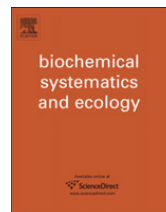




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Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Flavonoids, benzoic acids and cinnamic acids isolated from shoots and roots of Italian rye grass (*Lolium multiflorum* Lam.) with and without endophyte association and arbuscular mycorrhizal fungus

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ARTICLE INFO

Article history:

Received 6 July 2008

Accepted 27 March 2009

Keywords:

Lolium multiflorum Lam

Poaceae

Italian rye grass

Neotyphodium sp

Glomus intraradices

Flavonoids

Phenolic acids

ABSTRACT

Phenolic compounds present in *Lolium multiflorum* Lam. were isolated and characterized. Significant differences in their distribution were found, in shoots and roots of plants with (Lm+) and without (Lm-) endophyte association, grown with (Mic+) and without (Mic-) mycorrhizal fungi *Glomus intraradices*, indicating a systemic effect of these microorganisms on the phenolics metabolism of *L. multiflorum*.

We report the isolation of eight new flavonoids 7,8-dihydroxy-2-(4-hydroxyphenyl)-5-methoxy-4H-1-benzopyran-4-one (**1**), 3,7-dihydroxy-8-methoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one (**2**), 7,8-dihydroxy-2-(2,4-dihydroxyphenyl)-4H-1-benzopyran-4-one (**3**), 3,7-dihydroxy-2-(4,5-dihydroxy-3-methoxyphenyl)-4H-1-benzopyran-4-one (**4**), 5,6-dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-4H-1-benzopyran-4-one (**5**), 7-hydroxy-8-methoxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (**6**), 3,7,8-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (**7**) and 7,8-dihydroxy-2-(2,3-dihydroxyphenyl)-4H-1-benzopyran-4-one (**8**), and two new benzoic acids 2-hydroxy-4-methoxy-1,3-benzenedicarboxylic acid (**9**) and 3,6-dimethoxy-2-hydroxy-benzoic acid (**10**), found with the known deoxykaempferol (**11**), 2',7-dihydroxyflavone (**12**), 2',3,5',7,8-pentahydroxyflavone (**13**), 3',4'-dihydroxy-3-methoxyflavone (**14**), 3,4'-dihydroxy-3-methoxyflavone (**15**), galangin (**16**), gentisic acid (**17**), isoferulic acid (**18**), 4-hydroxy-1,2,3-benzenetricarboxylic acid (**19**), 3,4-dihydroxyphthalic acid (**20**), 2,5-dimethoxy-benzoic acid (**21**), p-methoxycinnamic acid (**22**) and 2,4-dimethoxyisophthalic acid (**23**). Compounds **11**, **12**, **13**, **14**, **15**, **18**, **20**, **21** and **23**, have not been isolated from plants before.

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1. Introduction

The family of phenolic substances comprises a variety of compounds including flavonoids and phenolic carboxylic acids, and they have frequently been associated with the chemical defence of plants against microbes and as signal molecules in plant microbe interactions (Lynn and Chang, 1990; Mayer, 2004; Shaw et al., 2006).

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Many antimicrobial phenolic compounds have been identified, associated with allelopathic interactions. They are usually classified as either phytoalexins or phytoanticipins (Van Etten et al., 1994; Hammerschmidt, 1999). Although the effect of constitutive phenolics on fungal pathogens is well documented (Friend, 1985), it is not always clear whether the active agent is a specific phenolic compound or its metabolite (Scervino et al., 2006).

Our previous studies show that infection of *Trifolium repens* with *Glomus intraradices* (a mycorrhizal symbiont) can significantly alter the composition of flavonoids accumulated in roots introducing systemic changes in flavonoid patterns (Ponce et al., 2004).

Also, recent evidence indicates that the symbiosis of grasses with the endophytic fungi *Neotyphodium* sp., which grows intercellularly causing asymptomatic infections with no sign of damage in the host tissues (Omacini et al., 2005), can induce significant changes in the functioning of soil microbial communities: endophyte infection may interfere with root colonization by arbuscular mycorrhizal fungus (AMF) (Guo et al., 1992; Müller, 2003; Novas et al., 2005), and may produce constitutive or induced secondary plant metabolites that are active against herbivores and microorganisms (Strobel et al., 2002; Harper et al., 2003; Mayer, 2004; Zhang et al., 2006).

As there is little known about the metabolic behaviour of plants when they are affected simultaneously by foliar and root microbial symbionts, and as part of our studies of changes produced in the pattern of plant secondary metabolites (particularly phenolics) by fungal symbiotic colonization (Ponce et al., 2004), we decided to study *Lolium multiflorum* grown in different symbiotic situations. *Lolium multiflorum* Lam. (Italian rye grass) is a cosmopolitan and dominant annual grass species that invades crops, pastures and grasslands, of which 90% of wild plants support two symbiotic organisms: arbuscular mycorrhizal fungi and endophytes. Accordingly, we studied the distribution of phenolic compounds in *L. multiflorum* cultivated under four different conditions: the presence or absence of the endophyte *Neotyphodium* sp. (Lm+/Lm–), and either inoculated or not inoculated with the mycorrhiza *Glomus intraradices* (Mic+/Mic–).

2. Materials and methods

2.1. General experimental procedures

UV spectra (Shimadzu UV-1203) were recorded in MeOH, whereas IR spectra (Nicolet 510P FT-IR) were obtained as a KBr disk film. ¹H NMR (Bruker AM-500, 500 MHz) and ¹³C NMR (Bruker AM-500, 125 MHz) spectra were acquired in DMSO-*d*₆ with TMS as internal standard, EIMS (Shimadzu QP-5000/Gc-17A/DI-50) and HREIMS (VG-ZAB-VSEQ) were recorded at 70 eV (ionising potential) using a direct inlet system. Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60F₂₅₄ aluminium sheets (0.2 mm thickness, Merck) eluted with EtOAc–CH₂Cl₂–HCO₂H (8:12:1). Chromatograms were visualized after drying (i) by UV light and (ii) by spraying with a solution containing 6 g vanillin and 3 ml H₂SO₄ in 197 ml of MeOH. Samples of quercetin, crysin and rutin were purchased from Aldrich. Luteolin, kaempferol, morin and apigenin were purchased from Sigma.

