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Agrobacterium-mediated transformation of the endophytic fungus *Acremonium implicatum* associated with *Brachiaria* grasses

Javier ABELLO[a], *Segenet KELEMU*[a,*], *Celsa GARCÍA*[b]

[a]*International Center for Tropical Agriculture (CIAT), A.A. 6713, Cali, Colombia*
[b]*Universidad Nacional de Colombia, Carrera 30 No. 45-03, Bogotá, Colombia*

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ABSTRACT

Acremonium implicatum is a seed-transmitted endophytic fungus that forms symbiotic associations with the economically significant tropical forage grasses, *Brachiaria* species. To take advantage of the endophyte's plant protective properties, we developed an efficient *Agrobacterium*-mediated transformation system for *Acremonium implicatum*, using green fluorescent protein (GFP) expression and vector pSK1019 (*trpC* promoter) or pCAMBIA1300 (CaMV35S promoter). We found that transformation efficiency doubled for both mycelial and conidial transformation as the co-cultivation period for *Agrobacterium tumefaciens* and *Acremonium implicatum* was increased from 48 to 72 h. Significantly, optimal results were obtained for either mycelial or conidial transformation with *Agrobacterium tumefaciens* strain AGL-1 and vector pSK1019 under the control of the *trpC* promoter. However, mycelial transformation consistently generated a significantly higher number of transformants than did conidial transformation. The mitotic stability of the transferred DNA was confirmed by growing ten transformants in liquid and agar media for six generations. In all cases, resistance to the selection pressure (hygromycin B) was maintained. Fluorescence emission was retained by the transformants and also expressed in *Brachiaria* tissues from plants inoculated with GFP-transformed *A. implicatum*. This technology will help in the transfer and expression of agronomically important genes in host plants.

Introduction

Acremonium implicatum is a seed-transmitted endophytic fungus that forms beneficial symbiotic associations with *Brachiaria* species (Dongyi & Kelemu 2004; Kelemu *et al.* 2001). The endophyte plays a role in protecting *Brachiaria* species from fungal pathogens, such as *Drechslera* spp., which cause leaf spots (Kelemu & Takayama 1998; Kelemu *et al.* 2001). Endophyte-infected grasses also possess several properties of applicable value, such as improved survival, growth stimulation, and drought tolerance (Arechavaleta *et al.* 1989).

Brachiaria is predominantly an African genus, comprising about 100 species, some of which have become commercially important forage grasses in tropical America. Several species of *Brachiaria* are apomictic and reproduce asexually through seed (Miles & do Valle 1991). Apomictic reproduction has key advantages for research on endophyte–host interactions and

* Corresponding author. Present address: International Livestock Research Institute (ILRI), BecA-ILRI Platform, Kabete Campus, Old Naivasha Road, P.O. Box 30709, 00100 Nairobi, Kenya.
E-mail address: s.kelemu@cgiar.org
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use. The practical implication of seed transmission of endophytes in *Brachiaria* is significant; once associated with the plant, the fungus can perpetuate itself through seed, especially in the apomictic genotypes of *Brachiaria*, provided that seed storage conditions do not reduce the endophyte's survival.

Several *Brachiaria* hybrids held at the forage breeding programme at the International Centre for Tropical Agriculture (CIAT, its Spanish acronym) were shown to harbour *A. implicatum*. Hence, we hope to exploit this association and its high seed transmission (Dongyi & Kelemu 2004) by using transgenic *A. implicatum* as a vehicle for producing and delivering gene products of agronomic interest into the host plant to enhance protective benefits and other traits.

The green fluorescent protein (GFP) gene was isolated from the jellyfish *Aequorea victoria*, and enhanced colour variants (ECFP for cyan, EGFP for green, and EYFP for yellow) have since been generated through mutagenesis. The gene and its variants are now some of the most widely used reporters in biological research (Lippincott & Patterson 2003; Patterson *et al.* 2001), having been expressed in a wide array of organisms, including plants and microbes (Mikkelsen *et al.* 2001; Pierce *et al.* 1997; Prasher *et al.* 1992; Tsien 1998). The fluorescent genes are useful for tracking proteins in living cells; as reporters to monitor promoter activity; as labels to visualize specific tissues, whole cells, or subcellular organelles; and for monitoring gene expression and protein localization (Tsien 1998). This protein is a successful reporter because it requires only UV or blue light and oxygen, and does not require cofactors or substrates for visualization, as do many other reporters. Therefore, the GFP gene was regarded as potentially useful as a reporter in the study of the interactions between *A. implicatum* and its host *Brachiaria*.

