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Fusarium equiseti LPSC 1166 and its *in vitro* role in the decay of *Heterostachys ritteriana* leaf litter

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Abstract The role of microorganisms in litter degradation in arid and semi-arid zones, where soil and water salinization is one of the main factors limiting carbon turnover and decay, remains obscure. *Heterostachys ritteriana* (Amaranthaceae), a halophyte shrub growing in arid environments such as "Salinas Grandes" (Córdoba, Argentina), appears to be the main source of organic matter in the area. Little is known regarding the microorganisms associated with *H. ritteriana*, although they are a potential source of enzymes such as cellulolytic ones, which might be important in biotechnological fields such as bioethanol production using ionic liquids. In the present study, by studying the microbiota growing on *H. ritteriana* leaf litter in "Salinas Grandes", we isolated the cellulolytic fungus *Fusarium equiseti* LPSC 1166, which grew and degraded leaf litter under salt stress. The growth of this fungus was a function of the C substrate and the presence of NaCl. Although *in vitro* the fungus used both soluble and polymeric compounds from *H. ritteriana* litter and synthesized extracellular β-1,4 endoglucanases, its activity was reduced by 10 % NaCl. Based on these results, *F. equiseti* LPSC 1166 can be described as a halotolerant cellulolytic fungus most probably playing a key role in the decay of *H. ritteriana* leaf litter in "Salinas Grandes".

Keywords Decay • β-1,4 Endoglucanase • Fungus • Halotolerant • Salinas Grandes

Introduction

Extreme environments are probably the most important sources of organisms with unknown metabolic pathways that allow growth under stressful conditions. An example of environments like this is the saline depression of Central Argentina called "Salinas Grandes" (64º 31` W, 29º 44` S), located in the northwestern limit of Córdoba Province (Reserva de Uso Múltiple "Salinas Grandes", Departments of Tulumba, Ischilín and Cruz del Eje, Argentina). This area is characterized by saline soils (Haplustol type) with a dry and warm climate: the annual precipitation is below 500 mm, the mean temperature is 19.9 ºC (maximum and minimum around 42 ºC and 6 ºC, respectively) and the annual potential evapotranspiration is 950, all of which lead to water deficit (Karlin et al. 2012). The vegetation of this area is composed of halophyte shrubs mainly belonging to the family Amaranthaceae such as *Heterostachys ritteriana* (Moq.) Moq. (Cabido et al. 2006). This plant is the most frequent one in the saline steppes of Central and South America, because it tolerates saline soils and periodic flooding events (Karlin et al. 2012). Besides, this plant is the main source of organic matter that stabilizes soils and increases their ability to retain water and nutrients, so it might be a biological tool for soil restoration (Cavanna et al. 2010). In addition, during periods of drought, it is a valuable source of fodder for goats, cows and other wild animals such as the plains viscacha rat (*Tympanoctomys barrerae)*.

The rate of litter degradation is dependent on both the composition of organic matter and the associated microbiota, the latter of which is the source of a wide array of decomposing enzymes that are also under the influence of environmental factors such as the soil and the climate (Sariyildiz et al. 2005). Studies regarding microbial degradation of leaf litter have been performed in cold, temperate and tropical environments. However, the role of these microorganisms in degradation in arid and semi-arid climates remains obscure (Lai et al. 2013). In xerophytic environments, the main factors limiting microbial degradation are water content and the osmotic stress caused by salt and soil alkalinization (Lai et al. 2013; Grover et al. 2016; McFarlane et al. 2016). Little is known regarding the microorganisms associated with *H. ritteriana* (Soteras et al. 2012), although they are a potential source of cellulolytic enzymes that degrade one of the main components of plant organic matter (Saparrat et al. 2007). Since these enzymes might tolerate stressful conditions such as high levels of salt, they might be important for biotechnology in fields such as bioethanol production using ionic liquids (Jurado et al. 2011; Amarasekara 2014; Wahlström et al. 2014).

The aim of this work was to identify a cellulolytic fungus from leaf litter of *H. ritteriana* present on the soil of a natural halophyte steppe of "Salinas Grandes" (Córdoba, Argentina) and assess its role in degradation processes under salt stress by evaluating its ability to degrade the plant debris *in vitro*.

Materials and methods

Litter sampling and physical-chemical analysis

Selected units of *H. ritteriana* litter ("artejos") were collected by using a composite random sampling method (Dick et al. 1996) during the wet season on January 15, 2011. Samples were taken from the canopy of shrubs of *H. ritteriana* growing at the Reserva de Uso Múltiple "Salinas Grandes", which is located on the northwestern limit of the Province of Córdoba (Departments of Tulumba, Ischilín and Cruz del Eje, Argentina). The sampling area has a saline soil that has been classified as Aridisol-Orthid typic Salorthid, with a sandy clay loam texture, pH of 7.8, electrical conductivity (EC) of 12.6 dS/m and low organic matter content (< 2.3 %; Soteras et al. 2012). The materials collected from different sites were pooled to form a composite sample, which was stored at 4 ºC until analyzed. A representative subsample was ground in an agate mill, sieved through a < 0.63 mm screen (Saparrat et al. 2010), washed with hot water to eliminate soluble compounds and then analyzed to determine the organic matter content and total nitrogen by using the micro-Kjeldahl method (Saparrat et al. 2007). Also, the EC of a water-soluble fraction (WSF), obtained at a litter to water ratio of 1:5 (w v^{-1}), was measured using an Altronix Model CTX-II conductivity meter (Brooklyn, NY, USA).

Cellulolytic microbial load and fungal isolation from litter

One hundred litter units ("artejos") were washed with sterile tap water according to Allegrucci et al. (2007) and inoculated at a rate of five units per plate on a mineral salt agar (MSA) medium (5 g $NH_4H_2PO_4$, 2.5 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 20 mg $Ca(NO_3)_2 \cdot 4H_2O$, 2 mg $FeCl_3 \cdot 6H_2O$, 0.5 mg H_3BO_3, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg KI, 0.4 mg $MnSO_4 \cdot 5H_2O$, 0.4 mg $ZnSO_4 \cdot 7H_2O$, 0.2 mg Na_2MoO_4 and 0.1 mg $CoCl_3$ per L of deionized water; Kreisel and Schauer 1987), supplemented with 0.5 % of sodium-carboxymethylcellulose (CMC, BDH Chemicals) as the sole C source either alone or amended with three concentrations of NaCl (1.25, 2.5 and 5 % w/v; Kogei et al. 2005), containing either 0.035 % (vol/vol) of Maxim XL fungicide (Syngenta; pH 7.0; to

inhibit fungal growth) or 0.05 % streptomycin sulfate and 0.025 % chloramphenicol (pH 5.0; to inhibit bacterial growth). Plates were incubated in the darkness at 25ºC for 7 days. Fungal colonies developed on each medium were identified based on cultural and morphological features according to Domsch et al. (1993) and Nelson et al. (1994). Both bacterial and fungal colonies with cellulolytic ability developed on each medium were detected according to the CMC-clearing method (Saparrat et al. 2007; Houfani et al. 2017).

A fungus was isolated from a representative colony developed from a litter unit inoculated on the CMC (0.5 %) agar (2 %) medium (pH 5.0) amended with 5 % NaCl and in the presence of the antibiotics. Stock cultures of this isolate were maintained on CMC (0.5 %) agar (2 %) medium (pH 5.0) with 5 % NaCl at 4 ºC. The fungus was deposited in the Culture Collection of the Instituto Spegazzini, Universidad Nacional de La Plata, La Plata, Argentina, as LPSC 1166.

Morphology of the fungal isolate LPSC 1166

A single spore culture of isolate LPSC 1166 was developed in 4 days on potato dextrose agar (PDA) at 25 ºC (Leslie and Summerell 2006). Texture, pigmentation, colony diameter and sporulation were recorded. Structures differentiated in these cultures such as sporodochia, macroconidia and chlamydospores were examined with an Olympus CX41 UC-MAD3 microscope.

DNA extraction, polymerase chain reaction (PCR), and sequencing

The fungus was cultured on PDA for 7 days at 25 °C and its DNA extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The quality and integrity of the DNA was verified by electrophoresis in 1 % agarose gel stained with ethidium bromide. The DNA was stored in a freezer at −20 °C until used. A standard PCR protocol was used to amplify the internal transcribed spacer (ITS) sequences using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White et al. 1990) and a partial sequence of the translation elongation factor 1-alpha (EF-1α) using primers ef1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and ef2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'; O'Donnell et al. 1998). Both reactions were performed in a 15-μL final volume containing 50 ng of template DNA, 50 ng of the forward and reverse primers, 1.5 μL 10X reaction buffer (500 mmol/L KCl; 100 mmol/L Tris-HCl, pH 9.0 a 25 ºC; 1 % Triton X-100), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs and 0.5 unit Taq

polymerase (Inbio Highway®, Buenos Aires, Argentina). Amplifications were performed in a PTC-0150 MiniCycler (MJ. Research. Watertown, MA, USA) under the following conditions: 2 min at 94 °C followed by 31 cycles of 45 s at 94 °C, 45 s at 56 °C, and 60 s at 72 °C, followed by final extension for 5 min at 72 °C and 15 °C hold. PCR products were purified and sequenced at Macrogen (Seoul, Korea) and both sequences of the ITS and EF-1α region were deposited at GenBank under the accession numbers KJ854378 and KJ854377, respectively.