2.2. Plant material

Seeds of *L. multiflorum* were collected from inland Pampas (Lm+), Buenos Aires province, Argentina (36°S, 61°5′W). Endophyte-free seeds were obtained by treating them with the systemic fungicide Baytan (Vila-Aiub et al., 2003).

All plants were grown in 1500 ml pots with steam-sterilized soil mixed with perlite (2:1, v:v). They were inoculated or not inoculated with the AMF *G. intraradices*, and grown in a greenhouse with supplementary light provided by Sylvania incandescent and cold-white lamps, 400 μE m⁻² s⁻¹, 400–700 nm, with a 16/8 h day/light cycle at 25/19 °C and 50% relative humidity. After 14 weeks, the plants were harvested and shoots were separated from roots. In each case, the plant material was dried in oven at 70 °C for 72 h prior to extraction.

2.3. Extraction and isolation

The dried plant material (see Table 1) was crushed and extracted exhaustively with EtOH at room temperature for 4 days (5 L per 20 g, 3 times). The extracts were filtered, evaporated to dryness (syrops: Lm+Mic+, 2.8 g from shoots and 0.308 g from roots; Lm–Mic+, 5.9 g from shoots and 0.726 g from roots; Lm+Mic–, 4.37 g from shoots and 0.363 g from roots; Lm–Mic–, 5.5 g from shoots and 1.03 g from roots), and suspended in water (150 ml). The aqueous solution was successively extracted with organic solvents [hexane (defatting), EtOAc and CH₂Cl₂ (3 × 50 ml)], these extracts were evaporated to dryness and the residues analysed by TLC; all flavonoids reported here were found in the EtOAc extract. The R_f values for TLC analysis of the authentic samples were: rutin (0), morin (0.45), luteolin (0.63), quercetin (0.67), crysin (0.88), apigenin (0.77), kaempferol (0.83) and isorhamnetin (0.88) (Bel Rhlid et al., 1993).

The components of the EtOAc extracts (syrops: Lm+Mic+, 14 mg from shoots and 4 mg from roots; Lm–Mic+, 153 mg from shoots and 23 mg from roots; Lm+Mic–, 236 mg from shoots and 32 mg from roots; Lm–Mic–, 55 mg from shoots and 37 mg from roots), were isolated by preparative TLC (Bel Rhlid et al., 1993) yielding flavonoids **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **11**, **12**, **13**, **14**, **15** and **16** (Fig. 1) and phenolic acids **9**, **10**, **17**, **18**, **19**, **20**, **21**, **22**, and **23** (Figs. 2 and 3).

Table 1

Summary of compounds found under different treatments.

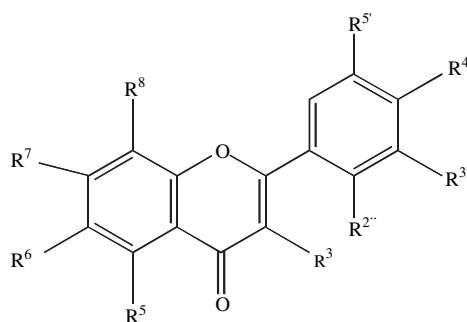
	Lm+Mic–		Lm+Mic+		Lm–Mic–		Lm–Mic+	
	S ^a	R	S	R	S	R	S	R
Dried plant material (g)	22	10	14	10	56	59	56	59
Flavonoids (mg/g) ^b	1.54	0.54	0.04	0.29	0.003	0.08	ND ^c	ND
Compounds (mg) ^d	1 (6.2) 2 (18) 11 (5.8) 12 (2) 13 (0.5) 16 (1.5)	2 (0.8) 3 (2.4) 14 (0.7) 15 (1.5)	2 (0.5)	4 (1.7) 5 (1.2)	6 (0.15)	7 (2.85) 8 (1.95)		
Benzoic acids (mg/g)	0.16	0.11	0.08	ND	0.02	0.03	ND	0.1
Compounds (mg)	17 (3.6)	9 (2.4)	17 (0.3) 19 (0.4) 20 (0.4)		10 (0.15) 21 (0.85)	9 (1.95)		9 (2.2) 23 (4)
Cinnamic acids (mg/g)	ND	ND	0.08	ND	0.03	ND	0.03	ND
Compounds (mg)			18 (1.1)		22 (1.75)		22 (1.75)	

^a S, shoots; R, roots.^b Total amount of flavonoids (mg), or benzoic/cinnamic acids, found per gram of dry material extracted.^c ND, not detected.^d For structures, see Figs. 1, 2, 3.

2.4. Identification

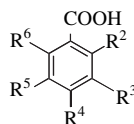
2.4.1. 7,8-Dihydroxy-2-(4-hydroxyphenyl)-5-methoxy-4H-1-benzopyran-4-one or 4',7,8-trihydroxy-5-methoxyflavone (**1**)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 252, 300; +NaOMe: 252, 368; IR, ν_{\max}^{film} cm⁻¹: 3500, 1630, 1602, 1492; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.79 (3H, s, 5-OCH₃), 6.26 (1H, s, H-6), 6.29 (1H, s, H-3), 6.78 (2H, d, $J = 8.6$ Hz, H-3' and H-5'), 7.49 (2H, d, $J = 8.6$ Hz, H-2' and H-6'); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in Table 2; EIMS 70 eV, m/z (rel. int.): 118 (22), 120 (100), 121 (43), 166 (19), 167 (16), 182 (9), 183 (13), 285 (12), 300 (11); HREIMS m/z : 300.0632 (calcd for C₁₆H₁₂O₆, 300.0634); $R_f = 0.77$.



