the phenolic profile, phenolic content, and antioxidant capacity of shoots of *L. perenne* as a function of endophyte infection tested with three strains of *N. lolii*. Infection with *N. lolii* significantly influenced *L. perenne* phenolic content. The change of total phenol content ranged between 3 and 17%, whereas antioxidant capacity changes were 7−8%. Changes in phenolic composition were merely quantitative. In contrast to earlier studies,[2,4,17] we found that endophyte infection does not necessarily increase the phenol content. Endophyte infection can have zero, positive, or negative effect on phenol content depending on the endophyte strain. Simple phenols, hydroxycinnamic acids, and flavonoid glycosides were determined in *L. perenne* extracts. Although *L. perenne* has a wide range of flavonol and flavone glycosides, chlorogenic acid and its derivatives were the major compounds responsible for antioxidant activity.

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ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CAE, caffeic acid equivalent; DAD, diode array detector; DPPH, 2,2′-diphenyl-1-picrylhydrazyl; DW, dry weight; E^+, endophyte infected; E^-, endophyte-free; E^{AR1}, *Neotyphodium lolii* strain AR1; E^{AR37}, *Neotyphodium lolii* strain AR37; E^{WT}, *Neotyphodium lolii* strain wild type; F, *Festuca*; GAE, gallic acid equivalent; L, *Lolium*; N, *Neotyphodium*; QE, quercetin equivalent; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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