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Nitrification Inhibitors from the Root Tissues of *Brachiaria humidicola*, a Tropical Grass

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Nitrification inhibitory activity was found in root tissue extracts of *Brachiaria humidicola*, a tropical pasture grass. Two active inhibitory compounds were isolated by activity-guided fractionation, using recombinant *Nitrosomonas europaea* containing *luxAB* genes derived from the bioluminescent marine gram-negative bacterium *Vibrio harveyi*. The compounds were identified as methyl-*p*-coumarate and methyl ferulate, respectively. Their nitrification inhibitory properties were confirmed in chemically synthesized preparations of each. The IC_{50} values of chemically synthesized preparations were 19.5 and 4.4 μM, respectively. The ethyl, propyl, and butyl esters of *p*-coumaric and ferulic acids inhibited nitrification, whereas the free acid forms did not show inhibitory activity.

KEYWORDS: ***Brachiaria humidicola*; nitrification inhibitor; methyl ferulate; methyl-*p*-coumarate**

INTRODUCTION

Nitrification is an important biological process in global nitrogen cycling whereby ammonia is converted to nitrite and nitrate by nitrifying bacteria (species of *Nitrosomonas* and *Nitrobacter*). The nitrification products are vulnerable to leaching and denitrification: an estimated 45% of applied fertilizer is lost by leaching (*1*) and 10−30% by denitrification (*2*). If the nitrification process is inhibited or slowed, then plants have adequate time to take up fertilizer N; N recovery and uptake are substantially improved and NO_3^- pollution problems are reduced (*3*). Considerable evidence shows that plants produce secondary metabolites that inhibit nitrification; e.g., grasslands display low nitrification rates and produce phenolic acids and flavonoids that inhibit nitrification (*3*).

Creeping signal grass or false creeping paspalum (*Brachiaria humidicola* [Rendle] Schweick) is a native pasture grass of Africa and is found from southern Sudan and Ethiopia in the north to South Africa and Namibia in the south. It is also grown widely in the humid-tropical countries of South America, the Pacific Islands, and South East Asia and in coastal Northern Australia (*4*). This tropical grass is grown widely not only as permanent pasture and as ground cover for the control of erosion and weeds but also for its adaptability to acid infertile soils and waterlogged habitats, particularly in the South American Cerrados (*5*). Lower levels of NO_3−N were found in fields of *B. humidicola* than in fields of other forage grasses (*Melinis minutiflora*, *Andropogon gayanus*, and *Brachiaria decumbens*) in the acid soils of Colombia; also, *B. humidicola* responded less well to the application of inorganic fertilizer N than did the other forage grasses (*5*, *6*). *Brachiaria humidicola* inhibits nitrification by suppressing the function of the nitrifying bacteria in the soil, and it keeps soil nitrogen in the NH_4^+ form (*7*). Recently, it was shown that compounds released from the roots of *B. humidicola* are mainly responsible for its inhibitory effect on soil nitrification (*8*) and that root tissue extracts of *B. humidicola* have substantial inhibitory effects on nitrification. Our investigation was aimed at isolating these nitrification inhibitors from the root tissues of *B. humidicola.*

MATERIALS AND METHODS

Chemicals. Ferulic acid and *p*-coumaric acid were purchased from Sigma (St. Louis, MO) and ICN Biomedicals (Aurora, OH), respectively. All the solvents and other chemicals used were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless otherwise stated.