This paper describes the establishment of a transformation protocol and expression of the GFP gene in an isolate of *A. implicatum*. Preliminary results of this work have already been published in abstract form (Kelemu *et al.* 2006).

Materials and methods

Plasmid

We used plasmid pSK1019, kindly provided by Seogchan Kang of the Department of Plant Pathology, University of Pennsylvania. The plasmid contains the *egfp* gene under the promoter of a gene that encodes glyceraldehyde-3-phosphate dehydrogenase (GPD) and was isolated from *Cochliobolus heterostrophus*. It also contains the hygromycin B resistance gene *hph*, controlled by the *Aspergillus nidulans trpC* promoter, and the *kan* gene for kanamycin resistance. Hygromycin B is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus*, and is used to select and maintain prokaryotic and eukaryotic cells transformed with the *hph* gene. Vector pCAMBIA1300, which carries the CaMV35S promoter and the *kan* and *hph* genes, was used as control.

Preparing Acremonium implicatum *cells*

Acremonium implicatum strain 6780-201v, isolated from *Brachiaria brizantha* CIAT 6780, was used to transform its conidia or mycelia. The fungus was grown on yeast–malt extract–glucose (YMG) agar (4 g yeast extract, 10 g malt extract, 4 g D-glucose, 10 g agar, 1 l distilled water, pH 6) medium for 8 d and incubated at 28 °C. Conidia were collected in a 1 ml solution of 0.15 M NaCl and cleaned by passing through a Whatman No. 1 filter paper. The conidia were then suspended in 50 ml YMG liquid medium and incubated while agitated (250 rev min^{-1}) for 4 h at 28 °C to induce conidial germination.

The conidia were then collected by filtration and resuspended in 20 ml of an induction medium with acetosyringone [IM + AS: 2.05 g K_2HPO_4; 1.45 g KH_2PO_4; 0.15 g NaCl; 0.5 g $Mg_2SO_4.7H_2O$; 0.07 g $CaCl_2.2H_2O$; 0.0025 g $Fe_2SO_4.7H_2O$; 0.5 g $(NH_4)_2SO_4$; 10 mM D–glucose; 0.5 % glycerol; and 40 mM MES (2-(*N*-morpholino) ethanesulphonic acid; pH 7) in 1 l distilled water]. Then, 200 μM acetosyringone were added just before use. The whole conidial suspension had a concentration of 1×10^6 conidia ml^{-1}.

To obtain mycelia for transformation, the protocol described above was used, except that incubation was extended from 4 to 48 h and the mycelial concentration in the IM + AS medium was adjusted to $OD_{600} = 0.35$.

Transformation of Acremonium implicatum

The protocol we used was based on the methods described by Mullins *et al.* (2001) for the transformation of the pathogenic fungus *Fusarium oxysporum*. Some modifications were introduced, for example, *Acremonium implicatum* is a slow-growing fungus. Hence, the recommended concentration of cefotaxime (200 μM) for inhibiting the growth of *Agrobacterium tumefaciens* was insufficient to prevent the bacterium's growth from impeding that of *Acremonium implicatum*. Experiments indicated that cefotaxime concentrations of 500 μM were sufficient to inhibit growth in *Agrobacterium tumefaciens* while allowing *Acremonium implicatum* putative transformants to grow on selection media. Instead of using minimal medium (MM), we used TYNG medium (for 1 l medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl, and 0.5 g $MgSO_4 \cdot 7H_2O$, pH 7.5), which reduced the time needed to reach the required bacterial concentration ($OD_{600} = 0.75$) from 48 to only 16 h. In addition, the TYNG medium eliminated the cell aggregation problem encountered with the growth of *Agrobacterium tumefaciens* (particularly with strain LBA4404 in MM), which interferes with the transformation process.