Comp.	R ³	R ⁵	R ⁶	R ⁷	R ⁸	R ^{2'}	R ^{3'}	R ^{4'}	R ^{5'}
1	H	OCH ₃	H	OH	OH	H	H	OH	H
2	OH	H	H	OH	OCH ₃	H	H	OCH ₃	H
3	H	H	H	OH	OH	OH	H	OH	H
4	OH	H	H	OH	H	H	OCH ₃	OH	OH
5	OCH ₃	OH	OH	H	H	H	H	OH	H
6	H	H	H	OH	OCH ₃	H	H	OH	H
7	OH	H	H	OH	OH	H	H	OH	H
8	H	H	H	OH	OH	OH	OH	H	H
11	OH	H	H	OH	H	H	H	OH	H
12	H	H	H	OH	H	OH	H	H	H
13	OH	H	H	OH	OH	OH	H	H	OH
14	OCH ₃	H	H	H	H	H	OH	OH	H
15	OH	H	H	H	H	H	H	OH	H
16	OH	OH	H	OH	H	H	H	H	H

Fig. 1. Structures of flavonoids.



Comp	R ²	R ³	R ⁴	R ⁵	R ⁶
9	OH	COOH	OCH ₃	H	H
10	OH	OCH ₃	H	H	OCH ₃
17	OH	H	H	OH	H
19	COOH	COOH	OH	H	H
20	COOH	OH	OH	H	H
21	OCH ₃	H	H	OCH ₃	H
23	OCH ₃	COOH	OCH ₃	H	H

Fig. 2. Structure of benzoic acids.

2.4.2. 3,7-Dihydroxy-8-methoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one or 3,7-dihydroxy-4',8-dimethoxyflavone (2)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 250, 304; IR ν_{\max}^{film} cm⁻¹: 3320–3490 (broad), 1625, 1601, 1490; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.81 (3H, s, 8-OCH₃), 3.82 (3H, s, 4'-OCH₃), 6.83 (1H, d, J = 8.6 Hz, H-6), 7.51 (2H, d, J = 8.6 Hz, H-3' and H-5'), 7.63 (1H, d, J = 8.65 Hz, H-5), 7.78 (2H, d, J = 8.8 Hz, H-2' and H-6'); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in Table 2; EIMS 70 eV, m/z (rel. int.): 118 (9), 119 (29), 120 (19), 121 (61), 132 (7), 135 (44), 148 (11), 151 (29), 165 (23), 166 (17), 167 (12), 299 (21), 313 (50), 314 (10); HREIMS m/z : 314.0788 (calcd for C₁₇H₁₄O₆, 314.0790); R_f = 0.69.

2.4.3. 7,8-Dihydroxy-2-(2,4-dihydroxyphenyl)-4H-1-benzopyran-4-one or 2',4',7,8-tetrahydroxyflavone (3)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 248, 310; IR ν_{\max}^{film} cm⁻¹: 3350–3515 (broad), 1615, 1600, 1475; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 6.65 (1H, d, J = 8.5 Hz, H-6), 6.75 (1H, s, H-3), 7.14 (1H, d, J = 1.5 Hz, H-3'), 7.37 (1H, dd, J = 8.5, J = 1.5 Hz, H-5'), 7.44 (1H, d, J = 8.5 Hz, H-6'), 7.75 (1H, d, J = 8.5 Hz, H-5); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in Table 2; EIMS 70 eV, m/z (rel. int.): 134 (6), 136 (9), 137 (6), 150 (8), 152 (10), 153 (9), 286 (0.6); HREIMS m/z : 286.0475 (calcd for C₁₅H₁₀O₆, 286.0477); R_f = 0.70.

2.4.4. 3,7-Dihydroxy-2-(4,5-dihydroxy-3-methoxyphenyl)-4H-1-benzopyran-4-one or 3,4',5',7-tetrahydroxy-3'-methoxyflavone (4)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 250, 302; IR ν_{\max}^{film} cm⁻¹: 3500, 1629, 1603, 1491; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.87 (3H, s, 3'-OCH₃), 6.69 (1H, dd, J = 8.6, 2.0 Hz, H-6), 6.90 (1H, d, J = 2.0 Hz, H-8), 7.73 (1H, d, J = 2.2 Hz, H-2'), 7.93 (1H, d, J = 8.6 Hz, H-5), 8.10 (1H, d, J = 2.2 Hz, H-6'); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in Table 2; EIMS 70 eV, m/z (rel. int.): 136 (2), 137 (5), 164 (1), 167 (2), 180 (13), 301 (0.4), 316 (0.5); HREIMS m/z : 316.0581 (calcd for C₁₆H₁₂O₇, 316.0583); R_f = 0.06.

2.4.5. 5,6-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-4H-1-benzopyran-4-one or 4',5,6-trihydroxy-3-methoxyflavone (5)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 252, 300, +NaOMe: 252, 358; IR ν_{\max}^{film} cm⁻¹: 3500, 1630, 1602, 1492; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.77 (3H, s, 3-OCH₃), 6.64 (1H, d, J = 8.4 Hz, H-8), 6.73 (2H, d, J = 8.4 Hz, H-3' and H-5'), 6.77 (1H, d, J = 8.4 Hz, H-7), 7.73 (2H, d, J = 8.4 Hz, H-2' and H-6'); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in Table 2; EIMS 70 eV, m/z (rel. int.): 300 (1), 285 (2), 118 (14), 119 (23), 121 (73), 148 (6), 152 (13), 153 (31), 180 (13); HREIMS m/z : 300.0632 (calcd. for C₁₆H₁₂O₆, 300.0634); R_f = 0.90.