Plant Materials. Seeds of *B. humidicola* (Rendle) Schweick (CIAT 679) were germinated, at 25 °C with RH 75%, in sand−vermiculite mixture (3:1) in trays and watered with distilled water. Two-week-old plants were transferred to aerated nutrient solution. The composition of the nutrient solution (mg L^{-1}) was 38.31:31.02:10.5:36.93:15.1:0.57:0.078:2.35:0.126:0.220 KH_2PO_4:K_2SO_4:$CaCl_2 \cdot 2H_2O$:$MgSO_4 \cdot 7H_2O$:FeEDTA:H_3BO_3:$CuSO_4 \cdot 5H_2O$:$MnSO_4 \cdot 6H_2O$:$Na_2MoO_4 \cdot 2H_2O$:$ZnSO_4 \cdot 7H_2O$. Nitrogen at 1 mmol L^{-1} was added as $(NH_4)_2SO_4$ to the nutrient solution. The pH of the nutrient solution was adjusted to 5.0, and the solution was constantly aerated. The nutrient solution was replaced with fresh solution at weekly intervals. The plants were grown in 50 L tanks on styrofoam blocks with 10 holes and 4 plants per hole, supported with sponge. Actively growing roots were cut from the plants once in

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3 months for 1 year, washed twice with ion-exchange water, and allowed to drain at room temperature for 30 min before being stored in a freezer at −20 °C until use.

Extraction of Nitrification Inhibition Activity. Roots of *B. humidicola* (from the freezer) were cut into small pieces (approximately 1 cm long) and blended in a mixer with 70% methanol (MeOH) in ultrapure water. The extracts were filtered through Whatman No. 1 filter paper, and the MeOH was removed in vacuo on a rotary evaporator for 45 min at 35 °C. The aqueous remainder was centrifuged at 15000*g* for 20 min, and the supernatants were partitioned repeatedly against ethyl acetate (EtOAc). The organic phases were combined, and the EtOAc was removed in vacuo on a rotary evaporator at 35 °C. The residues were dissolved in a minimum volume of MeOH and stored in a freezer at −20 °C; aliquots of these samples (100 μL) were further concentrated on a centrifugal evaporator (model CVE-200D, Eyela, Tokyo, Japan) to 25 μL in dimethyl sulfoxide (DMSO) for bioassay.

Nitrification Inhibition Bioassay. The nitrification inhibition (NI) activity of the samples was determined using a modified bioassay that utilizes recombinant luminescent *Nitrosomonas* (*8*, *9*). The detailed methodology for the detection, quantification, and expression of NI activity has been described previously (*8*). The NI activity of the samples is expressed in units defined in terms of the action of a standard inhibitor, allylthiourea (AT); the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one AT unit of activity (*8*).

Isolation of NI Activity. Crude extracts containing NI activity were dissolved in 5% MeOH before being loaded onto a reversed-phase column (25 cm × 2.8 cm, Wakosil 40C18, Wako). The column was eluted with 200 mL each of 5%, 10%, 20%, 40%, 60%, 80%, and 100% MeOH. All the above fractions were dried in vacuo at 35 °C and the residues were collected in MeOH (1 mL); aliquots of these fractions (50 μL) were used for bioassay. The active fractions were further purified by an HPLC on a Jasco Gulliver HPLC system consisting of a PU-1580 reciprocal pump, UV-1570/1575 UV detector, and 807-IT integrator with TSKgel Super-ODS (4.6 mm × 100 mm or 10 mm × 100 mm) columns (Tosoh, Japan); monitoring was performed at a wavelength of 220 nm. The column was eluted with a linear-gradient mobile-phase system (10−30% acetonitrile), and all the peaks and troughs were checked for activity.

Instrumental Analyses. The UV absorption spectra of the compounds in methanol were recorded on a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). The mass (MS) spectra were recorded on a GCMS-QP2010 spectrometer (Shimadzu) by direct electron ionization (EI) at an ionization energy of 70 eV. The ^{1}H NMR, ^{13}C NMR, nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra at 298 K were recorded on an Avance 500 spectrometer (Bruker Biospin, Karlsruhe, Germany) equipped with a CryoProbe; the pulse sequences and software provided by the manufacturer were used.