Sequence alignments and phylogenetic analyses

To assess the taxonomic position of LPSC 1166, a phylogenetic analysis using the ITS and EF-1α sequences under both Maximum-parsimony (MP) and Maximum-likelihood (ML) criteria was carried out. Since both morphological and nucleotide BLAST (NCBI, *http://blast.ncbi.nlm.nih.gov/Blast.cgi*) analyses suggested that the isolate belonged to the genus *Fusarium*, sequences of representatives of the *F. oxysporum* species complex (FOSC), the *Gibberella* (*Fusarium*) *fujikuroi* species complex (GFSC), the *F. incarnatum-F. equiseti* species complex (FIESC), the *F. solani* species complex (FSSC), the *F. sambucinum* species complex (FSAMSC), the *F. chlamydosporum* species complex (FCSC), the *F. dimerum* species complex (FDSC) and the *F. tricinctum* species complex (FTSC), as well as *Trichoderma harzianum* as an outgroup, were included in the phylogenetic analyses. All sequences were taken from GenBank (www.ncbi.nlm.nih.gov; Table 1).

Sequences were aligned with MEGA 5.10 (Tamura et al. 2011) using the default parameters of the ClustalW algorithm (gap opening penalty 15, gap extension penalty 6.66). MP-based phylogenetic analyses were performed using the PAUP* (Phylogenetic Analysis Using Parsimony) 4.0b10 software (Swofford 2002). MP trees were inferred with the heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions. Characters were treated as unweighted and gaps were treated as missing data. Clade stability was assessed via 1000 bootstrap replications, using the heuristic search option described above but with 10 random addition sequence replicates. To determine whether DNA sequences from the ITS and EF-1α genes could be concatenated into a single data set, the congruence was assessed with the Partition Homogeneity Test (Farris et al. 1995) under the same settings. As for the ML analysis, best-fit models of nucleotide substitution were assessed with the jModelTest 2 software (Darriba et al. 2012) by using Akaike Information Criterion (Akaike 1974). Parameters of the chosen models were used in the PhyML 3.1 software (Guindon and

Gascuel 2003) to find the most-likelihood trees, whose branch support was estimated via 1000 bootstrap replicates.

Fungal growth and extracellular cellulolytic activity under a range of NaCl concentration

Growth and cellulolytic activity were determined on MSA basal medium with 0.5 % of CMC (pH 5.0) as the sole C source in the presence of 5 and 10 % (w/v) NaCl. Plates were inoculated with a 6-mm diameter mycelial plug grown on MSA basal medium with 0.5 % of CMC at 25 ± 2 ºC for 7 days. Five replicates per treatment, including control plates free of NaCl, were incubated in the dark at 25 ± 2 ºC for 6 days. Growth was estimated by measuring colony diameter. Extracellular cellulolytic activity was measured according to the CMC-clearing method by staining with Congo Red (Saparrat et al. 2007).

C substrates used as source of fungal growth in two saline conditions

The ability of the fungus to use glucose versus CMC as the sole C source was evaluated under two saline conditions on MSA basal medium either alone and amended with glucose or CMC at 0.5 % either unsupplemented or supplemented with 10 % NaCl. Inoculated plates were incubated in the dark at 25 ± 2 ºC for 4 days. Growth was estimated as described before. The experiment was a completely randomized design with four treatments and the number of replicates per treatment was five plates.

Solid-state fermentation (SSF), analytical determinations, and enzyme assays

A sample of *H. ritteriana* litter was washed with hot water (80 ºC) three times to eliminate soluble compounds like salts and readily available C sources, then dried in an oven at 80 ºC overnight, and sterilized at 121 ºC for 20 min (Saparrat et al. 2010). Fungal growth on washed litter units ("artejos") under conditions of SSF was evaluated in 100-mL Erlenmeyer flasks containing 1 g of sterile dry material, supplemented with 1.4 mL of either water or 10 % NaCl solution (sterilized at 121 ºC for 20 min). Each flask was inoculated with 2 mL of a 0.05 % (mass/vol) mycelial suspension obtained from 7-day-old cultures, grown on 2 % malt extract agar (Dorado et al. 1999). Inoculated flasks were incubated at 25± 1.5 ºC in the darkness for 14 days in a humidified chamber, as previously (Saparrat et al. 2010). Sterile uninoculated litter incubated under the same conditions for

the same period of time was used as control. The number of replicates per treatment was three. Fourteen days after inoculation, degradation was measured as percentage litter mass reduction (percentage reduction of inoculated substrate in relation to the uninoculated one; Saparrat et al. 2010). The activity of β-1,4 endoglucanase (E.C.3.2.1.4) of the WSF, obtained from inoculated litter, was assessed as described in Saparrat et al. (2008). Also, the pH and the EC (dS/m) as well as the polymerization/polydispersity index (the ratio of optical density at 465 nm to that at 665 nm; Dorado et al. 1999) of the WSF of control and inoculated litter were determined.

Fourier transform infrared (FTIR) spectroscopy

The chemical composition of uninoculated and inoculated *H. ritteriana* litter alone (control) and supplemented with NaCl 10 % was analyzed after a 14-day incubation period by FTIR spectroscopy, as previously (Saparrat et al., 2010). Each sample was dried in an oven at 80 ºC overnight, ground in an agate mill, and sieved through a < 0.63 mm screen (Saparrat et al. 2010). Briefly, the litter powder was dried at 40 ºC overnight and embedded in KBr 13-mm pellets (2 mg of sample and 20 mg of infrared grade KBr). A representative FTIR spectrum per replicate (n=3) per treatment was collected on a Thermo Nicolet Nexus 470 spectrometer (Thermo-Electric Corporation, Chicago, IL, USA) over a 4000-400/cm range. The spectrum was the result of 64 scans, which were co-added at a resolution of 4/cm for Fourier transformation processing, and corrected by automatic subtraction of the air background. The absorbance spectrum was calculated by means of the OMNIC software (Thermo Nicolet, Madison, WI, USA) and saved in JCAMP-DX format for further analysis. Spectra of each treatment were then saved in ASCII Data File-DAT format using win-das software (Wiley, New York), and then normalized at a value of 1 at 1060 nm according to Schwanninger et al. (2004) and averaged using the Excel program. Bands selected as index peaks reflecting functional groups associated with the cell walls were assigned as described in Saparrat et al. (2010). Values of relative intensities for lignin-associated bands and carbonyl ones were ratioed against carbohydrate reference bands to provide relative changes in the composition of the structural components relative to each other, according to Dorado et al. (1999).

X-ray diffraction (XRD) analysis

XRD analysis was performed with the same litter powder samples used for FTIR, as well as on commercial crystalline cellulose (Sigmacell, type 20), which was used as a reference control. The analysis was performed at 25 ºC with a diffractometer (Philips, Netherlands) equipped with a generator and a vertical goniometer (PW 1732/0 and PW 1050/70, respectively) using Cu Kα radiation (λ = 1.5406 Å) and a Ni filter at 40 kV and 20 mA. The diffraction patterns were obtained within 2θ = 3 ° and 60 ° at a scan rate of 2 °/min and steps of 0.1 °. On each XRD spectrum, the relative crystallinity index (RCI) was determined according to Harris and De Bolt (2008), using the heights (intensities) fixed at 2θ equal to 18.0 ° and 22 ° for the amorphous cellulose and crystalline regions respectively, according to the following equation:

$$\mathrm{RCI} = (\mathrm{I22°} - \mathrm{I18°})/\mathrm{I22°}$$

where $I22^{o}$ and $I18^{o}$ represent the maximum heights of the signals at 2θ = 22 ° and 18 °, respectively.

Statistical analysis

Except for β-1,4 endoglucanase activity, data were analyzed by ANOVA and means of the treatments were contrasted by the Tukey Test (Statistix 8.0). The means of β-1,4 endoglucanase activity of the inoculated treatments were contrasted by means of Student's t test (Statistix 8).

Results

Heterostachys ritteriana litter had 43.4 % (w/w) of organic matter, 25.2 ±5 % of organic C, 1.685 ±0.005 % of total N (Kjeldahl), a 14.94 C/N ratio, and an EC of 2.9 ± 0.23 dS/m.

All washed litter units that were inoculated on basal agar medium with CMC as the sole C source and were added with NaCl 1.25, 2.5 and 5 % hosted culturable bacteria and fungi of the genus *Fusarium* section Gibbosum that developed in the media tested (Fig. 1a,b). Plates amended with Congo Red showed clear halos around colonies, indicating CMC hydrolysis. Therefore, this litter carried cellulolytic microorganisms (data not shown). Although culturing both bacteria and fungi at increasing concentrations of NaCl resulted in a reduction in colony size, fungi were less sensitive to salt stress. A fungus, designated as isolate LPSC 1166, was obtained from a representative colony developed on litter-inoculated CMC medium supplemented with NaCl 5 %.