2.4.6. 7-Hydroxy-8-methoxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one or 4',7-dihydroxy-8-methoxyflavone (6)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 248, 306, +NaOMe: 248, 371; IR ν_{\max}^{film} cm⁻¹: 3480, 1625, 1598, 1496; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.79 (3H, s, 8-OCH₃), 6.68 (1H, s, H-3), 6.71 (1H, d, J = 8.7 Hz, H-2' and H-6'), 7.61 (1H, d, 8.5 Hz, H-5), 6.80 (1H, d, J = 8.5 Hz, H-6), 7.77 (2H, d, J = 8.7 Hz, H-3' and H-5'); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in

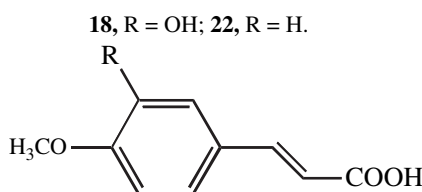


Fig. 3. Structure of cinnamic acids.

Table 2
¹³C NMR spectroscopic data (ppm) of flavonoids.

C	1	2	3	4	5	6	7	8
2	162.9	147.5	160.9	146.9	147.0	162.9	147.5	161.8
3	105.1	139.0	113.3	139.0	141.9	106.7	139.0	113.3
4	181.5	173.6	177.3	173.0	177.6	177.3	173.6	177.3
4a	102.9	117.9	115.2	118.6	103.5	119.2	114.5	115.2
5	154.8	122.4	121.5	126.5	150.5	122.2	121.8	121.5
6	100.4	115.2	117.0	115.2	142.9	114.8	117.5	117.0
7	153.6	154.9	155.6	162.7	125.3	155.0	155.5	155.6
8	127.6	135.6	133.9	103.5	121.5	135.2	134.3	133.9
8a	155.6	147.6	151.1	156.6	154.1	147.4	150.1	151.1
1'	123.0	126.6	113.1	121.6	122.7	123.9	125.7	120.1
2'	127.0	128.1	153.7	105.9	126.6	126.9	127.2	146.4
3'	115.9	113.7	106.8	147.9	115.8	115.9	116.3	144.8
4'	160.8	163.8	163.5	138.8	160.6	160.8	160.7	118.2
5'	115.9	113.7	110.1	144.0	115.8	115.9	116.3	121.1
6'	126.9	128.0	124.2	109.3	126.6	126.9	127.2	116.3
CH ₃ O C-3					59.5			
CH ₃ O C-3'				56.2				
CH ₃ O C-5	55.9							
CH ₃ O C-8		60.8						
CH ₃ O C-4'		55.4						

Table 2; EIMS (70 eV) 118 (8), 121 (16), 134 (5), 151 (9), 166 (5), 167 (4), 284 (2); HREIMS *m/z*: 284.0683 (calcd for C₁₆H₁₂O₅, 284.0685); *R*_f = 0.60.**2.4.7. 3,7,8-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one or 3,4',7,8-tetrahydroxyflavone (7)**

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 250, 304, +NaOMe: 250, 364; IR ν_{\max}^{film} cm⁻¹: 3500, 1630, 1602, 1492; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 7.66 (2H, *d*, *J* = 7.8 Hz, H-2' and H-6'), 7.13 (2H, *d*, *J* = 7.8 Hz, H-3' and H-5'), 7.49 (2H, *d*, *J* = 8.0 Hz, H-5), 7.09 (2H, *d*, *J* = 8.0 Hz, H-6); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in **Table 2**; EIMS 70 eV, *m/z* (rel. int.): 118 (6), 121 (24), 134 (13), 152 (6), 153 (7), 286 (2); HREIMS *m/z*: 286.0475 (calcd for C₁₅H₁₀O₆, 286.0477); *R*_f = 0.08.

2.4.8. 7,8-Dihydroxy-2-(2,3-dihydroxyphenyl)-4H-1-benzopyran-4-one, or 2',3',7,8-tetrahydroxyflavone (8)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 251, 300; IR ν_{\max}^{film} cm⁻¹: 3300–3500 (broad), 1620, 1605, 1495; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 6.72 (1H, *d*, *J* = 8.45 Hz, H-4'), 6.78 (1H, *s*, H-3), 6.81 (1H, *d*, *J* = 8.7 Hz, H-6), 7.43 (1H, *dd*, *J* = 7.75, 7.1 Hz, H-5'), 7.50 (1H, *d*, *J* = 8.7 Hz, H-6'), 7.61 (1H, *d*, *J* = 8.7 Hz, H-5); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in **Table 2**; EIMS 70 eV, *m/z* (rel. int.): 121 (21), 134 (22), 137 (11), 152 (13), 153 (8), 286 (1); HREIMS *m/z*: 286.0475 (calcd for C₁₅H₁₀O₆, 286.0477); *R*_f = 0.67.

2.4.9. 2-Hydroxy-4-methoxy-1,3-benzenedicarboxylic acid (9)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 215, 232, 320; IR ν_{\max}^{film} cm⁻¹: 3500, 1670, 1612, 1444, 818, 859; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.79 (3H, *s*, 4-OCH₃), 6.79 (1H, *d*, *J* = 8.35 Hz, H-5), 7.77 (1H, *d*, *J* = 8.3 Hz, H-6); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in **Table 3**; EIMS 70 eV, *m/z* (rel. int.): 55 (56), 65 (32), 69 (38), 71 (22), 105 (19), 107 (33), 109 (18), 121 (60), 150 (4), 167 (4), 169 (4), 212 (1); HREIMS *m/z*: 212.0319 (calcd for C₉H₈O₆, 212.0321); *R*_f = 0.67.