A. tumefaciens strains AGL-1 and LBA4404 were transformed with vector pSK1019 or pCAMBIA1300, using the methods described by Den Dulk-Ras & Hooykaas (1995). The transformed bacteria were grown in TYNG medium, supplemented with kanamycin (100 μg ml^{-1}), and incubated at 28 °C in the dark for 16 h to an OD_{600} of 0.75. This bacterial cell concentration was subsequently diluted with IM + AS to $OD_{600} = 0.1$ and further incubated for 4 h to induce virulence genes. Once the incubation was completed, the bacterial cell concentration was adjusted to $OD_{600} = 0.2$.

The *A. implicatum* preparations described previously and these *Agrobacterium tumefaciens* transformant cells were mixed together in equal volumes (100 μl each). Each mixture was placed, at 200 μl, on a nitrocellulose membrane with a 0.45 μm pore size and 45 mm diam (Whatman LabSales, Hillsboro, OR), and plated on IM+AS agar medium (glucose

Table 1 – Number of putative *Acremonium implicatum* transformant colonies per Petri dish of selection medium, showing the effect of the co-cultivation period for *Agrobacterium tumefaciens* strains AGL–1 and LBA4404 and *Acremonium implicatum* on transformation efficiency

Agrobacterium tumefaciens strain		AGL-1[a]				LBA4404			
Promoter		*trpC*		CaMV35S		*trpC*		CaMV35S	
Recipient fungal structure[b]		M	C	M	C	M	C	M	C
Co-cultivation period (h)	48	542	271	1.7	2.0	0.7	0.3	0	0
	60	836	318	1.3	3.3	1.0	1.0	0	0
	72	1084	542	0	0	1.3	1.7	0	0

a *Agrobacterium tumefaciens* strain AGL–1 contains plasmid pSK1019, which has the enhanced green fluorescent protein (*egfp*) gene under the promoter for the gene that encodes glyceraldehyde-3-phosphate dehydrogenase (GPD) and was isolated from the fungus *Cochliobolus heterostrophus*. It also contains the hygromycin B resistance gene *hph*, controlled by the *Aspergillus nidulans trpC* promoter.
b M, mycelia; C, conidia. The values represent the mean number of transformants for three plates.

content reduced to 5 mM). These mixtures were incubated for 48, 60, and 72 h. The membranes were subsequently transferred to Petri plates, carrying YMG agar media that contained hygromycin B (100 μg ml^{-1}) and cefotaxime (500 μM), and incubated further at 28 °C. Putative transformant *Acremonium implicatum* cells became apparent on the selection media after 5 d of incubation.

Control *A. implicatum* cells were treated the same way, except that they were co-cultivated with strains of *Agrobacterium tumefaciens* that had not been transformed with the plasmid vectors.

PCR amplifications

DNA isolated (Kelemu *et al.* 2003) from putative transformant bacteria and fungi, as well as controls, was analysed using PCR, using primers with sequences from *egfp* and/or *hph*: glGFP3 (5′-GCCGAGCTCAGATCTCACTTGTACAGCTCGTCCATGCC-3′) and glGFP5 (5′-GCCGGAATTCATGAGCAAGGGCGAGGAACTG TTC-3′) (Fitzegerald *et al.* 2003); and hph122U (5′-TTCGATG TAGGAGGGCGTGGAT-3′) and hph725L (5′-CGCGTCTGCTGC TCCATACAAG-3′) (Malonek & Meinhardt 2001).