On PDA, the fungus developed whitish-pink colonies with a 7-cm diameter after a 6-day incubation period at 25 °C. The morphological characteristics analyzed confirmed that the fungus was *F. equiseti*, the type species of the section Gibbosum. It produced thick-walled falcate macroconidia that presented a distinctive curvature, mainly in the apical cell, which could be whip-like, and foot-shaped basal cells. Also, the fungus developed thick-walled chlamydospores (8-20 μm) of an ochraceous color that were produced in chains (data not shown).

We amplified and sequenced the ITS (which codes for the 3´ end of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and the 5´ end of the 28S rDNA) and the EF-1α gene. The ITS fragment was 569-bp long and 99 % homologous to representatives of the genus *Fusarium*, specifically *F. equiseti*, whereas EF-1α was 718-bp long and 100 % homologous to that of *F. equiseti*. The partition homogeneity test revealed that both the ITS and EF-1α genes should not be combined (P value = 0.001), so we performed a phylogenetic analysis based on MP and ML criteria using separate data sets of the two loci from 24 taxa, including representatives of several *Fusarium* species complexes and *Trichoderma harzianum* CBS 226.95 as an outgroup. A monophyletic group was obtained using a ML analysis based on EF-1α data, which clustered the isolate LPSC 1166 with *F. equiseti* at a high bootstrap value (Fig. 2). Similar tree topologies were found when the MP analysis was performed with both EF-1α and ITS data. However, isolate LPSC 1166 appeared in an unresolved position in the FIESC (Online Resource 1,2). Also, the ML analysis with the ITS sequences confirmed that isolate LPSC 1166 was closely associated with the unresolved FIESC (Online Resource 3). Therefore, we concluded that the isolate LPSC 1166 belongs to the FIESC, specifically *F. equiseti*.

Although fungal growth on MSA medium with 0.5 % CMC was drastically reduced by the presence of salt in the medium, extracellular cellulolytic activity was also detected under both saline conditions (Table 2; Fig. 3). At 10 %, NaCl reduced the size of the colonies by 72 % in a 6-day period of incubation. Concomitantly with this, NaCl 10 and 5 % reduced cellulolytic halos around the colonies by 66 and 24 %, respectively, when compared with those found in the control medium.

Fungal growth was affected by the C source and the salt content in the media (Table 3; Online Resource 4). In the presence of Na^{+1} and Cl^{-1} ions at 0.04 and 0.85 mg/L respectively, colonies grew more on CMC than on glucose, whereas in NaCl 10 %, growth was unaffected by the C source.

We also studied the ability of LPSC 1166 to grow and to transform *H. ritteriana* litter under SSF conditions and the effect of NaCl on the process. Both unamended and NaCl-amended litter was colonized by the fungus, since a velvet-like mycelium covered the debris that later developed conidia (data not shown).

Concomitantly, in a 14-day incubation period, the fungus degraded *H. ritteriana* litter, either amended or not with NaCl 10 %, by 10.4 and 21.1 % respectively, when comparing the inoculated dry mass of substrate with the uninoculated one ($p < 0.05$; Table 4; Online Resource 5). The FTIR spectra of untransformed and transformed substrates under both conditions presented a typical pattern of lignocellulosic materials. Several peaks were in the fingerprint region (800-1800/cm; Fig. 4). However, the ratio between the peak around 1504/cm, which is related to aromatic skeletal vibration from lignin, vs the peak at 896/cm, which is related to the C1-H vibration in cellulose and hemicellulose, was higher in inoculated samples than in uninoculated ones (Online Resource 6,7). In addition, the fungus caused the loss of cellulose crystallinity in unamended litter but not in NaCl-amended one (Table 5; Online Resource 8). Furthermore, fungal activity increased the pH of the WSF, independently of whether the litter was amended or not with NaCl, although under higher ionic strength changes were less pronounced (Table 4). Still it was evident that NaCl inhibited the fungal transformation of WSF, which was estimated through the $Abs._{465\ nm}/Abs._{665\ nm}$ ratio (Table 4). Furthermore, β-1,4 endoglucanase activity changed from 86.3 ±36.5 mU/mL WSF in NaCl-unsupplemented inoculated litter to 29.3 ±11.2 mU/mL WSF in NaCl-supplemented inoculated litter (Student's t test $p < 0.05$).

Discussion

Heterostachys ritteriana (Amaranthaceae), a halophyte shrub growing in arid environments such as "Salinas Grandes", Córdoba, Argentina (Soteras et al. 2012), appears to be the main source of organic matter in the area. Therefore, this plant litter is critical for the C bulk and fertility of these soils, and may contribute to increasing the ability of the soil to retain water below ground horizons (Karlin et al. 2012).

The chemical composition of live plants and their leaf litter vary among different plant species, and are influenced by the environment. This might in turn influence the plant-microbiota interactions. Unlike other representatives of the family Amaranthaceae, which have laminate leaves (Kadereit et al. 2003), and other sources of litter such as *Atriplex* species, which have high content of C (Cepeda-Pizarro 1993; Alvarez et al. 2008), *H. ritteriana* has succulent leaves and lower C content. We did not measure ash content in *H. ritteriana* litter, but Medina et al. (2008) reported 24.7 % of ash content and Na concentrations > 4000 mmol/kg in leaves of this species growing on a clayey-sandy saline soil in the Caribbean coast of Venezuela, which is typical of plants living in environments with low water potential. Another Amaranthaceae, *Haloxylon ammodendron*, which grows in sandy deserts of Middle and Central Asia, also has a low content of organic matter (Zhou et al.

2012). Compared to other members of the Amaranthaceae such as *Alternanthera philoxeroides* and *Chenopodium acuminatum* (Li et al. 2011; Zhang et al. 2014), *H. ritteriana* litter has a low C/N ratio. This might benefit growth of the prevailing microbial populations since colonization of organic matter and its degradation are a function of N, a nutrient whose availability in arid environments is limited (Perroni-Ventura et al. 2010). In line with this, our results suggest that, in "Salinas Grandes", *H. ritteriana* debris is a source of life, including halotolerant bacteria and fungi, most probably linked to cellulose degradation. Among these organisms, fungi have proved to adapt better than bacteria to high contents of salt, which is agreement with the finding of Romaní et al. (2006), who reported a higher ability of fungi to decompose plant polymers. Furthermore, fungi that recycle nitrogen generally assimilate organic substrate more efficiently than bacteria (Hodge et al. 2000).

In the present study, a cellulolytic fungus was isolated from *H. ritteriana* litter when applied on agar medium in the presence of NaCl 5 %. Morphological characters as well as the phylogenetic analyses using the ITS and EF-1α markers confirmed that the fungus was *F. equiseti* (LPSC 1166). Several species of *Fusarium*, including *F. equiseti*, had already been related to environments with low water potential (Jurado et al. 2005; Smolyanyuk et al. 2013). Isolate LPSC 1166 proved to degrade CMC in the presence of 5 and 10 % NaCl, suggesting that the metabolic pathways of this fungus are active even under high ionic strength. Because of this, we hypothesized that this fungus might play a key role in degrading cellulose in saline soils such as those of "Salinas Grandes". Previous findings suggest a relationship between isolation sources of the fungi and their metabolic pathways, including plasticity and synthesis of lytic enzymes such as cellulases, which have adaptive and ecological significance (Fenice et al. 1997; Colpaert et al. 2004). Since Pacheco et al. (2016) demonstrated that the salt content in the isolation medium tolerated by fungi of the genus *Fusarium* is directly related to the salt content in the environment of isolation, this may explain the cellulolytic ability of *F. equiseti* LPSC 1166 under salt stress. However, this depends on the available NaCl concentration.

Although isolate LPSC 1166 on agar cultures nursed itself from substrates such as glucose, CMC and probably endogenous C sources or other exogenous materials as well as gases (e.g. CO_2) and volatiles from the atmosphere, as reported for several fungi, including representatives of *Fusarium* (Parkinson et al. 1991; Liu and Chen 2003), its growth was dependent on the C source and NaCl concentration. Glucose proved to be a poor C source for this fungus under low NaCl level. Cochrane et al. (1963) and Diniz Maia et al. (1999) reported that, *in vitro*, species within *Fusaria* like *F. oxysporum* f. *lini* and *F. solani* can grow better on C compounds other than glucose. However, when saline stress was imposed, *F. equiseti* LPSC 1166 growth on glucose was the same as that on CMC. Such response might be due to the osmotic stress that probably affected the uptake of C sources

(Ayar-Kayall and Tarchan 2004). The efficiency of the fungus to assimilate cellulose changed concomitantly with the availability of salt, probably due to the higher demand of C and energy for the synthesis of degradative enzymes, osmoregulation (osmolytes) and/or growth even under such stress. Breuer and Harms (2006) suggested that lignocellulolytic fungi under stress develop alternative mechanisms of nutrition and/or adjustment of the internal osmotic potential to survive. Furthermore, salt tolerance might be also related to the activation of transport processes involving both efflux and influx of protons, sodium, and potassium ions and/or the accumulation of compatible solutes (Dijksterhuis et al. 2013). Salt-dependent regulation of genes encoding enzymes involved in osmoadaptation and glycolytic flux has been reported in other fungi (Petelenz-Kurdziel et al. 2013). Although the method used to estimate colony size might prevent us from detecting small differences between data from each salt-supplemented medium, we hypothesized that the saline stress might have enhanced the ability of isolate LPSC 1166 to grow on C sources other than cellulose, probably by inhibiting the synthesis and/or activity of depolymerizing enzymes.