2.4.10. 3,6-Dimethoxy-2-hydroxy-benzoic acid (10)

UV $\lambda_{\max}^{\text{MeOH}}$ nm (log ϵ): 213, 2.25, 318; IR ν_{\max}^{film} cm⁻¹: 3500, 1678, 1602, 1474, 838, 855; ¹H NMR spectral data (500 MHz, ((CD₃)₂-SO)): δ 3.78 (3H, *s*, 3-OCH₃), 3.79 (3H, *s*, 6-OCH₃), 6.80 (1H, *d*, *J* = 8.5 Hz, H-4), 7.50 (1H, *d*, *J* = 8.7 Hz, H-5); ¹³C NMR spectral data (500 MHz, ((CD₃)₂-SO)): data are shown in **Table 3**; EIMS 70 eV, *m/z* (rel. int.): 55 (100), 63 (16), 91 (30), 122 (6), 198 (10), 153 (9), 167 (4), 181 (4), 183 (6); HREIMS *m/z*: 198.0526 (calcd for C₉H₁₀O₅, 198.0528); *R*_f = 0.60.

3. Results and discussion

This is the first comparative study of shoot and root phenolic composition (flavonoids and acids) of Italian rye grass either in the presence or absence of *Neotyphodium* sp., and grown with or without the AMF *G. intraradices*. Analysis of the EtOAc extracts of plants cultivated under the different conditions gave the following results (**Figs. 1–3**). Methanol was not used at any stage of these analysis, so all methylated congener compounds found (**2–3**, **9–23** and **17–21**) should not be considered artifacts.

The analysis of these extracts revealed variations in the patterns of flavonoids and carboxylic acids with growing conditions as summarized in **Table 1**; these variations are in agreement with our previous study, when AMF colonized roots of plants of *Trifolium repens* (Ponce et al., 2004).

Table 3¹³C NMR spectroscopic data (ppm) of benzoic acids.

C	9	10
1	110.3	109.8
2	170.9	155.8
3	109.9	144.1
4	161.6	112.2
5	111.1	109.7
6	130.9	151.4
COOH C-1	170.4	169.2
COOH C-2		
COOH C-3	169.5	
CH ₃ O C-2		
CH ₃ O C-3		56.1
CH ₃ O C-4	55.7	
CH ₃ O C-5		
CH ₃ O C-6		55.7

As a result of this work, we here report eight new flavonoids (**1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**), two new phenolic acids (**9**, **10**), five known flavonoids: deoxykaempferol (**11**, 3,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), 2',7-dihydroxyflavone (**12**, 7-hydroxy-2-(2-hydroxyphenyl)-4H-1-benzopyran-4-one), (2',3,5',7,8-pentahydroxyflavone) (**13**, 3,7,8-trihydroxy-2-(2,5-dihydroxyphenyl)-4H-1-benzopyran-4-one), 3',4'-dihydroxy-3-methoxyflavone (**14**, 3-methoxy-2-(3,4-dihydroxyphenyl)-4H-1-benzopyran-4-one), 3,4'-dihydroxyflavone (**15**, 3-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), galangin (**16**, 3,5,7-trihydroxy-2-4H-1-benzopyran-4-one), and seven known phenolic acids: gentisic acid (**17**, 2,5-dihydroxy-benzoic acid), isoferulic acid (**18**, 3-(3-hydroxy-4-methoxyphenyl)-2-propenoic acid), 4-hydroxy-1,2,3-tricarboxylic acid (**19**), 3,4-dihydroxyphthalic acid (**20**, 3,4-dihydroxy-1,2-benzenedicarboxylic acid), 2,5-dimethoxybenzoic acid (**21**), *p*-methoxycinnamic acid (**22**, 3-(4-methoxyphenyl)-2-propenoic acid), and 2,4-dimethoxyisophthalic acid (**23**, 2,4-dimethoxy-1,3-benzenedicarboxylic acid). Compounds **11**, **12**, **13**, **14**, **15**, **19**, **20**, **21** and **23** have not been isolated from plants before. All compounds were isolated by conventional methods, and their structures elucidated by spectroscopic analyses (IR, UV, ¹H NMR, ¹³C NMR, MS and HRMS; see Section 2).

Compound **1**, $R_f = 0.77$, gave a molecular ion [M^+] at m/z 300 in the EI mass spectrum and at m/z 300.0632 in the HREIMS, corresponding to the molecular formula $C_{16}H_{12}O_6$ (calcd 300.0634). The UV absorption maxima of **1** in MeOH at 252 and 300 nm were typical of a flavonoid derivative. Addition of NaOMe caused a shift in the UV absorption maxima of +68 nm in agreement with the presence of the HO group as a substituent at C-4. The EIMS of **1** displayed two retro-Diels–Alder fragmentations, at m/z 118 [$HOC_6H_4C=O$] and at m/z 182 [$(OH)_2C_6H(CH_3O)OCO$], indicating the presence of a methoxyl group in ring-A. The absence of the signal at m/z 148 [$HOC=CC_6H_4OCH_3$] agrees with the presence of a hydroxyl group in ring-A. The positive ferric chloride test and the absorption bands at 3350–3515 (broad) cm^{-1} indicated that compound **1** had free hydroxyl groups. A sharp singlet at δ 6.29 integrating for one proton was assigned to H-3. A sharp three-proton resonance for the one methoxyl group at δ 3.79 was also observed. The structure of **1** was thus deduced as shown (Fig. 1).

Compound **2** ($R_f = 0.69$) was obtained as a colourless solid. The IR absorption bands at 3320–3490 (broad), 1625, 1601 and 1490 cm^{-1} , and positive ferric chloride test indicated that compound **2** had free hydroxyl groups. It gave [M^+] peaks at m/z 314 and at m/z 314.0788 in its EIMS and HREIMS, respectively consistent with the molecular formula $C_{17}H_{14}O_6$ (calcd 314.0790) further supported by the presence of 17 carbon signals in its ¹³C NMR spectrum (Table 2). The ¹H NMR spectrum of **2** showed signals for two *para*-coupled doublets with $J = 8.8$ Hz at δ 7.78 and 7.51, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively. Another two sharp three-proton singlets integrating each one for three hydrogens each at δ 3.82 and 3.81 were also observed and were assigned to two methoxyl groups at C-4' and C-8 respectively. The EIMS of **2** has two retro-Diels–Alder fragments at m/z 166 [$M^{+\bullet} - CH_3OPhC=COH$], 148 [$CH_3OPhC=COH$] and 135 [$CH_3OPhC=O$] consistent, together with the ¹H NMR spectral data, with the presence of a methoxyl group at the C-4' position in ring-B and another methoxyl group in ring-A. Thus, from the foregoing spectral studies the structure of compound **2** was established as 3,7-dihydroxy-4',8-dimethoxyflavone (Fig. 1).

