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An explicit AFLP-based marker for monitoring *Fusarium oxysporum* f.sp. *strigae* in tropical soils

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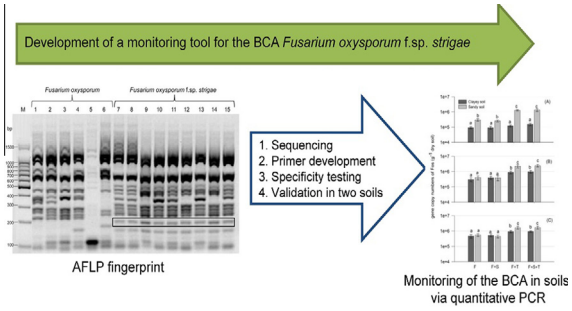
HIGHLIGHTS

- An AFLP marker was developed to quantify the BCA Fos via quantitative PCR in soils.
- Validity of AFLP marker was approved in two tropical soils inoculated with Fos.
- The AFLP marker enables prospective Fos quantification under field conditions.
- The AFLP marker allows quality control during Fos inoculum production.

GRAPHICAL ABSTRACT



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ABSTRACT

Our objective was to develop an explicit AFLP-marker to quantify the abundance of *Fusarium oxysporum* f.sp. *strigae* (Fos), an effective soil-borne biocontrol agent (BCA) against the parasitic weed *Striga hermonthica*, in tropical soils. The specificity of the AFLP-marker to Fos was confirmed on basis of comparison of *Fusarium* isolates of differing relatedness to Fos. These consisted of 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum* isolates retrieved from tropical and temperate ecosystems. The robustness of the AFLP-marker for monitoring Fos was validated in a controlled incubation experiment. In this experiment, we inoculated the known Fos-BCA "Foxy-2" as model organism via seed coating ($1.15 * 10^5$ colony forming units per seed) of a tropical maize (*Zea mays* L.) variety to a tropical clayey (Humic Nitisol) and a sandy (Ferric Alisol) soil, both with no indigenous Fos infestation. The proliferation of "Foxy-2" was followed at 14, 28 and 42 days after start of experiment by Fos-specific quantitative PCR using the AFLP-marker. Moreover, the experimental set-up considered two additional factors: (1) presence of *S. hermonthica*, and (2) application of *Tithonia diversifolia* residues as nitrogen-rich resource for supporting "Foxy-2" proliferation. The explicit AFLP-marker was appropriate to reveal that soil type and organic resource availability exhibited distinct effects on abundance of inoculated "Foxy-2". Negative PCR signals, which confirmed the Fos-specificity of the developed AFLP-marker, were retrieved from control soils not inoculated with "Foxy-2". Hence, we verified the applicability of the explicit AFLP-marker for Fos in two soils, but recommend additional broad scale field studies to approve its suitability as Fos monitoring tool for differently managed soils in contrasting tropical agro-ecological zones.

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1. Introduction

The parasitic weed *Striga hermonthica* (Del.) Benth. is a major constraint to cereal production in Sub-Saharan Africa affecting the livelihood of about 100 million people (Ejeta, 2007). *S. hermonthica* lives parasitically on cereal crops such as millet (*Pennisetum americanum* L. Leeke), sorghum (*Sorghum bicolor* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.) (Elzein and Kroschel, 2004; Marley et al., 2004). It causes annual crop damage ranging from 30% to 90% deemed equivalent to approximately 9 billion US dollars financial loss (Parker and Riches, 1993; van Mourik, 2007; Watson et al., 2007). Control of *S. hermonthica* remains challenging due to its extremely high seed production per plant with seed survival rates in soils of more than 10 years (Parker and Riches, 1993; van Mourik, 2007).

Current *S. hermonthica* control strategies include hand pulling, catch crops (e.g., Sudan grass (*Sorghum sudanense* L.)) and trap crops (e.g., soybean (*Glycine max* L.), lucerne (*Medicago sativa* L.), cotton (*Gossypium* spp.)), as well as the use of tolerant crop varieties. But these approaches were shown to be ineffective when applied individually. Hence, integrated approaches are postulated as control strategies against *S. hermonthica* (Atera et al., 2012; Hearne, 2009; Menkir and Kling, 2007).

Combination of biological control agents (BCAs) such as *Fusarium oxysporum* f.sp. *strigae* (Fos) strains along with tolerant crop varieties has shown respectable control success in field experiments conducted in Burkina Faso, Benin and Nigeria (Schaub et al., 2006; Venne et al., 2009). In this respect, the Fos strain "Foxy-2" was approved to be superior in suppressing all development stages of *S. hermonthica* ranging from germination to flowering (Elzein and Kroschel, 2004; Ndambi et al., 2011). In addition, Ndambi et al. (2011) reported that "Foxy-2" colonized saprophytically the roots of cereals, where the biocontrol activity of "Foxy-2" was initialized after *S. hermonthica* attacked the root system. Hence, integration of Fos with tolerant crop varieties is, in comparison to the use of agrochemicals, an environmentally friendly and durable strategy to combat *S. hermonthica*.