NI Activity of Free Acids and Synthesized Esters. C_1−C_4 alkyl esters of *p*-coumaric acid and ferulic acid were synthesized by acid esterification (*10*). Briefly, *p*-coumaric acid or ferulic acid (0.5 g) was dissolved in 10 mL of acidified (0.1 N HCl) methanol, ethanol, 1-propanol, or 1-butanol. The solution was incubated at 37 °C for 24 h. The volatiles were removed thoroughly with a centrifugal evaporator. The reaction product was then purified using a Sep-Pack C18 cartridge (Waters, Milford, MA; purity >95%, HPLC), and the molecular mass was confirmed by EI-MS. The NI activity of the free acids (*p*-coumaric acid and ferulic acid) and various ester forms (C_1−C_4 alkyl esters of *p*-coumaric acid and ferulic acid) was assayed, and the inhibitory concentration 50% (IC_{50}) values (mean results of three replications) were calculated.

RESULTS AND DISCUSSION

Roots of *B. humidicola* were extracted initially with 70% MeOH. This was followed by solvent partitioning to obtain 35−40 AT units of NI activity g^{-1} of dried roots. The crude extracts with NI activity were further fractionated by activity-guided fractionation. Upon fractionation by reversed-phase column chromatography, definite activity was detected in the 60% MeOH effluent. This active fraction was further purified by reversed-phase HPLC using a 10−30% acetonitrile gradient mobile phase system. Finally, two compounds (**1** and **2**) were isolated as having NI activity. The dry weights of **1** and **2** were 0.8 and 0.4 mg, respectively, and in the bioassay 1 mg of **1** and **2** was equivalent to 885 and 535 AT units of NI activity (21% and 6% recovery), respectively.

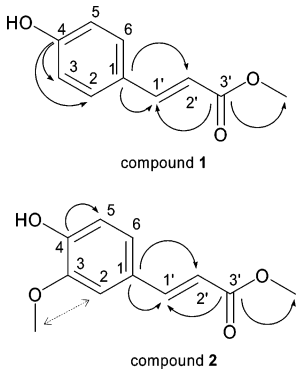

Figure 1. Chemical structures of methyl-*p*-coumarate (**1**) and methyl ferulate (**2**). Arrows indicate important NMR correlations. Solid arrows are selected HMBC correlations (from C to H), and the broken arrow is a NOESY correlation.

The physicochemical properties of compounds **1** and **2** were as follows:

Compound **1**: pale yellow gum; UV λ_{max} (CH_3OH) nm 308; ^{1}H NMR (500.1 MHz, CD_3OD) δ 3.76 (3H, *s*, OCH_3), 6.33 (1H, *d*, J = 16.0 Hz, H-2′), 6.80 (2H, *brd*, J = 9.0 Hz, H-3 and H-5), 7.45 (2H, *brd*, J = 9.0 Hz, H-2 and H-6), 7.61 (1H, *d*, J = 16.0 Hz, H-1′); ^{13}C NMR (125.8 MHz, CD_3OD) δ 51.7 (OCH_3), 114.6 (C-3 and C-5), 116.5 (C-2′), 126.8 (C-1), 130.9 (C-2 and C-6), 146.3 (C-1′), 161.1 (C-4), 169.5 (C═O); EIMS *m*/*z* (%) 178 $[M]^+$ (66), 147 (100), 119 (41), 91 (34), 65 (21). Assignments of the chemical shifts of ^{1}H and ^{13}C NMR spectra were confirmed by 2D-NMR analyses, NOESY, HMBC, and HSQC. These data agreed well with the data as previously reported (*11*, *12*, *13*). Therefore, compound **1** was identified as methyl (*E*)-3-(4-hydroxyphenyl)prop-2-enoate [methyl-*p*-coumarate, **1**, **Figure 1**].