Compound **3** ($R_f = 0.70$) was isolated as a colourless solid. The IR showed absorption bands at 3350–3520 (broad), 1615, 1600 and 1475 cm^{-1} and the positive ferric chloride test indicated that compound **3** had free hydroxyl groups. The UV absorption maxima of **3** in MeOH at 248 and 310 nm was typical of a flavonoid derivative. It gave [M^+] peaks at m/z 286 and at m/z 286.0475 in its EIMS and HREIMS respectively, consistent with the molecular formula $C_{15}H_{10}O_6$ (calcd 286.0477) and further supported by the presence of 15 carbon signals in its ¹³C NMR spectrum (Table 2). The ¹H NMR spectrum of **3** showed signals for two *ortho*-coupled doublets with $J = 8.5$ Hz at δ 7.75 and 6.65, each integrating for one proton, which were assigned to the coupled H-5 and H-6, respectively; two other one-proton signals at δ 6.75 and 7.14 ($J = 1.5$ Hz) were assigned to hydrogens at C-3 and C-3' respectively. It presents another proton signal at 7.44–7.37 with $J = 8.5$ and 1.5 Hz assigned to protons 5' and 6'. The EIMS of **3** has two retro-Diels–Alder fragments at m/z 152 [$M^{+\bullet} - (HO)_2PhC=CH$], 134 [$(HO)_2PhC=CH$], 137 [$(HO)_2PhC=O$] consistent with the analysis of the ¹H NMR spectral data. Thus, from the foregoing spectral studies the structure of compound **3** was established as 7,8-dihydroxy-2',4'-dihydroxyflavone (Fig. 1).

Compound **4** ($R_f = 0.06$) was isolated as a colourless solid. It gave a molecular ion $[M^+]$ at m/z 316 in the EI mass spectrum and at m/z 316.0581 (calcd 316.0583) in HREIMS consistent with the molecular formula $C_{16}H_{12}O_7$, which was further supported by the presence of 16 carbon signals in its ^{13}C NMR spectrum (Table 2). The IR absorption bands at 3500 (broad), 1629, 1603 and 1491 cm^{-1} , and a positive ferric chloride test indicated that compound **4** had free hydroxyl groups. The 1H NMR spectrum of **4** showed two typical *meta*-signals at δ 8.10 and 7.73 with $J = 2.2\text{ Hz}$ assigned to protons 6' and 2'; a signal with $J = 8.5\text{ Hz}$ assigned to *ortho*-coupled proton 5, another *meta*- and *ortho*-coupled proton signal at δ 6.69 with $J = 8.5$ and 2.0 Hz assigned to proton 6 and a *meta*-coupled signal at δ 6.90 with $J = 2.0\text{ Hz}$ assigned to proton 8. A sharp three-proton singlet at δ 3.87 was also assigned to a methoxyl group at C-3'. The EIMS of **4** displayed two retro-Diels–Alder fragments at m/z 301 $[M^+ - CH_3]$, 136 $[M^+ - CH_3O(OH)_2PhC=COH]$, 180 $[(CH_3O(OH)_2PhC=COH)]$ and 167 $[CH_3O(OH)_2PhC=O]$, which together with analysis of the 1H NMR spectroscopy data, indicated the presence of a methoxyl group at C-3' in ring-B; its deduced structure is shown in Fig. 1.

Compound **5** ($R_f = 0.90$) was isolated as a colourless solid, showed $[M]^+$ peaks at m/z 300 and at m/z 300.0632 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{16}H_{12}O_6$ (m/z calcd 300.0634) which was further supported by analysis of its ^{13}C NMR spectrum (Table 2). The IR absorption bands at 3500 (broad), 1630, 1602 and 1492 cm^{-1} , and a positive ferric chloride test indicated that compound **5** had free hydroxyl groups. The UV absorption maxima of **5** in MeOH at 252 and 300 nm were typical of a flavonoid derivative. Addition of NaOMe caused a shift in the UV absorption maxima of +58 nm in agreement with the presence of the HO group as a substituent at C-4'. The 1H NMR spectrum of **5** showed two *para*-coupled doublets with $J = 8.45\text{ Hz}$ at δ 7.73 and 6.73, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively. Two typical *ortho*-coupled doublets with $J = 8.65\text{ Hz}$ at δ 6.77 and 6.64, each integrating for one proton assigned to the coupled H-7 and H-8 together with a sharp three-proton resonance for the one methoxyl group at δ 3.77 were also observed. The EIMS of **5** had two retro-Diels–Alder fragments at m/z 152 (ring-A), 121 (HOC_6H_4CO , fragment B₂) and 148 ($CH_3OC=CC_6H_4OH$, ring-B) consistent with the presence of a methoxyl group at the C-3 position in the ring A (Fig. 1).