In vitro, the fungus degraded *H. ritteriana* litter and modified the physical and chemical properties of the WSF. FTIR analysis showed higher concentration of lignin-type compounds relative to polysaccharides in plant cell walls treated with the fungus than in untreated ones. This and the detection of β-1,4 endoglucanase activity in the WSF suggest that the fungus selectively degrades holocellulose. In addition, through XRD, we demonstrated a reduction in the crystallinity of cellulose in the litter treated with the fungus. Several Ascomycetes fungi, including *Fusaria*, cause soft-rot decays in lignocellulosic materials, where the amount of cellulose degraded is also significantly larger than that of lignin (Ma et al. 2013). Although in the present study salt reduced the ability of the fungus to degrade litter, mostly polysaccharides, probably due to a reduction in the extracellular β-1,4 endoglucanase activity, we found a relative increase in the cellulose crystalline structure. This might be due, at least in part, to the effect of Na^+ ions that contributed to a re-organization of the residual amorphous cellulose. Yue et al. (2013) reported the generation of new crystalline structures in amorphous regions from cotton fibers by NaOH treatment, a process that was dependent on the NaOH concentration. Although we only added Na^+ ions in form of a neutral salt to the culture system, both high available levels of Na^+ ions and the alkalinity caused by the fungal activity on the litter WSF might have led to the conditions for mercerization of cellulose by NaOH, increasing in this way the relative crystallinity index.

To our knowledge, this is the first report regarding the isolation of *F. equiseti* from *H. ritteriana* litter. *F. equiseti* LPSC 1166 is a halotolerant cellulolytic fungus that plays a key role in the degradation of *H. ritteriana* litter under salt stress such as that prevailing in the soils of "Salinas Grandes", Córdoba, Argentina.

Therefore, this fungus may be a biological tool for the restoration of desert areas with saline soils with important consequences in soil fertility and plant productivity. Also, *F. equiseti* LPSC 1166 might be an interesting source for future studies on biotechnological research such as bioethanol production using ionic liquids.

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Figure legends:

Fig. 1 Bacterial (a) and fungal (b) colonies from washed *Heterostachys ritteriana* litter material grown on selective media with carboxymethylcellulose (CMC) as sole C source (control, I) and also supplemented with NaCl: 1.25 (II), 2.5 (III) and 5 (IV) % after 7 days of incubation at 25 ºC.

Fig. 2 Maximum-likelihood phylogram of the partial sequence of the EF-1α gene; most-likelihood tree (-lnL = -5177.85400) under best-fit nucleotide substitution model (TIM2+G) selected by Akaike Information Criterion from among 88 competing models (-Ln likelihood = 5177.8538; base freq: A = 0.2286, C = 0.3039, G = 0.2199, T = 0.2476; rates of substitution = [AC] = 1.7157, [AG] = 3.4239, [AT] = 1.7157, [CG] = 1.0000, [CT] = 5.9048; gamma shape = 0.5660); percentage bootstrap support values from 1000 replicates are shown; the scale bar indicates substitutions per site.

Fig. 3 Extracellular cellulolytic activity of *Fusarium equiseti* LPSC 1166 grown on CMC agar medium supplemented with 5 (left) and 10 (right) % NaCl: plates after staining with Congo red solution and washing with 1 mol/L NaCl and 5 % acetic acid solutions, showing clear zones around the growth of the isolate.

Fig. 4 Average FTIR absorption spectra normalized at the peak 1060/cm of unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *F. equiseti*: uninoculated litter (I), inoculated litter (II), uninoculated litter + NaCl (III) and inoculated litter + NaCl (IV).

Table 1 Isolates and GenBank accession numbers used in this study for sequence alignments and phylogenetic analyses.

Species name	Strain number/ID	ITS[a]	EF-1α[a]
F. acuminatum	PUF035	HQ165938.1	HQ165866.1
F. acuminatum	PUF036	HQ165939.1	HQ165867.1
F. armeniacum	NRRL 43641	GQ505462.1	GQ505430.1
F. avenaceum	PUF034	HQ165937.1	HQ165865.1
F. concolor	NRRL 13459	GQ505763.1	GQ505674.1
F. delphinoides	PUF027	HQ165930.1	HQ165858.1
F. delphinoides	PUF028	HQ165931.1	HQ165859.1
F. equiseti	NRRL 26419	GQ505688.1	GQ505599.1
F. equiseti	MAFF 236723	AB587000.1	AB674278.1
F. equiseti	**LPSC 1166**	**KJ854378**	**KJ854377**
F. equiseti	MAFF 236434	AB586999.1	AB674277.1
F. incarnatum	MAF 236521	AB586988.1	AB674267.1
F. incarnatum	11	KF181239.1	KJ194168.1
F. incarnatum	PUF030	HQ165933.1	HQ165861.1
F. sporotrichioides	PUF032	HQ165935.1	HQ165863.1
F. cf. oxysporum	PUF017	HQ165920.1	HQ165848.1
F. cf. solani	PUF007	HQ165910.1	HQ165838.1
F. cf. solani	PUF008	HQ165911.1	HQ165839.1
F. cf. solani	PUF009	HQ165912.1	HQ165840.1
Gibberella fujikuroi	PUF022	HQ165925.1	HQ165853.1
Gibberella intermedia	PUF018	HQ165921.1	HQ165849.1
Gibberella intermedia	PUF020	HQ165923.1	HQ165851.1
Gibberella intermedia	PUF021	HQ165924.1	HQ165852.1
Hypocrea lixii	CBS 226.95	AY605713.1	AF534621.1

[a]GenBank accession number

Table 2 Growth and cellulolytic ability of *Fusarium equiseti* LPSC nº 1166 on agar-CMC plates in the presence of NaCl after 6 days of incubation[a].

	BM[c] (Control)	BM + 5 % NaCl	BM + 10 % NaCl
Colony diameter (cm)	7.4 ± 0.11 a[d]	5.9 ± 0.29 b	2.1 ± 0.17 c
Cellulolytic halo[b] (cm)	7.9 ± 0.06 a	6.0 ± 0.38 b	2.7 ± 0.04 c

[a]data are means of four replicates ± SD (standard deviation); [b]diameter of halo area where CMC was degraded; [c]basal medium; [d]data with the same letter are not significantly different (Tukey test $p < 0.05$)

Table 3 Growth of *Fusarium equiseti* LPSC nº 1166 on MSA medium supplemented with several C sources either alone or in the presence of 10 % NaCl after 4 days of incubation[a].

	Colony diameter (cm)
Basal medium	3.63 ± 0.25 b
+ CMC[b]	4.22 ± 0.09 a
+ Glucose	3.23 ± 0.06 c
Basal medium + 10 % NaCl	1.68 ± 0.08 e
+ CMC	2.07 ± 0.15 d
+ Glucose	2.38 ± 0.10 d

[a]mean ± SD of three replicates; data followed by the same letter are not significantly different (Tukey test $p < 0.05$); [b]*CMC* carboxy-methylcellulose

Table 4 *Heterostachys ritteriana* litter treatment with *Fusarium equiseti* LPSC nº 1166 and its response to NaCl at 10 %: litter mass and physical-chemical parameters in the WSF[a].

Parameter	UL	IL	UL-NaCl	IL-NaCl
Leaf mass (g)	0.95 ± 0.01 a	0.75 ± 0.01 b	0.96 ± 0.01 a	0.86 ± 0.01 c
pH	7.68 ± 0.01 a	9.23 ± 0.10 b	6.89 ± 0.05 c	8.08 ± 0.05 d
EC (dS/m)	1.72 ± 0.11 a	2.36 ± 0.11 a	12.66 ± 0.75 b	12.80 ± 0.25 b
Abs. $_{465\ nm}$/Abs. $_{665\ nm}$	8.79 ± 0.23 a	6.90 ± 0.38 b	8.46 ± 0.56 a	8.50 ± 0.21 a

[a]means ± SD of three replicates; data followed by the same letter are not significantly different (Tukey test $p < 0.05$); *UL* uninoculated litter; *IL* inoculated litter; *UL-NaCl* uninoculated litter + NaCl; *IL-NaCl* inoculated litter + NaCl