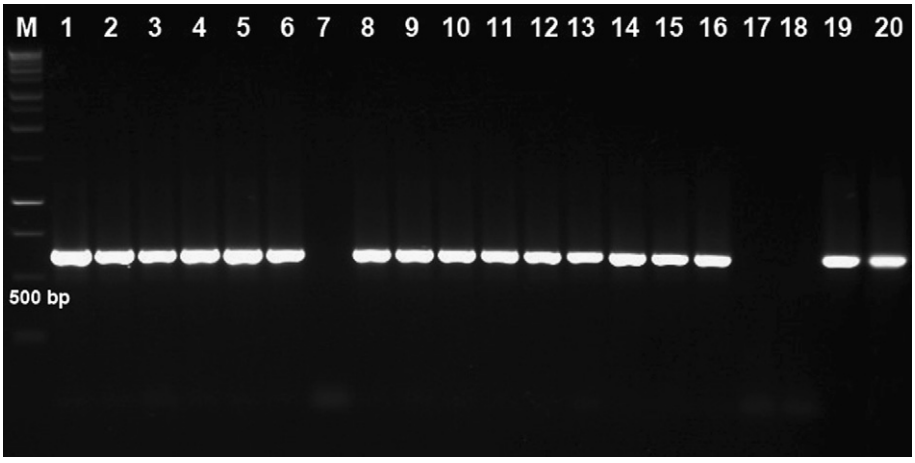

Fig 1 – Primers specific for sequences of the hygromycin B resistance gene (*hph*) were used for PCR amplifications of template DNA isolated from *Acremonium implicatum* transformants. Lane M = molecular marker; lane 1 = conidia transformed with pSK1019 in *Agrobacterium tumefaciens* strain LB4404, co-cultivated for 72 h and maintained without antibiotic selection pressure; lanes 2–3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1, co-cultivated for 48 and 60 h, and maintained without or with antibiotic selection pressure, respectively; lane 4 = conidia transformed with pCAMBIA1300 in strain AGL-1, co-cultivated for 72 h and maintained without antibiotic selection pressure; lane 5 = mycelia transformed with pSK1019 in strain LB4404, co-cultivated for 60 h and maintained without antibiotic selection pressure; lane 6 = mycelia transformed with pSK1019 in strain AGL-1, co-cultivated for 72 h and maintained with antibiotic selection pressure; lane 7 = negative control (water); lanes 8–11 = mycelia transformed with pSK1019 in strain AGL-1, co-cultivated for 48 (lanes 8–10) and 72 h, and maintained with antibiotic selection pressure or without it (lane 10); lane 12 = conidia transformed with pSK1019 in strain LB4404, co-cultivated for 72 h and maintained with antibiotic selection pressure; lane 13 = conidia transformed with pSK1019 in strain LB4404, co-cultivated for 72 h and maintained without antibiotic selection pressure for six generations; lanes 14–16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1, co-cultivated for 48 and 60 h (lane 16), and maintained with antibiotic selection pressure; lanes 17–18 = negative controls *Phaeoisariopsis griseola* and wild-type *Acremonium implicatum* strain 6780-201v, respectively; lanes 19–20 = positive controls pSK1019 and pCAMBIA1300, respectively.

