Endophytic fungus *Acremonium implicatum* is seed transmitted in *Brachiaria* spp.

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ABSTRACT

Several species of *Brachiaria*, a pan-tropical grass genus comprising about 100 species, are forages of economic importance in tropical America and elsewhere. *Acremonium implicatum* forms a symbiotic endophytic association with at least some of these economically important *Brachiaria* species. We sought to ascertain whether endophytic *A. implicatum* could be seed-transmitted in *Brachiaria*. Twenty tillers were vegetatively propagated from a single, endophyte-infected mother plant. Ten tillers were treated with the fungicide Folicur® to eliminate the endophyte while the remaining ten tillers were untreated. Seeds were harvested individually from these genetically identical plants, with or without the endophyte. Some of the seeds were germinated and seedlings grown in the glasshouse. A polymerase chain reaction (PCR)-based method developed previously uses a pair of endophyte-specific primers to amplify a single DNA fragment of about 500 bp. DNA both from remnant seeds and from 2-month-old seedlings was amplified with these primers to detect presence of the endophyte. The diagnostic DNA fragment was consistently amplified in DNA of seeds harvested from the endophyte-infected plants and DNA from seedlings grown from seeds harvested from endophyte-infected plants, but not from seeds or seedlings originating from fungicide treated endophyte-free plants. We conclude that *A. implicatum* can be transmitted through seeds.

INTRODUCTION

Brachiaria is a pan-tropical genus of grasses with about 100 species. The fungus *Acremonium implicatum* can develop an endophytic association that is mutually beneficial with *Brachiaria* species.

DNA from isolates of *A. implicatum* was amplified using 10-base random primers. Primer OPAK 10 (Operon Technology Inc.) amplified bands including a 500-bp product common to all of the isolates tested. This fragment has been cloned and sequenced. Based on this sequence data, several primers were designed and synthesized. A primer pair designated P1 (5'-TTCGAATGATAAGGCAGATC-3' and P4 (5'-ACGCATCCACTGTATGCTAC-3') amplified a 500-bp product with template DNA from isolates of *A. implicatum* in pure cultures and in tissues of *Brachiaria* infected with *A. implicatum*. No amplification product was detected in plants free from *A. implicatum* or using DNA of non-endophytic fungi or the bacterium *Xanthomonas campestris* pv. *graminis*, a pathogen of species of *Brachiaria* (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118).

This primer pair was used to conduct seed transmission studies in plants with and without *A. implicatum*. We report here the results of *A. implicatum* transmission studies in seeds of *Brachiaria*. The primer pair amplified a 500-bp product with template DNA of seeds harvested from *A. implicatum* infected *Brachiaria* plants, but no amplified products were observed with DNA of seeds from endophyte-free plants.

MATERIALS AND METHODS

Endophyte elimination

The fungicide Folicur® was used to generate endophyte-free *Brachiaria* clones. Twenty or more plantlets were propagated from a mother plant naturally or artificially infected with the endophyte. Half of these plantlets were soaked in a solution of 0.6 mL/L of Folicur® (250 g a.i./L) for 6 h to eliminate the endophyte, and the other half were left untreated to serve as controls. All plantlets were individually planted in small pots and placed in the greenhouse. Plants were examined 4-6 weeks after treatment for the presence or absence of *A. implicatum*.

DNA isolations

Fresh mycelia of endophyte isolates cultured on potato dextrose agar (PDA) plates, endophyte-infected or endophyte-free plant leaves, or seeds were collected and macerated in liquid nitrogen for genomic DNA isolation. Genomic DNA was extracted using the DNeasy™ Plant Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

PCR Amplifications

Specific primers P1 (5'-TTCGAATGATAAGGCAGATC-3') and P4 (5'-ACGCATCCACTGTATGCTAC-3') were used in the PCR reactions. Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc.), programmed with 44 cycles for genomic DNA of endophyte pure cultures or plant leaves, and 54 cycles for DNA from *Brachiaria* seeds, of a 30 sec denaturation step at 94°C (3 min for the first cycle), follwed by 1 min at 65°C, and primer extension for 1 min (10 min in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad), stained with ethidium bromide and photographed under UV lighting.

Seed samples were collected from plants confirmed to be endophyte-infected or endophyte-free using the PCR tests with template DNA isolated from plant tissues, and fungal endophyte isolation on culture media.