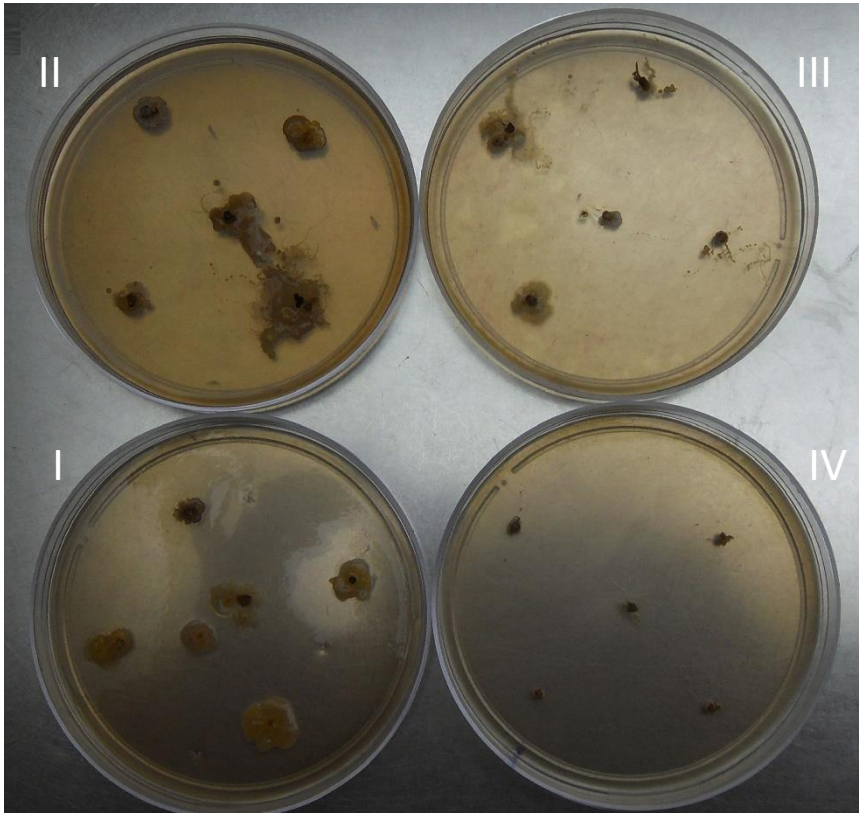
Table 5 RCI of unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *Fusarium equiseti*.

	RCI (%)[a]
UL	77 ±1.7 a
UL-NaCl	74 ±0.7 a
IL	60 ±2.1 b
IL-NaCl	90 ±3.2 c

[a]means ± SD of three replicates; data followed by the same letter are not significantly different (Tukey test $p < 0.05$); *UL* uninoculated litter; *IL* inoculated litter; *UL-NaCl* uninoculated litter + NaCl; *IL-NaCl* inoculated litter + NaCl

Fig. 1

a



b

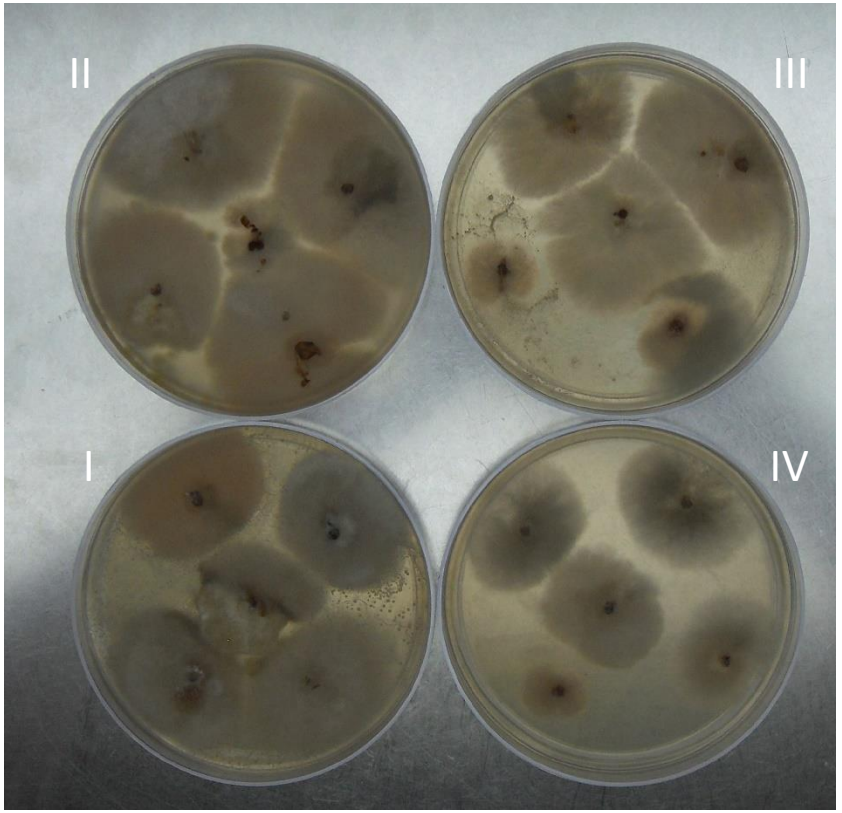


Fig. 2

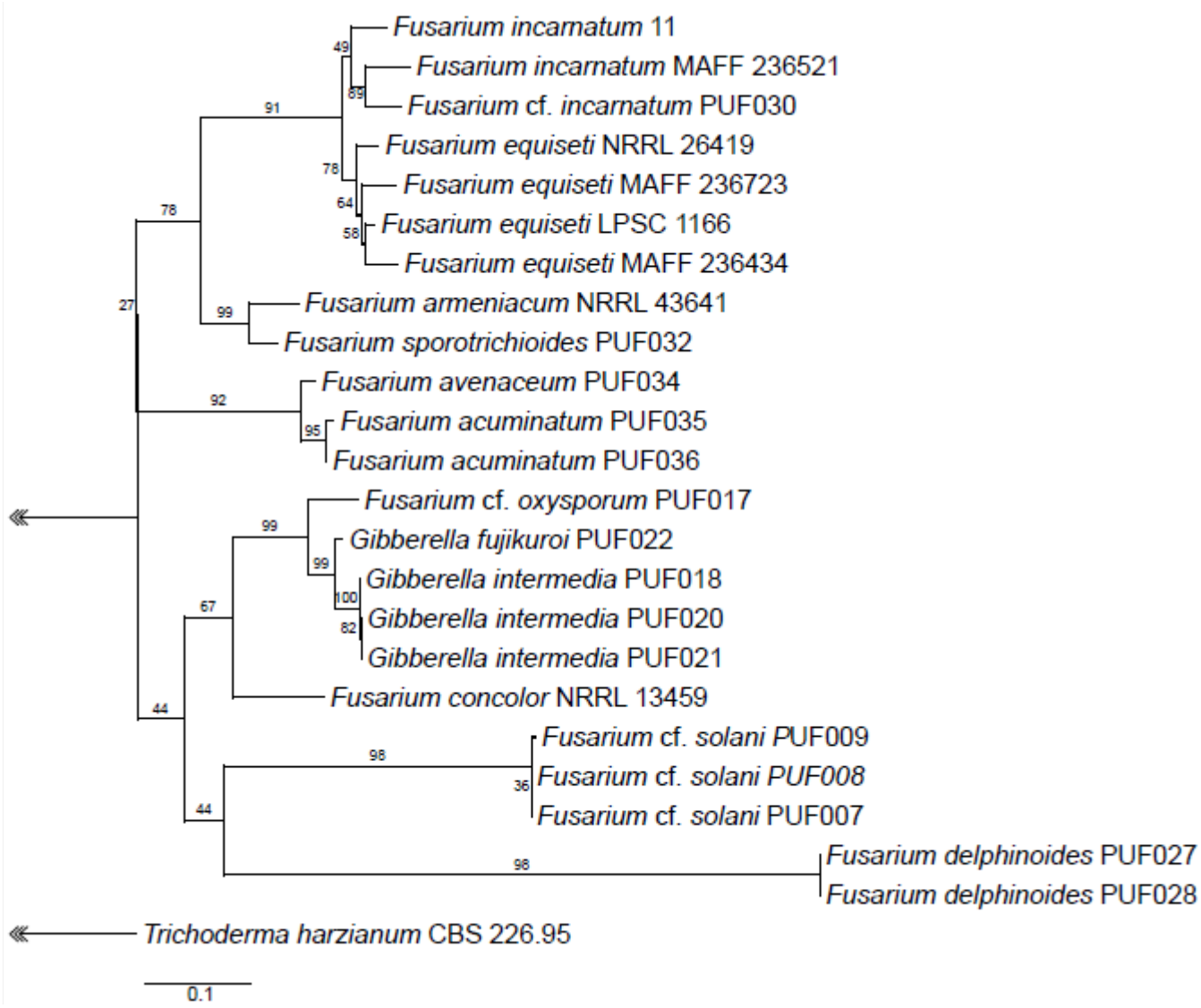

Fusarium incarnatum 11
Fusarium incarnatum MAFF 236521
Fusarium cf. incarnatum PUF030
Fusarium equiseti NRRL 26419
Fusarium equiseti MAFF 236723
Fusarium equiseti LPSC 1166
Fusarium equiseti MAFF 236434
Fusarium armeniacum NRRL 43641
Fusarium sporotrichioides PUF032
Fusarium avenaceum PUF034
Fusarium acuminatum PUF035
Fusarium acuminatum PUF036
Fusarium cf. oxysporum PUF017
Gibberella fujikuroi PUF022
Gibberella intermedia PUF018
Gibberella intermedia PUF020
Gibberella intermedia PUF021
Fusarium concolor NRRL 13459
Fusarium cf. solani PUF009
Fusarium cf. solani PUF008
Fusarium cf. solani PUF007
Fusarium delphinoides PUF027
Fusarium delphinoides PUF028
Trichoderma harzianum CBS 226.95
0.1