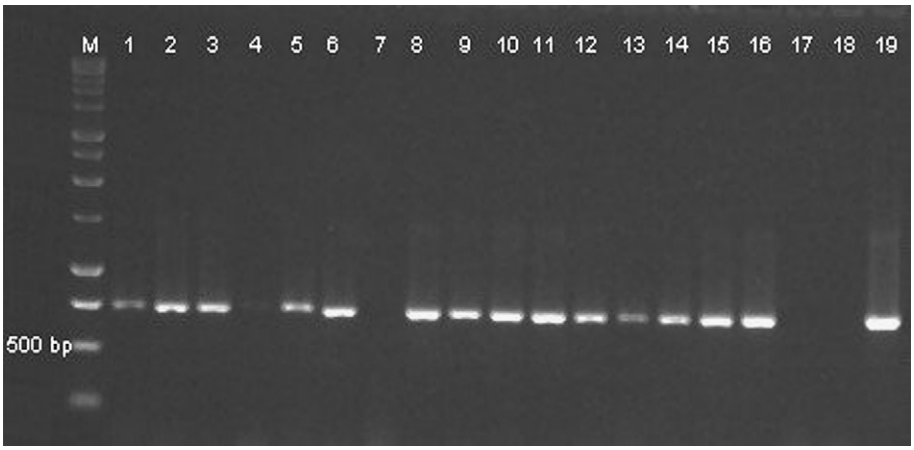

Fig 2 – Primers specific for sequences of the enhanced GFP (*egfp*) gene were used for PCR amplifications of template DNA isolated from *Acremonium implicatum* transformants. Lane M = molecular marker; lane 1 = conidia transformed with pSK1019 in *Agrobacterium tumefaciens* strain LB4404, co-cultivated for 72 h and maintained without antibiotic selection pressure; lanes 2, 3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1, co-cultivated for 48 and 60 h, and maintained without or with antibiotic selection pressure, respectively; lane 4 = conidia transformed with pCAMBIA1300 in strain AGL-1, co-cultivated for 72 h and maintained without antibiotic selection pressure; lane 5 = mycelia transformed with pSK1019 in strain LB4404, co-cultivated for 60 h and maintained without antibiotic selection pressure; lane 6 = mycelia transformed with pSK1019 in strain AGL-1, co-cultivated for 72 h and maintained with antibiotic selection pressure; lane 7 = negative control (water); lanes 8–11 = mycelia transformed with pSK1019 in strain AGL-1, co-cultivated for 48 (lanes 8–10) and 72 h (lane 11), and maintained with antibiotic selection pressure or without it (lane 10); lane 12 = conidia transformed with pSK1019 in strain LB4404, co-cultivated for 72 h and maintained with antibiotic selection pressure; lane 13 = conidia transformed with pSK1019 in strain LB4404, co-cultivated for 72 h and maintained without antibiotic selection pressure for six generations; lanes 14–16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1, co-cultivated for 48 and 60 h (lane 16), and maintained with antibiotic selection pressure; lanes 17–18 = negative controls *Phaeoisariopsis griseola* and *Acremonium implicatum* strain 6780-201v, respectively; lane 19 = positive control pSK1019.

Amplifications were carried out in a programmable thermal controller (MJ Research, Waltham, MA) programmed to 35 cycles, comprising a 45 s denaturation step at 94 °C (4 min for the first cycle), followed by 1 min at 60 °C, and a primer extension for 1.5 min (10 min in the final cycle) at 72 °C. The amplification products were separated by electrophoresis in a 1 % agarose gel (Bio-Rad Laboratories, Hercules, CA), stained with ethidium bromide, and photographed under UV light.

Southern blot analysis

The DNA of 19 randomly selected putative *Acremonium implicatum* transformants was analysed using Southern blot analysis. The hygromycin B resistance gene *hph* was used as a probe. Southern hybridization was carried out, using standard procedures as described in Sambrook *et al.* (1989). Labelling and detection were carried out, using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Basel).

Microscope examination

The putative GFP-expressing transformants were examined under a Leica fluorescence microscope fitted with two filters: one Leica D, with an excitation range between 355 and 425 nm (UV/violet), and one Leica H3, with an excitation range between 420 and 490 nm (violet/blue).

Plant inoculations

Brachiaria seedlings were inoculated with a few selected *Acremonium implicatum* transformants, using the method described earlier by Kelemu *et al.* (2001).

Voucher material

All strains of the fungus *Acremonium implicatum* described in the study are stored at the culture collection maintained by the Plant Pathology Section, Tropical Forages Program, of the International Center for Tropical Agriculture (CIAT), Cali, Colombia.

Results and discussion

The endophytic fungus *Acremonium implicatum* was successfully transformed with the enhanced GFP (*egfp*) gene. Transformation efficiency increased on using *Agrobacterium tumefaciens* strain AGL-1 (Table 1). Although *Acremonium implicatum* transformants containing either pSK1019 (*trpC* promoter) or pCAMBIA1300 (CaMV35S promoter) were obtained, a significantly higher number of transformants were obtained with vector pSK1019 (Table 1). The colony size of transformants obtained for both vectors was, however, similar, with an average

diameter of 19 mm after 12 d of incubation at 28 °C on the selection medium. The results also indicated that 'the transformation efficiency is directly influenced by the length of the co-cultivation period for *Agrobacterium tumefaciens* and *Acremonium implicatum* (Table 1). As the co-cultivation period increased from 48 to 72 h, efficiency increased from 542 transformant colonies to 1084 for the mycelial transformation protocol; and from 271 to 542 for conidial transformation (Table 1). Similar results have been reported for the transformation of *Magnaporthe grisea* (Rho *et al.* 2001) and *Fusarium oxysporum* (Mullins *et al.* 2001). The efficiency of transformation also differed according to whether we used mycelia or conidia for transformation (Table 1). Optimal transformation results were obtained with *Agrobacterium tumefaciens* strain AGL-1 and plasmid pSK1019 under the control of the *trpC* promoter, with either mycelial or conidial transformation. However, mycelial transformation consistently generated significantly higher numbers of transformants than when conidia were used (Table 1).