Compound **2**: Pale yellow gum; UV λ_{max} (CH_3OH) nm 325 and 295 (shoulder); ^{1}H NMR (500.1 MHz, CD_3OD) δ 3.76 (3H, *s*, $COOCH_3$), 3.89 (3H, *s*, $ArOCH_3$), 6.36 (1H, *d*, J = 15.9 Hz, H-2′), 6.80 (1H, *d*, J = 8.1 Hz, H-5), 7.07 (1H, *dd*, J = 2.0, 8.1 Hz, H-6), 7.18 (1H, *d*, J = 2.0 Hz, H-2), 7.61 (1H, *d*, J = 15.9 Hz, H-1′); ^{13}C NMR (125.8 MHz, CD_3OD) δ 52.0 ($COOCH_3$), 56.0 ($ArOCH_3$), 111.5 (C-2), 115.0 (C-2′), 116.3 (C-5), 123.9 (C-6), 127.8 (C-1), 146.8 (C-1′), 149.2 (C-3), 150.4 (C-4), 169.8 (C═O); EIMS *m*/*z* (%) 208 $[M]^+$ (100), 177 (66), 145 (55), 117 (24), 89 (20), 77 (13). Assignments of the chemical shifts of ^{1}H and ^{13}C NMR spectra were confirmed by 2D-NMR analyses, NOESY, HMBC, and HSQC. These data agreed well with the data as previously reported (*12*, *13*). Hence, compound **2** was identified as methyl (*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate [methyl ferulate, **2**, **Figure 1**].

Compounds **1** and **2** exhibited definite NI activity, but it was not possible to estimate their IC_{50} values because of the small amounts purified. To confirm the NI properties of these two phenolic compounds, we chemically synthesized both through esterification of the corresponding free acids. These preparations were also found to have definite NI activity, with IC_{50} values of 19.5 and 4.4 μM, respectively. Thus, it was proved that compounds **1** and **2** were both NI-active.

Table 1. IC_{50} (in μmol) of the Chemically Synthesized Methyl-*p*-coumarate, Methyl Ferulate, and Other Ester Forms and Free Acids

compound	IC_{50} (in μM)[a]
p-coumaric acid	>10000
methyl-*p*-coumarate	19.5 ± 0.50
ethyl-*p*-coumarate	16.0 ± 0.50
propyl-*p*-coumarate	16.0 ± 1.00
butyl-*p*-coumarate	36.0 ± 1.50
ferulic acid	>10000
methyl ferulate	4.4 ± 0.10
ethyl ferulate	0.2 ± 0.08
propyl ferulate	2.2 ± 0.10
butyl ferulate	4.2 ± 0.06

[a] Mean ± standard deviation.

As demonstrated in **Table 1**, various alkyl esters (C_1-C_4) of *p*-coumarate and ferulate exhibited NI activity. Among the esters tested, ethyl and/or propyl esters showed the highest NI activity, but their free acids were not active. It is well-known that in *Nitrosomonas europaea*, the nitrification enzymes and related electron carrier system are located in the periplasm or inner membrane (*14*). Ethyl or propyl esters may have suitable structures or polarity for penetrating the bacterial cell. In contrast, the presence of a polar −COOH group in the free acids might make their permeability too low to approach the action point. Similarly, Daayf et al. has reported that the methyl esters of phenolic acids are more toxic to fungi than are their corresponding free acids (*15*), and Kikuzaki et al. observed an increase in antioxidant activity when ferulic acid was esterified (*16*).

Ferulic acid and *p*-coumaric acid play important roles in the structural integrity of the plant cell wall matrix and represent factors that partly limit the biodegradability of nonlignified plant cell-wall polysaccharides (*17*). Both of these acids are found widely distributed in nature, e.g., in root exudates of *Arabidopsis thaliana* (*18*) and in the rhizomes and roots of black cohosh (*Actaea racemosa* L., *19*), as secondary metabolites of plants, and exhibit considerable antimicrobial effects against fungi, bacteria, and viruses. However, the methyl ester form is rare and is found only occasionally in some herbaceous and medicinal plants (*20*).