Fig. 3

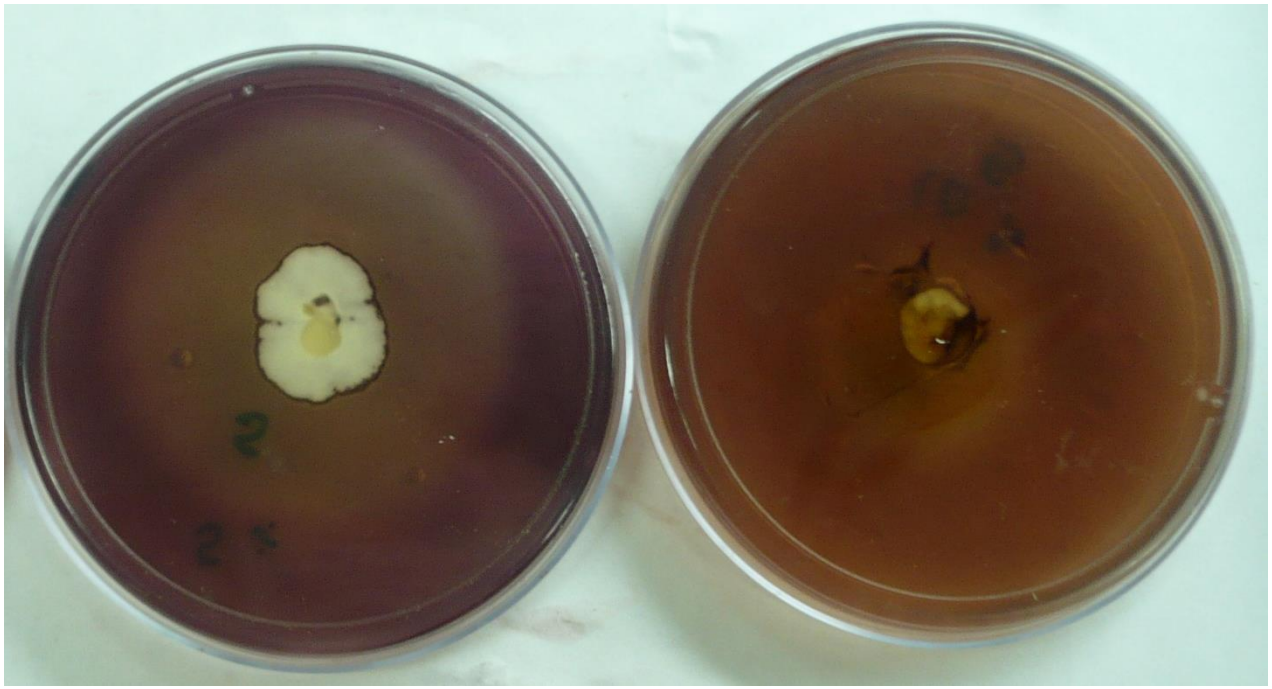
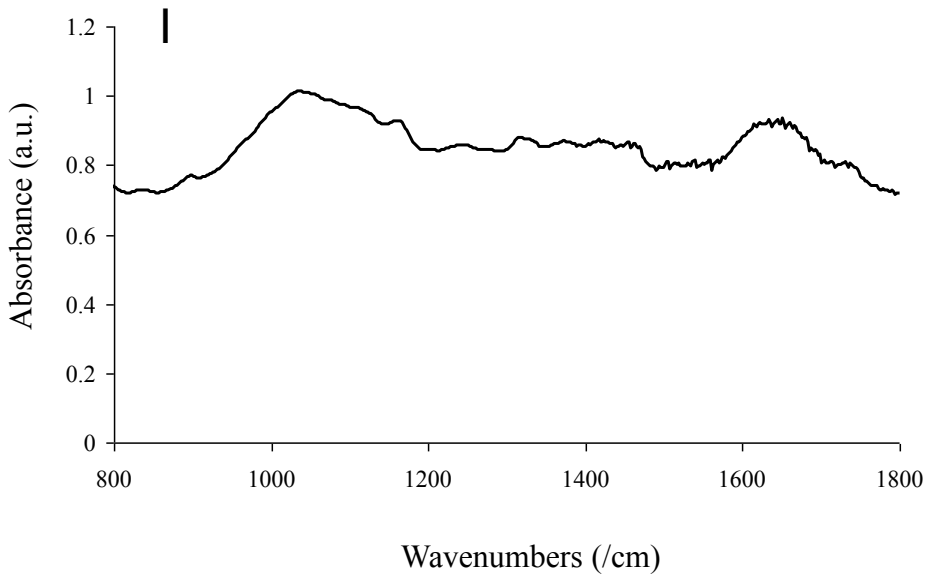
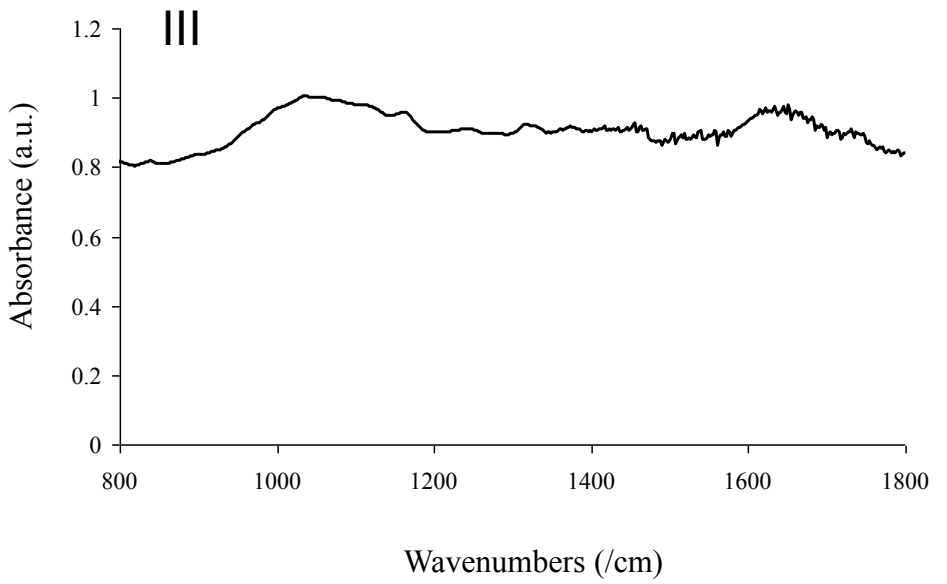
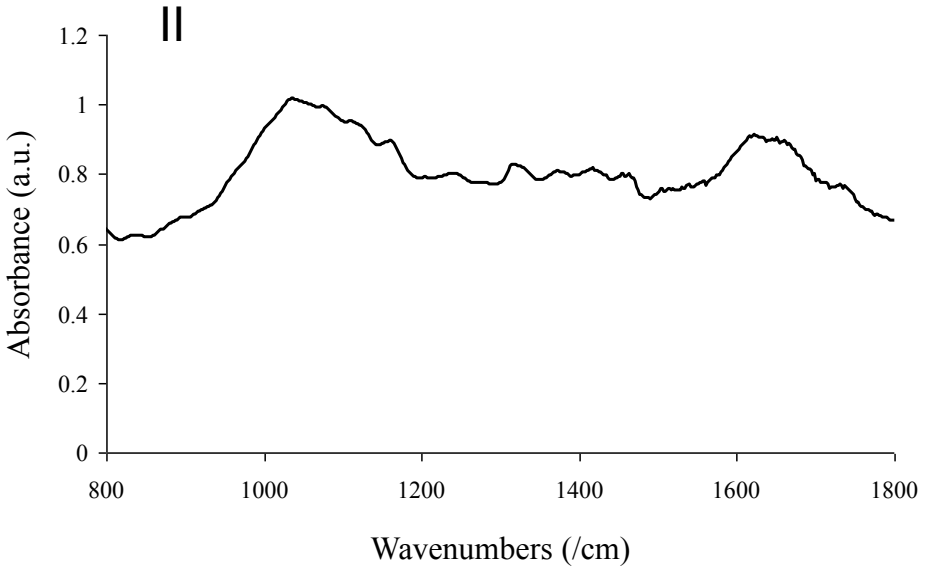
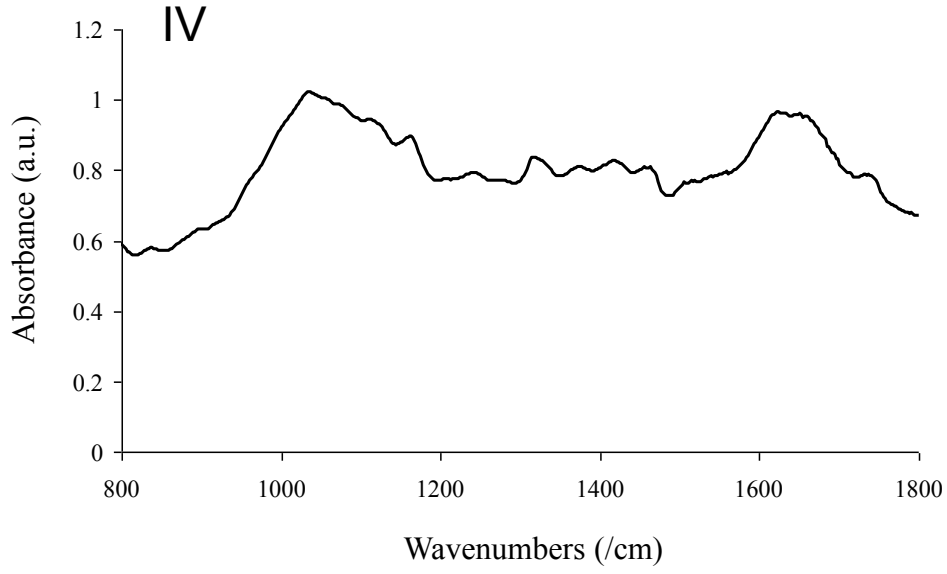