As discussed above, the putative *Acremonium implicatum* transformants selected on agar media containing hygromycin B were further examined, using a fluorescence microscope, PCR, and Southern blot analysis. The PCR method allowed us to quickly examine and further confirm putative transformants that were selected on antibiotic selection media (Figs 1 and 2). To determine the copy number of the transferred T–DNA, genomic DNA from19 randomly picked transformants from each experimental condition was digested with *Hin*dIII and analysed with Southern blot. The results exhibited genomes with inserts ranging from one to five inserts (data not shown), whereas the negative control, untransformed *A. implicatum*, showed no hybridization. No correlation existed between the average copy number of T-DNA per genome and the co-cultivation period, the mycelial or conidial transformation, or other variables introduced into the experiments.

Microscopic examinations of selected transformants demonstrated strong expression of the *egfp* gene, as evidenced by intense fluorescence emission. All parts of the fungal structure, including conidia, mycelia, and germinating conidia showed emission. These results demonstrate that the fungal promoter with glyceraldehyde-3-phosphate dehydrogenase (GPD), isolated from *Cochliobolus heterostrophus*, functions well for the expression of genes in the endophytic fungus *A. implicatum* (Figs 3 and 4).

The mitotic stability of the transferred DNA was examined by growing ten transformants in liquid and agar media for six