Compound **6** ($R_f = 0.60$) was obtained as a colourless solid, and showed $[M]^+$ peaks at m/z 284 and at m/z 284.0683 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_{16}H_{12}O_5$ (m/z calcd 284.0685) which was further supported by analysis of its ^{13}C NMR spectrum (Table 2). Compound **6** had free hydroxyl groups. As we have seen for **1** and **5**, it had a positive ferric chloride test and its UV absorption maxima in MeOH at 248 and 306 nm, typical of a flavonoid derivative, suffered a bathochromic effect in the UV absorption maxima of +65 nm upon addition of NaOMe, in agreement with the presence of the HO group as a substituent at C-4'. The IR showed absorption bands at 3480 (broad), 1625, 1598 and 1496 cm^{-1} . The 1H NMR spectrum of **6** showed two typical *para*-coupled doublets with $J = 8.7\text{ Hz}$ at δ 7.77 and 6.71, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively. A sharp three-proton resonance for the methoxyl group at C-8, δ 3.79 and a singlet for the hydrogen at C-3, δ 6.68 were observed. Also present was a typical *ortho*-coupled signal for protons at C-5 and C-6 with $J = 7.75\text{ Hz}$ at δ 7.61 and 6.80 respectively. The EIMS of **6** had two retro-Diels–Alder fragments at m/z 166 (ring-A), 121 (HOC_6H_4CO , fragment B₂) and 134 ($HOC=CC_6H_4OH$, ring-B) consistent with the presence of a methoxyl group at the C-8 position in ring-A (Fig. 1).

Compound **7** ($R_f = 0.08$) was obtained as a colourless solid. It gave a molecular ion $[M^+]$ at m/z 286 in the EI mass spectrum and at m/z 286.0475 in the HREIMS, corresponding to the molecular formula $C_{15}H_{10}O_6$ (calcd 286.0477). The EIMS of **7** displayed two retro-Diels–Alder fragmentations, at m/z 118 [$HOC_6H_4C=O$] and at m/z 152 [HOC_6H_3OCO]. The presence of the signal at m/z 134 [$HOC=CC_6H_4OH$] agrees with the presence of an hydroxyl group at C-3 in ring-A. The positive ferric chloride test indicated that compound **7** had free hydroxyl groups. The 1H NMR spectrum showed signals for two *para*-coupled doublets with $J = 7.8\text{ Hz}$ at δ 7.66 and 7.13, each integrating for two protons, which were assigned to the coupled H-2' and H-6', and H-3' and H-5', respectively and a typical *ortho*-coupled signal for protons at C-5 and C-6 with $J = 8\text{ Hz}$ at δ 7.49 and 7.09 (Fig. 1). All this, together with the 15 carbon signals in its ^{13}C NMR spectrum (Table 2), is consistent with the structure proposed for **7**.

Compound **8** ($R_f = 0.67$) was isolated as a colourless solid. The IR absorption bands at 3300–3500 (broad), 1620, 1605 and 1495 cm^{-1} , and positive ferric chloride test indicated that it had free hydroxyl groups. It gave $[M]^+$ peaks at m/z 286 and at m/z 286.0475 in its EIMS and HREIMS, respectively consistent with the molecular formula $C_{15}H_{10}O_6$ (calcd 286.0477) further supported by the presence of 15 carbon signals in its ^{13}C NMR spectrum (Table 2). The 1H NMR spectrum of **8** showed signals for three aromatic protons: a double doublet at δ 6.61 $J = 7.75, 7.1$, assigned to H-5', a doublet at δ 6.72, $J = 8.45$, assigned to H-4' and another doublet at δ 7.50 $J = 8.7$, assigned to H-6'. A sharp one-proton peak at δ 6.78, assigned to H-3, and another typical *ortho*-coupled signal for protons at C-5 and C-6 with $J = 8.7\text{ Hz}$ at δ 7.61 and 6.81 were present. The EIMS of **8** has two retro-Diels–Alder fragments at m/z 152 $[M^+ - (HO)_2PhC=CH]$, 137 [$HOPhC=O$] and 134 $[(HO)_2PhC=CH]$ consistent with the 1H NMR spectral data. Thus, from the foregoing spectral studies the structure of compound **8** was established as 2',3',7,8-tetrahydroxyflavone (Fig. 1).

Compound **9**, $R_f = 0.67$, was obtained as a colourless solid. It gave a molecular ion $[M^+]$ at m/z 212 in the EI mass spectrum and at m/z 212.0319 in the HREIMS, corresponding to the molecular formula $C_9H_8O_6$ (m/z calcd 212.0321), which was further supported by analysis of its ^{13}C NMR spectrum (Table 3). The IR bands at 3500 (broad), 1670, 1612, 1444, 818, 859 cm^{-1} and the UV absorption at 320 nm were indicative of an aromatic skeleton with hydroxyl substituents. The 1H NMR showed signals for aromatic protons *ortho*-coupled with $J = 8.3\text{ Hz}$ at δ 7.77 and 6.79. The fragment peaks at m/z 197 $[M^+ - CH_3]$, 195 $[M^+ - OH]$, 167 $[M^+ - COOH]$ in the EIMS of **9** agree with a carboxylic acid structure with methoxyl and hydroxyl substituents (Fig. 2).

Compound **10** ($R_f = 0.6$), isolated as a colourless solid, showed $[M]^+$ peaks at m/z 198 and at m/z 198.0526 in its EIMS and HREIMS, respectively, consistent with the molecular formula $C_9H_{10}O_5$ (m/z calcd 198.0528) which was further supported by analysis of its ^{13}C NMR spectrum (Table 3). The IR spectrum displayed absorption bands at 3500 (broad), 1678, 1602, 1474, 838, 855 cm^{-1} , and compound **10** gave a positive ferric chloride test which indicated that it had a free hydroxyl group. The 1H NMR spectrum of **10** showed two typical *ortho*-coupled doublets with $J = 8.7$ Hz at δ 7.50 and 6.80, each integrating for one proton, which were assigned to H-5 and H-4, respectively. It also showed two sharp three-proton singlets for methoxyl groups at δ 3.78 and 3.79. The fragment peaks at m/z 183 [$M^+ - CH_3$], 181 [$M^+ - OH$], 167 [$M^+ - CH_3O$], 153 [$M^+ - COOH$] in the EIMS of **10** agree with a carboxylic acid structure with the methoxyl and hydroxyl substituents shown (Fig. 2).