Fig. 4


I
Absorbance (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
800
1000
1200
1400
1600
1800
Wavenumbers (/cm)


III
Absorbance (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
800
1000
1200
1400
1600
1800
Wavenumbers (/cm)


II
Absorbance (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
800
1000
1200
1400
1600
1800
Wavenumbers (/cm)


IV
Absorbance (a.u.)
1.2
1
0.8
0.6
0.4
0.2
0
800
1000
1200
1400
1600
1800
Wavenumbers (/cm)

Fusarium equiseti LPSC 1166 and its *in vitro* role in the decay of *Heterostachys ritteriana* leaf litter

Folia Microbiologica

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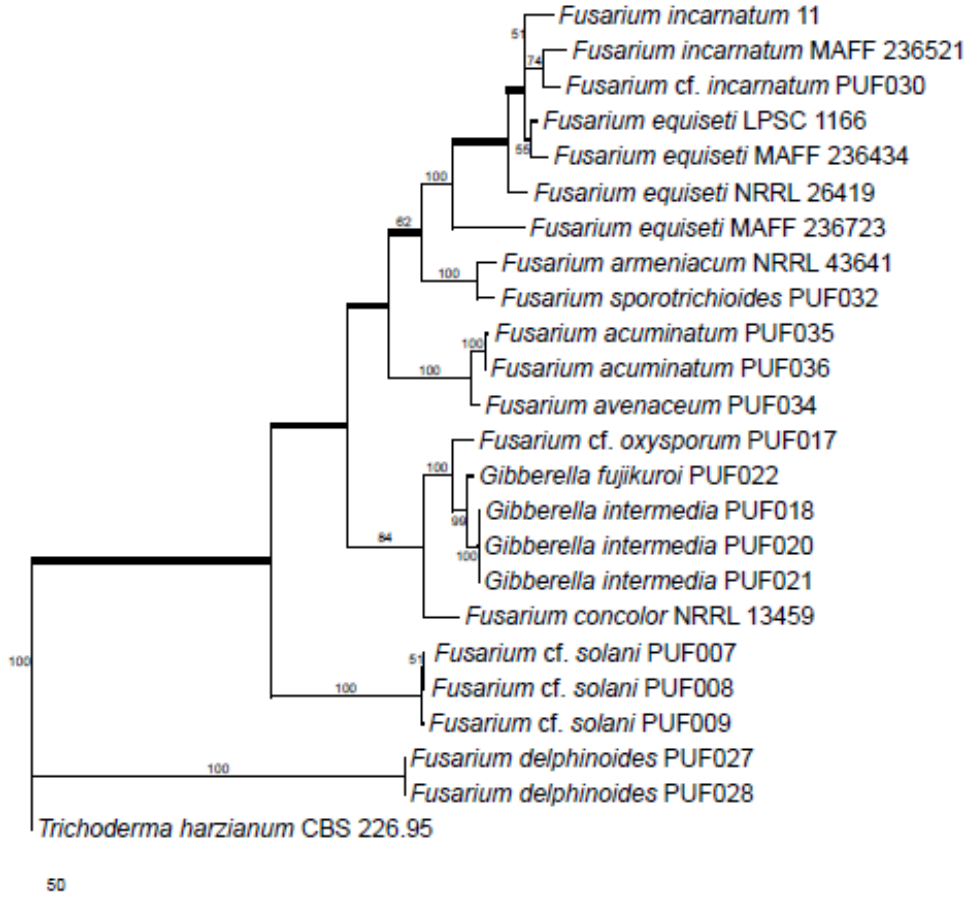
Electronic supplementary material 1 Maximum-parsimony phylogram of EF-1α gene partial sequences based on 661 characters derived from 24 taxa (including the outgroup); one of the sixteen most parsimonious trees (tree length = 1135 steps; consistency index = 0.6899; retention index = 0.7910; rescaled consistency index = 0.5457); percentage bootstrap support values from 1000 replicates are shown; the scale bar indicates nucleotide changes (steps); thickened branches indicate branches that collapse in the strict consensus tree; while 146 characters were constant, 191 variable characters were parsimony-uninformative and 324 characters were parsimony-informative.



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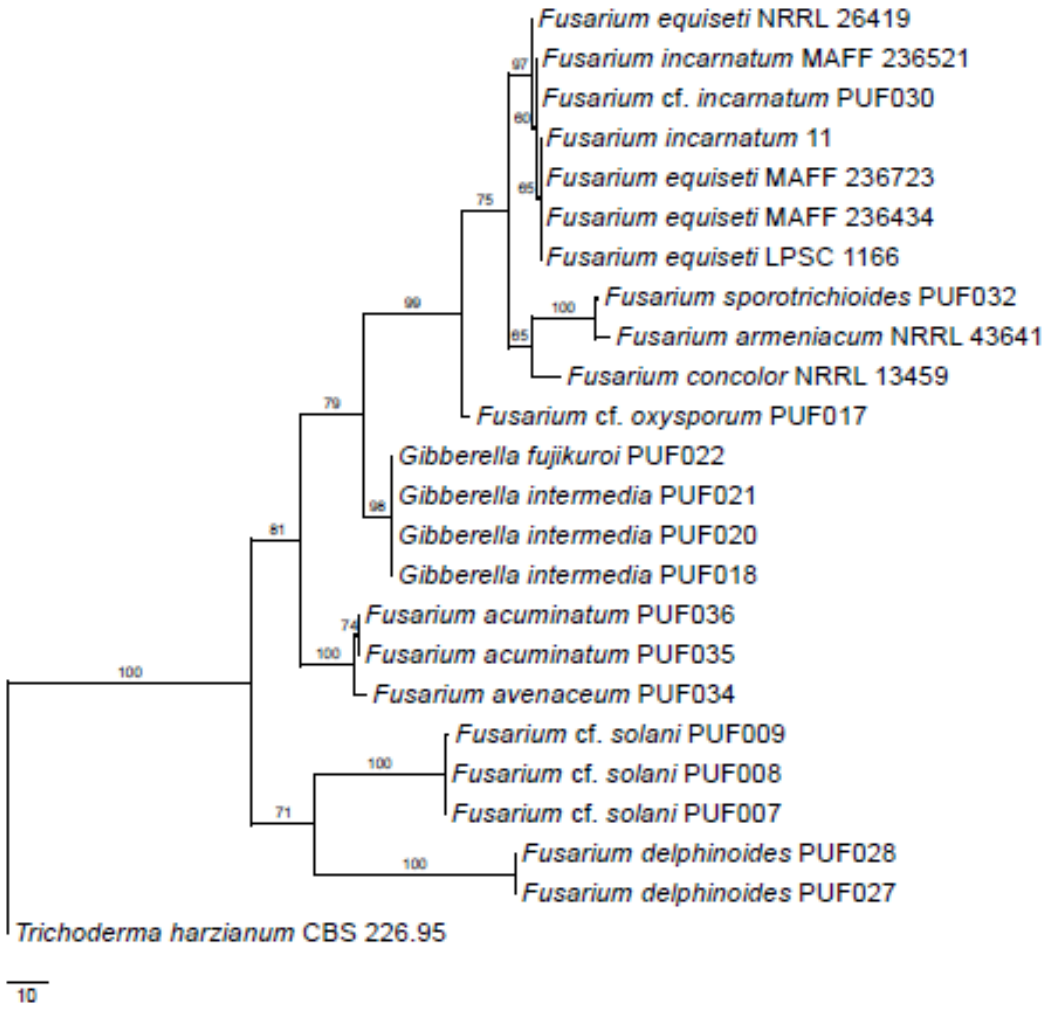
Electronic supplementary material 2 Maximum-parsimony phylogram of ITS partial sequences based on 528 characters derived from 24 taxa (including the outgroup); one of the two most parsimonious trees with identical topology (tree length = 285 steps; consistency index = 0.8035; retention index = 0.9065; rescaled consistency index = 0.7284); percentage bootstrap support values from 1000 replicates are shown; the scale bar indicates nucleotide changes (steps); while 358 characters were constant, 35 variable characters were parsimony-uninformative and 135 characters were parsimony-informative.