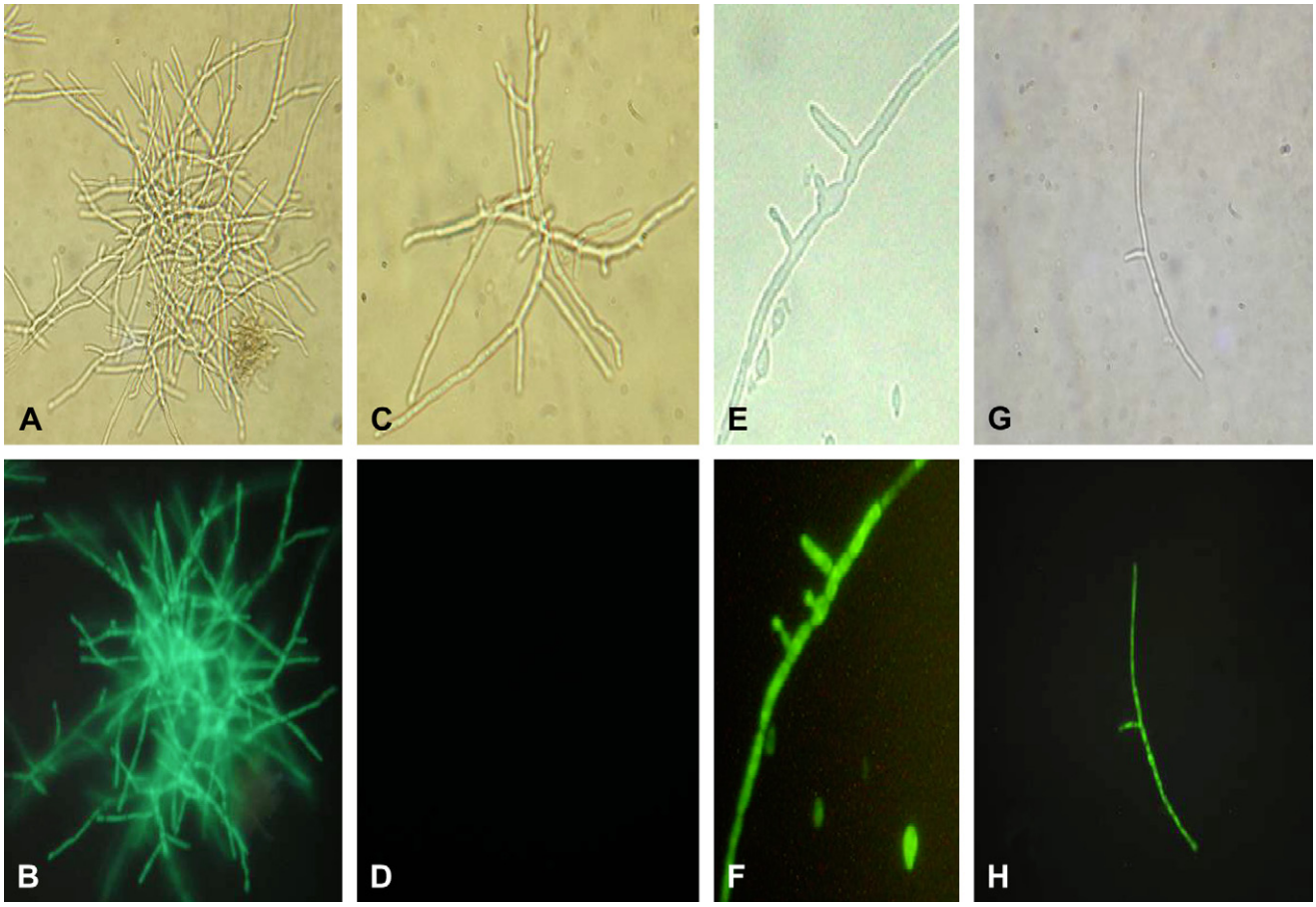

Fig 3 – Structures of *Acremonium implicatum* strain 6780-201v transformed with the enhanced GFP (*egfp*) gene and observed microscopically under UV light. (A, C, E, G) Under normal light; (B) fluorescence emission from transformed mycelia, using a Leica D filter (355 and 425 nm); (C–D) control, untransformed *A. implicatum* strain 6780–;201v without and with UV light, respectively; (F and H) transformed structures emitting green fluorescence under UV light, using a Leica H3 filter (420 and 490 nm).

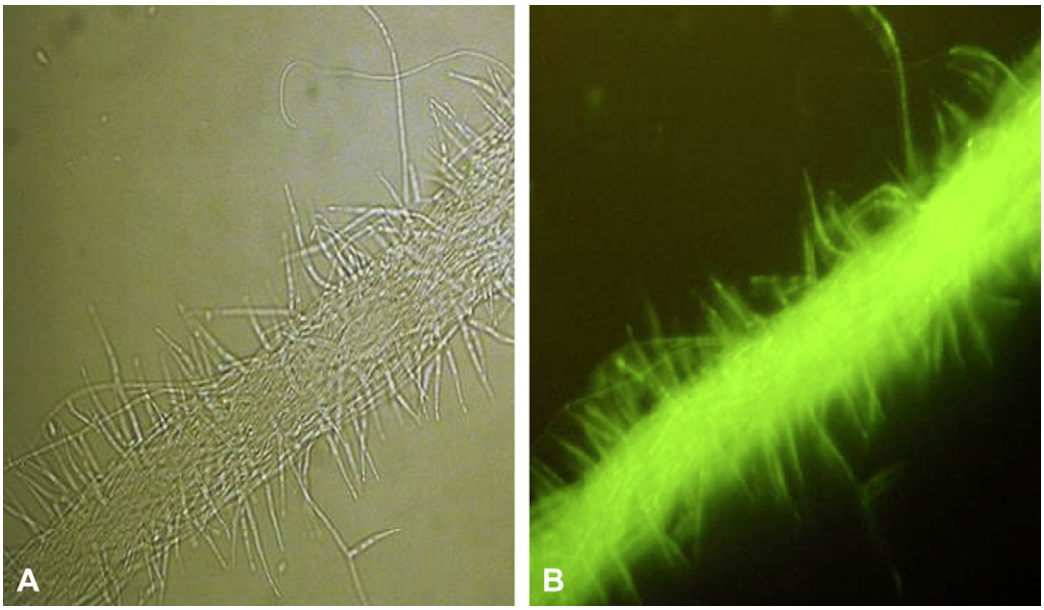

Fig 4 – Mycelium of *Acremonium implicatum* transformed with the enhanced GFP (*egfp*) encoding gene: (A) mycelium observed microscopically (×40) under normal light; and (B) the same mycelium demonstrates fluorescence emission when observed under UV light.

generations without selection pressure. Then, on testing, all cases demonstrated maintenance of resistance to hygromycin B, indicating that the transferred DNA was stable. They also all retained emission of fluorescence. The PCR method further confirms that transformants that were maintained without selection pressure still produced the diagnostic amplification product (Figs 1 and 2). Meiotic stability could not be determined because the fungus cannot be crossed.