The structures of all the other known compounds were consistent with literature data: compound **11** (Grael et al., 2005); compound **12** (Sargenti and Vichnewski, 2000); compound **13** (Park et al., 2007); compound **14** (Smith and Read, 1997); compound **15** (Souleman et al., 1998); compound **16** (van Acker et al., 2000); compound **17** (Krishnamachari et al., 2004); compound **18** (Jiang et al., 2003); compound **19** (Martin and Gonzalez Vila, 1988); compound **20** (Suemori et al., 1995); compound **21** (Negoro et al., 2003); compound **22** (Rigano et al., 2006) and compound **23** (Tachibana and Sumimoto, 1982).

As far as we know, no previous studies have been reported about the relation of any of these compounds with *Glomus intraradices* (AMF) and/or *Neotyphodium* (endophyte) colonization; the data presented here suggest that these microorganisms regulate flavonoid and phenolic acid metabolism with a systemic effect on the plant.

As can be seen in Table 1, infection by *Neotyphodium*, although it only colonizes shoots, increases the flavonoid content of the whole plants (both shoots and roots) grown in the absence of *Mycorrhiza* (Lm–Mic– vs Lm+Mic–), not only in quantity but also in variety. This also occurs in plants whose roots are colonized by *Glomus intraradices* (Lm–Mic+ vs Lm+Mic+). On the other hand, the colonization of roots by *Mycorrhiza* reduces the variety and the amount of flavonoids not only in roots but also in shoots, either with endophytes (Lm+Mic+ vs Lm+Mic–) or without them (Lm–Mic+ vs Lm–Mic–). The corresponding effects on the benzoic acids fractions is not so marked, it is the amounts of acids and not their variety which seem to be mainly affected. Thus both *Neotyphodium* and *Glomus intraradices* may be said to have systemic effects on the metabolism of flavonoids and benzoic acids of *L. multiflorum*.

Several authors (Bécard et al., 1992; Chabot et al., 1992) have related the activity of flavonoids to their chemical structure: by comparison of the effect of different flavonoids on AMF they attributed this activity to the hydroxyl group on position 3 of the C aromatic ring, as a glycosidation on this position resulted in a complete loss of activity. Nonetheless, we have already shown that the loss of the C-3 hydroxyl does not affect this activity, it is the glycosidation of the hydroxyls at C-3 and/or C-7 which results in the loss of activity (Scervino et al., 2006). Our present findings are in agreement with these results: 7 of the new flavonoids have a hydroxyl in C-7 (**1**, **2**, **3**, **4**, **6**, **7** and **8**), of which three also have a hydroxyl in C-3 (**2**, **4** and **7**); of the six known flavonoids, four have a C-7 hydroxyl (**11**, **12**, **13** and **16**), three of which also have a C-3 hydroxyl (**11**, **13** and **16**) and one is hydroxylated at C-3 but not at C-7 (**15**).

If we consider that from the ethyl acetate extract of the plant control Lm–Mic–, we obtained **6**, **7**, **8**, **9**, **10**, **21** and **22**, the rest of the compounds (**1**, **2**, **3**, **4**, **5**, **11**, **12**, **13**, **14**, **15**, **16**, **17**, **18**, **19** and **20**) could be considered as responses to the interaction between fungi and plant.

The benzoic acids are found both in shoots and roots of non-AMF colonized plants, while in AMF colonized plants this varies according to the endophyte presence: those with *Neotyphodium* had benzoic acids only in shoots, whilst in those without endophyte they were found only in roots. It is of note that compound **9** (and its methylated congener **23**) was the only carboxylic acid found in roots, but not in shoots. Both **9** and **22** were present only in Lm–Mic– and in greater amounts in Lm–Mic+, where **23** was also found; this could be in response to the AMF *Glomus intraradices*, and thus these compounds could be considered phytoanticipins.

Apparently the presence of *Neotyphodium* enhances not only the quantity but the variety of phenolic compounds obtained from *Lolium*, both with and without AMF. In this case compounds **1**, **2**, **3**, **4**, **5**, **11**, **12**, **13**, **14**, **15**, **16**, **17**, **18**, **19** and **20**, which are absent in the controls, could be classified as phytoalexins. As compounds **2** and **17** are the only ones present in the two groups of plants with the endophyte, Lm+Mic–, Lm+Mic+, they may both be considered to be the response to the infection by the endophyte.

While phenolic compounds are known to be fungal and bacterial inhibitors (Siqueira et al., 1991a), there are also reports of considerable increases in the amount of phenolic compounds in hosts when they are colonized by AMF such as *Glomus mosseae* and bacteria such as *Rhizobium* (Ling Lee et al., 1977; Devi and Reddy, 2002). This is true in this study only for benzoic acids; conversely flavonoids were greatly reduced in the presence of AMF. This means that the role of these substances in disease suppression still has not been systematically understood, although there can be little doubt that phenolics, both constitutive and induced play an important role in the protection of plants against both herbivores and fungal pathogens.

The present report shows that there are clear differences in secondary metabolism development when the association between Italian rye grass (*Lolium multiflorum* Lam.) and AMF *Glomus intraradices* and/or *Neotyphodium* sp. takes place. We agree with previous reports (Ling Lee et al., 1977; Siqueira et al., 1991b; Devi and Reddy, 2002; Ponce et al., 2004) that changes in quantity and quality of the phenolic composition are the final result of physiological interactions between microorganisms and plants, although we are unable yet to give a comprehensive interpretation of the metabolic changes observed as a response to the mycorrhiza–endophyte–plant interaction; whether the changes in the phenolic composition are the consequence or the cause of colonization by fungi still has to be elucidated and awaits further studies.

Acknowledgements

This work was supported partially by UBA (UBACyT X028), CONICET (PIP5003) and ANCYPT (PICT 13404). J.M. Scervino, E.J. Chaneton and A.M. Godeas are research members of CIC (CONICET). We are grateful to E.A. Hughes for the proofreading of this manuscript.

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