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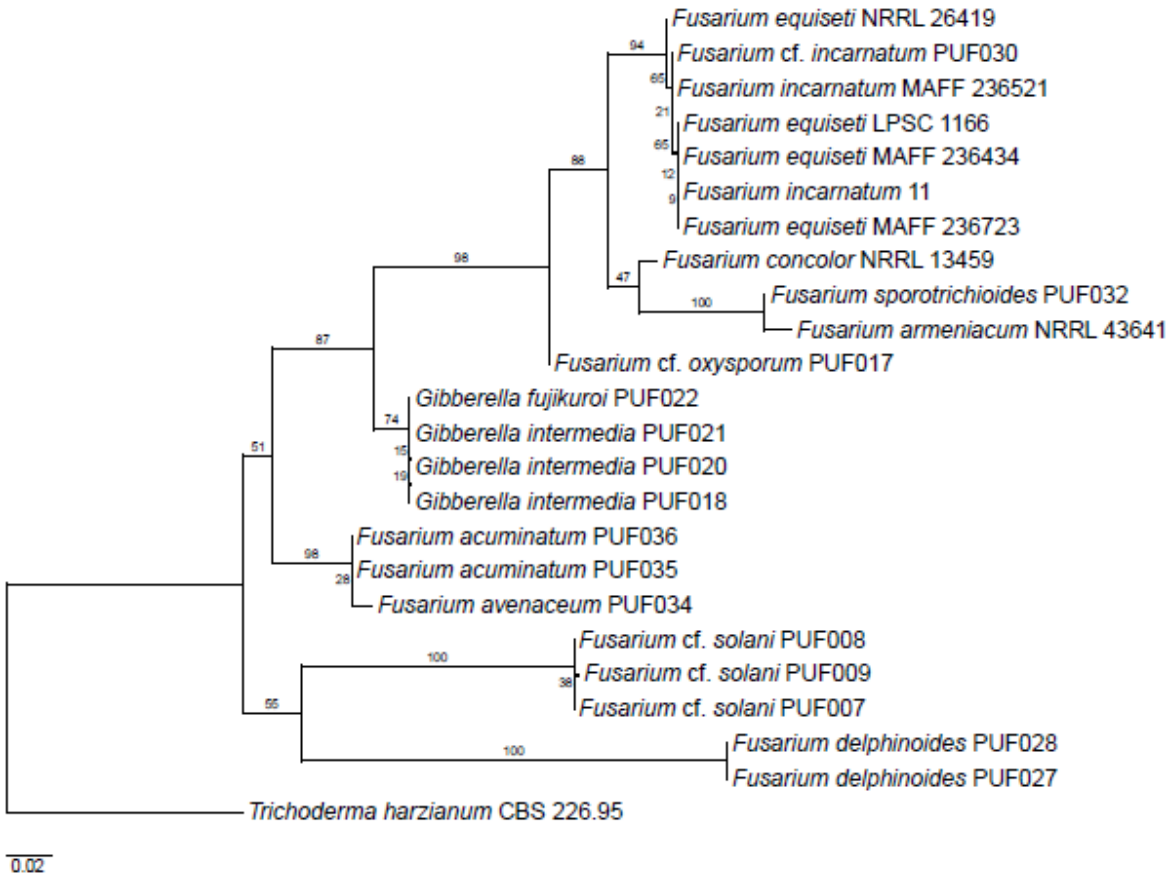
Electronic supplementary material 3 Maximum-likelihood phylogram of ITS partial sequence; most-likelihood tree (-lnL = -2053.23848) under best-fit nucleotide substitution model (TIM2+G) selected by AIC from 88 competing models (-Ln likelihood = 2053.2384; base freq: A = 0.2484, C = 0.2931, G =0.2385, T = 0.2200; rates of substitution= [AC] = 1.6894, [AG] = 1.1855, [AT] = 1.6894, [CG] = 1.0000, [CT] = 2.9262; gamma shape = 0.3490); percentage bootstrap support values from 1000 replicates are shown; the scale bar indicates substitutions per site.



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Electronic supplementary material 4 ANOVA for growth when the fungus was cultured on MSA basal medium supplemented with several C sources and two NaCl levels as well as the interaction between these variables.

		Colony diameter	
Factor	df	F value	*p*
C source	2	101.7	<0.000000
NaCl level	1	972.4	<0.000000
C source x NaCl level	2	117.1	<0.000000

df degree of freedom

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Electronic supplementary material 5 ANOVA for substrate mass and WSF parameters in unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *Fusarium equiseti* as well as the interaction between these variables.

		Leaf mass		pH		EC		Abs. $_{465\ nm}$/Abs. $_{665\ nm}$	
Factor	df	F value	*p*	F value	*p*	F value	*p*	F value	*p*
Treatment	1	1202.0	<0.000000	1717.3	<0.000000	2.9	ns	17.9	<0.002886
NaCl level	1	185.9	<0.000001	868.9	<0.000000	2104.6	<0.000000	8.5	<0.019301
Treatment x NaCl level	1	120.7	<0.000004	29.2	<0.000645	1.2	ns	19.5	<0.002257

df degree of freedom; *ns* not significant

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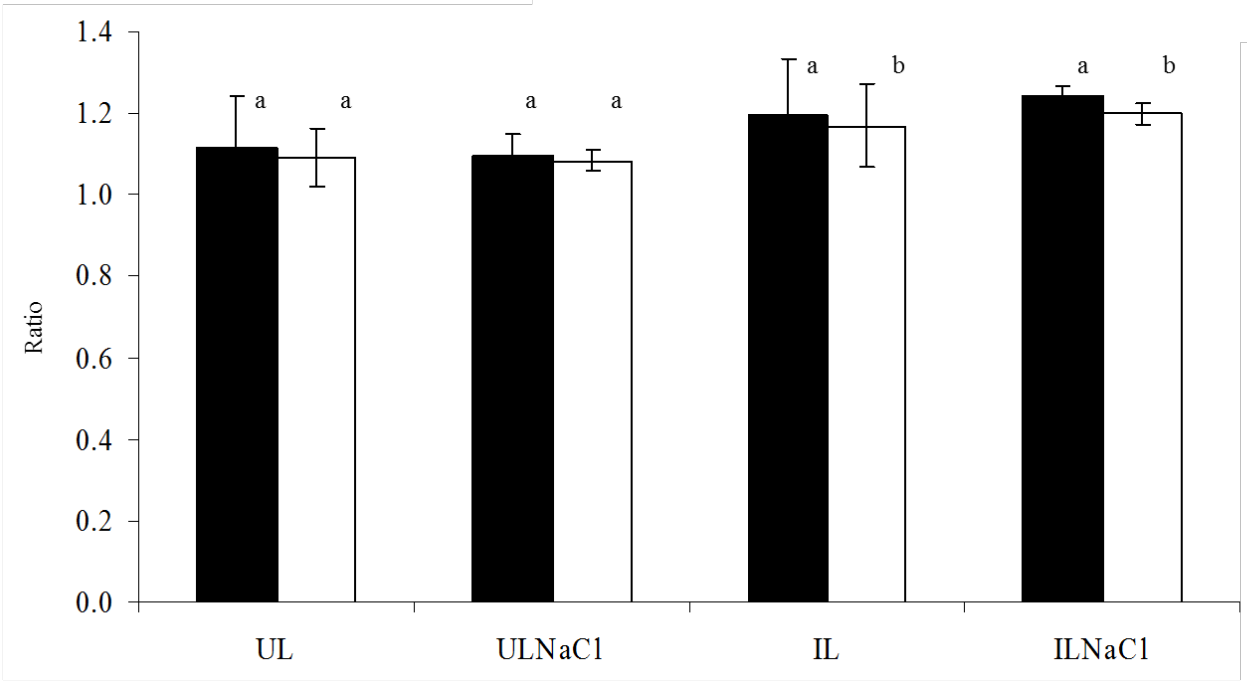
Electronic supplementary material 6 Relative intensity of selected bands in the FTIR spectra of unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *Fusarium equiseti*: ratio between 1737/cm and 896/cm (black bars) and between 1504/cm and 896/cm (white bars); *UL* uninoculated litter; *IL* inoculated litter; *UL-NaCl* uninoculated litter + NaCl; *IL-NaCl* inoculated litter + NaCl; all values are means of three replicates and error bars correspond to SD; bars for each ratio with the same letter are not significantly different (Tukey test $p < 0.05$).



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Electronic supplementary material 7 ANOVA for the ratios of the intensity of FTIR absorption bands associated with specific functional groups (896/cm for polysaccharide; 1504/cm for lignin; 1737/cm for carbonyl group) in unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *Fusarium equiseti* as well as the interaction between these variables.

		Abs. $_{1504/cm}$/ Abs. $_{896/cm}$		Abs. $_{1737/cm}$/Abs. $_{896/cm}$	
Factor	df	F value	*p*	F value	*p*
Treatment	1	6.6	<0.033477	4.0	ns
NaCl level	1	0.1	ns	0.1	ns
Treatment x NaCl level	1	0.2	ns	0.3	ns

df degree of freedom; *ns* not significant

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Electronic supplementary material 8 ANOVA for the RCI in unsupplemented and 10 % NaCl-supplemented leaf litter both untreated and treated with *Fusarium equiseti* as well as the interaction between these variables.

		RCI	
Factor	df	F value	*p*
Treatment	1	0.4	ns
NaCl level	1	125.6	<0.000004
Treatment x NaCl level	1	868.2	<0.000001

df degree of freedom; *ns* not significant