RESULTS AND DISCUSSION

The primer pair, P1/P4, allows the precise and rapid detection of *A. implicatum* in *Brachiaria* plants and permits a differentiation between endophytic and non-endophytic fungi (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118).

A single band of about 500-bp in all examined isolates of *A. implicatum* was amplified. Endophyte-containing and endophyte-free plants were also consistently differentiated using this primer combination (Fig. 1). Seeds were collected from plants whose tissue samples were used in Fig.1 as well as other plants. All seed DNA from endophyte-containing plants had a 500-bp amplified product. No amplification product was detected with seed DNA from endophyte-free plants (Fig. 2).

Seedlings generated from seed samples of endophyte-containing and endophyte-free plants had consistently tested positive or negative, respectively, for the diagnostic 500-bp amplified product (Fig. 3). From these results, we concluded that *A. implicatum* maintains its symbiotic association with species of *Brachiaria* through seed transmission.

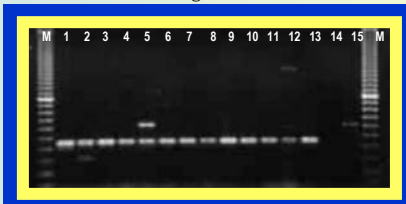

Figure 1. Specific detection of *Acremonium implicatum* in tissues of *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1-12**, template DNA of *Brachiaria* hybrid plants *BROONO*(#7), *BROONO* (#8), *BROONO* (#14), *BROONO* (#22), *BROONO* (#23), *BROONO* (#29), *BROONO* (#37), *BROONO* (#39), *BROONO* (#42), *BROONO* (#44), *BROONO* (#48), *BROONO* (#52), respectively; **lane 13**, *B. brizantha* CIAT 26110 (#15), artifically inoculated with endophyte isolate EB6780 (201); **lanes 14 and 15**, *B. brizantha* CIAT 16320 (#32-25) and *B. brizantha* CIAT 16320 (#32-29), respectively, (these were plants derived from naturally endophyte infected tillers and whose endophytes were successfully eliminated by treatment with fungicide Folicur® (0.6mL/L) for 5 hr.); **lanes M**, 100-bp ladders.

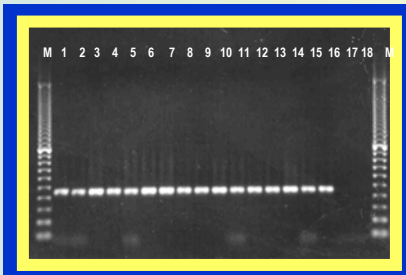

Figure 2. Specific detection of *Acremonium implicatum* in seeds harvested from endophyte-infected *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1-16**, template DNA extracted from seeds of endophyte infected *Brachiaria* hybrids SX99/3488 (8), SX99/0275 (14), BR99NO/4132 (22), FM9201/1873 (29), BR99NO/4015 (37), BR99NO/4132 (39), *B. decumbens* CIAT 606 (42), BRUZ4X/4402 (44), FM9201/1873 (48), SX99/0731 (52), *B. brizantha* CIAT 16320 (32a), FM9503/S046/024 (45), *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (68), and *B. brizantha* CIAT 6780 (111), respectively. **Lanes 17,18**, DNA extracted from seeds of endophyte-free plants of *B. brizantha* CIAT 16320 (32-25) and *B. brizantha* CIAT 16320 (32-29); **lanes M**, 100-bp ladders. *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (68), *B. brizantha* CIAT 6780 (111) were artificially infected. All others were naturally infected.

Figure 3. Specific detection of *Acremonium implicatum* in seedlings generated from seeds of endophyte-infected and endophyte-free *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1-7**, seedlings from seeds harvested from naturally endophyte-infected plants SX99/3488 (8), BRN99NO/4132 (22), BRN99NO/4132 (39), *B. decumbens* accession CIAT 606 (42), BRUZ4X/4402 (44), FM9201/1873 (48), SX99/073 (52), respectively; **lanes 8-17**, seedlings generated from seeds of ten artificially infected *B. brizantha* CIAT 26110 (15) plants; **lanes 18-25**, seedlings generated from seeds of eight naturally infected *B. brizantha* CIAT 16320 (32) plants; **lanes 26 & 27**, seedlings generated from seeds of two endophyte-free *B. brizantha* CIAT 16320 (32-25) plants; **lanes M**, 100-bp ladders.