Nevertheless, some literature is available on compounds **1** and **2**. For example, Seifert and Unger (*21*) isolated compound **1** from the aerial parts of woad (*Isatis tinctoria* L., a medicinal plant) and showed that it has insecticidal activity against termites (*Reticulitermes santonensis*) and the larvae of the house longhorn beetle (*Hylotrupes bajulus*) and fungicidal activity against brown-rot fungus (*Coniophora puteana*). Rhizomes of crepe ginger (*Costus speciosus*, a herbaceous and medicinal plant) and leaves of cucumber (*Cucumis sativus* L.) also contain compound **1**, which has been shown to have antifungal activity against many plant pathogens, including *Aspergillus niger*, *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Curvularia* sp., *Penicillium* sp., *Cladosporium cucumerinum*, *Botrytis cinerea*, *Pythium ultimum*, and *Pythium aphanidermatum* (*15*, *20*, *22*). Compound **1** has also been reported in aloe leaves (*Aloe vera*, *23*), the aerial parts of the herb *Psoralea plicata* (a grazing and medicinal plant, *24*), and the leaves of *Lonchocarpus yucatanensis* and *Lonchocarpus xuul* (endemic trees growing on the Yucatan Peninsula of Mexico, *25*). Nuntanakorn et al. (*19*) reported compound **2** in extracts of the rhizomes and roots of black cohosh, used as antioxidants and for their anticancer activity.

To our knowledge, this is the first time that specific nitrification inhibitors have been isolated and characterized from *B. humidicola*; moreover, no previous authors have ever reported the NI activity of compounds **1** and **2**. There have been reports of some natural products that act as nitrification inhibitors; examples are neem (*Azadirachta indica* Juss.) cake or an acetone/alcohol extract of neem (*26*, *27*) and karanj (*Pongamia glabra* vent.) seed, bark, and leaves (*28*). Nitrification inhibitors such as gallocatechin, epigallocatechin, catechin, and epicatechin have also been isolated from the roots of *Leucaena leucocephala*, a multipurpose tree from tropical America introduced into most of the tropics (*29*). Crude extracts from the roots of *Astragalus mongholicus* have been reported to inhibit nitrification in soil (*30*).

Nitrification is an essential biological process during which nitrifying bacteria (species of *Nitrosomonas* and *Nitrobacter*) convert ammonia to hydroxylamine by ammonia monooxygenase (AMO); the hydroxylamine is converted into nitrite by hydroxylamine oxidoreductase (HAO, *3*). Many synthetic nitrification inhibitors, such as allylthiourea, and some phenolic nitrification inhibitors, such as diphenyliodonium (known to irreversibly inactivate flavoproteins) and phenylacetylene (known for its high solubility and low volatility but high potency), inhibit the AMO activity of *N. europaea* but do not inhibit HAO activity (*14*, *31*, *32*, *33*). Compounds **1** and **2**, also phenolic compounds having similar chemical structure to diphenyliodonium and phenylacetylene, perhaps also inhibit AMO activity and thus inhibit the process of nitrification. However, to support this hypothesis, further experiments on, for example, the effects of compounds **1** and **2** against isolated AMO activity are required.

Our findings demonstrate that *B. humidicola* roots produce two methylated phenolic acids, methyl-*p*-coumarate and methyl ferulate, that have inhibitory effects on nitrification. The isolation of these nitrification inhibitors from roots has many implications in our quest for understanding the biologically produced nitrification inhibitors in *B. humidicola* pastures. It is estimated that nearly 30% of the root mass is turned over annually in *B. humidicola* pastures, and this amounts to an annual addition of 1 t of root tissue ha^{-1} (on a dry weight basis) to the soils in these systems (*34*). Thus, substantial amounts of nitrification inhibitors are added annually to the soil during the process of root turnover (i.e., through decomposition of the old roots), and this could have important additive effects over time in influencing soil nitrification in these pastoral systems. This is perhaps one of the main reasons for the observed low nitrification rates in soils where *B. humidicola* is grown.

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