Data also showed that tissues taken from *Brachiaria* plants inoculated with GFP-transformed *A. implicatum* expressed fluorescence emission (Fig 5). Fig 4 shows the GFP-expressing transgenic *A. implicatum* that was used to inoculate the *Brachiaria* plants. The implications of these findings are that we will be able to study the endophyte–*Brachiaria* interaction, endophyte distribution within plant tissues, and stability in seeds. We will then be able to examine the potential use of

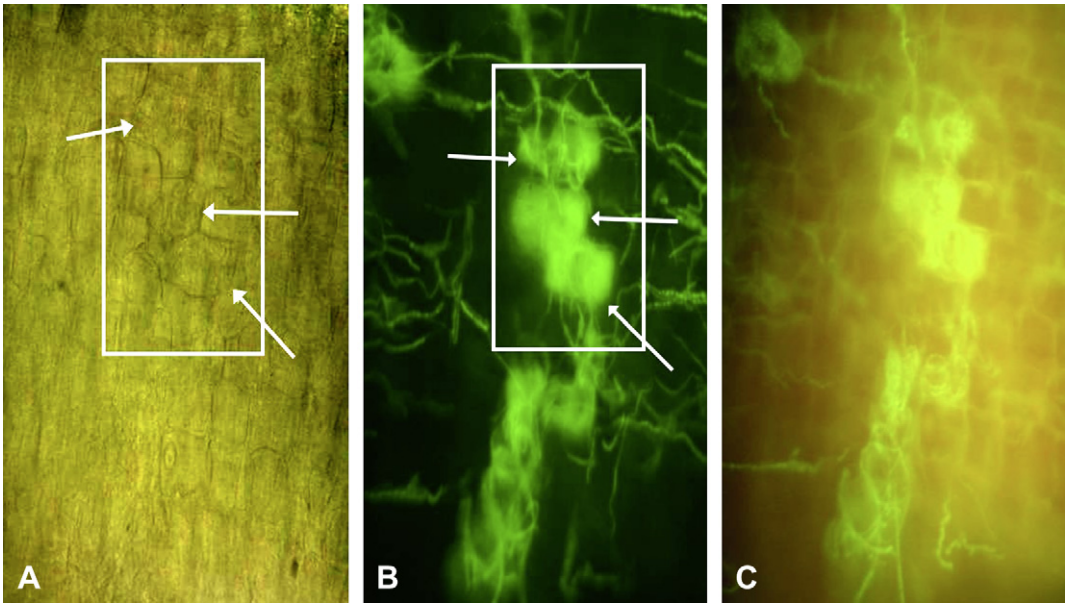

Fig 5 – *Brachiaria* tissues taken from plants inoculated with *Acremonium implicatum* strain 6780-201v transformed with the enhanced GFP (*egfp*) gene (see transformed strain in Fig 3). (A) Under normal light; (B) fluorescence emission under UV light, using a Leica D filter; and (C) fluorescence emission under UV light, using a Leica H3 filter.

this endophyte as a gene delivery and expression system in plants.

Although various transformation systems have been developed and reported for many fungi, including endophytes (Mikkelsen *et al.* 2001; Murray *et al.* 1992), the successful application of this technology is still not routine for many species. Our study describes the successful transformation and expression of the GFP-encoding gene in an isolate of *A. implicatum*, an endophyte in *Brachiaria* species. We also demonstrated that both the mycelia and conidia of *A. implicatum* can be efficiently transformed, using *Agrobacterium tumefaciens*. *A. tumefaciens*-mediated transformation has long been applied to transfer foreign genes to a wide range of plants. Recently, it has also been used to transform a wide range of fungi, enabling efficient genetic manipulations of the recipient organisms. The presence of acetosyringone is important for successful *A. tumefaciens*-mediated transformation. We believe that this is the first report on the *Agrobacterium*-mediated transformation of this endophytic fungus.

The stable integration and expression of the introduced gene into the genome of the recipient fungus indicate that the endophyte may become an excellent tool for delivering and expressing genes of agronomic importance (e.g. disease and insect resistance) to host plants. For this to be successful, the practical implication of high seed transmission of *Acremonium implicatum* in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed, especially in the apomictic genotypes of *Brachiaria*, provided that seed storage conditions do not reduce the endophyte's survival (Dongyi & Kelemu 2004). That such a strategy may be feasible was indicated when several *Brachiaria* hybrids obtained from CIAT's tropical forage breeding programme were shown to harbour *A. implicatum*. We also want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its *Brachiaria* hosts.

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