Schaub et al. (2006) and Venne et al. (2009) showed, however, that the success of "Foxy-2" in controlling *S. hermonthica* under field conditions is variable, presumably as a consequence of differing environmental conditions across agro-ecological zones with different soil types, as well as rainfall and temperature patterns. Thus, if a prospective broad scale application of BCA Fos in agricultural fields infested by *S. hermonthica* is anticipated, its proliferation and survival potential in differently managed soil ecosystems requires a thorough evaluation. However, a specific monitoring tool is still lacking allowing the robust quantification of Fos abundance in target environments and also quality assessment during inoculum production.

Conventional techniques for monitoring fungi in soils include the estimation of colony forming units (cfu) on selective media and immunological assays (e.g., ELISA) (Lievens and Thomma, 2005). These methods are generally time-consuming, require taxonomical expertise, and often lack in specificity for the organism of interest (Ward et al., 2004). Molecular, DNA-based techniques compensate many of known limitations of such conventional analyses. Recently, application of quantitative polymerase chain reaction (qPCR) emerged as a suitable method for specific monitoring of microorganisms in soils (Edel-Hermann et al., 2011; Jiménez-Fernández et al., 2010; Providenti et al., 2009). A number of qPCR assays were developed previously for *Fusarium* spp. (Bluhm et al., 2004; Pasquali et al., 2006; Waalwijk et al., 2004). Edel-Hermann et al. (2011) developed a strain-specific qPCR assay based on a sequence-characterized amplified region marker (SCAR) for quantification of BCA "Fo47", a soil-borne *F. oxysporum* strain.

Amplified fragment length polymorphisms (AFLP; Vos et al., 1995) was developed as one of the most recognized DNA fingerprinting techniques to analyze fungal populations including *Fusarium* spp. in soils (e.g., Abd-Elsalam et al., 2004; Kiprop et al., 2002; Leslie et al., 2005; Silva et al., 2013). The reproducibility and resolution of AFLPs are superior to those of other markers (e.g., random amplified polymorphic DNAs, restriction fragment length polymorphisms, microsatellites) (Mueller and Wolfenbarger, 1999). AFLP-markers for Fos as necessary prerequisite for monitoring its proliferation in soils are yet to be developed.

Our objective was thus to develop an explicit AFLP-marker for monitoring the abundance of Fos in soils via qPCR. The developed AFLP derived marker was tested for its specificity against 40 Fos, 17 *Fusarium* spp. and 68 *F. oxysporum* isolates and validated in a controlled incubation experiment. This experiment included two contrasting tropical soils (sandy Ferric Alisol versus clayey Humic Nitisol), with and without *S. hermonthica* infestation, to which "Foxy-2" was inoculated and its proliferation monitored during 42 days. Soils were not sterilized to validate the specificity of the monitoring tool against the indigenous soil fungal population and to monitor the "Foxy-2" proliferation under natural conditions. Additionally, an organic fertilization treatment with *Tithonia diversifolia* residues, a widely used green manure in Sub-Saharan Africa (Gachengo et al., 1998; Jama et al., 2000), was included to investigate its stimulating effect on the saprophytic survival of "Foxy-2" in studied soils.

2. Material and methods

2.1. AFLP marker development

2.1.1. Fungal strains, growth conditions and DNA extraction

The model Fos isolate "Foxy-2" was obtained from *S. hermonthica* collected from North Ghana (Abbasher et al., 1995). Taxonomic identification of the isolate was confirmed by Julius-Kühn-Institut (JKI, Berlin, Germany), where it is deposited under accession number "BBA-67547-Ghana". Details on the origin of *Fusarium* spp. and *F. oxysporum* isolates involved in this study are given in Table 1. Fos isolates CAV 6021–CAV 6236 (Table 1) were recently collected from diseased *S. hermonthica* plants in Kenya and Nigeria, and pathogenicity tests were conducted to confirm their pathogenicity to *S. hermonthica* plants (H. Wainwright and A. Elzein, unpublished data). Fos isolates were further characterized by vegetative compatibility group (VCG) testing and phylogenetic analysis of Tef-1α and MtSSU DNA sequences (M. de Klerk, unpublished data). Strains were propagated in potato dextrose broth at 28 °C for 3 days, followed by DNA extraction (UltraClean Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA). Concentration and quality of isolated DNA were determined on a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA).

2.1.2. Amplified fragment length polymorphism (AFLP) procedure

A selection of 9 Fos and 6 *F. oxysporum* isolates out of 125 isolates (Table 1) were included in AFLP fingerprinting to reduce labor and costs. All primers (Biomers GmbH, Ulm, Germany) used are listed in Table 2.

AFLP fragments were generated as described by Vos et al. (1995) with minor modifications. Digestion was performed at 37 °C for 3 h using 300 ng genomic DNA extracted from each fungal isolate, 1× NEBuffer 4 (New England Biolabs Inc. (NEB), Ipswich, MA, USA), 1 U *Mse*I (NEB), 1 U *Eco*RI (NEB), 0.1 mg ml^{-1} bovine serum albumin (NEB), and then adding water to a total volume of 50 µl. Preparation of the *Eco*RI adapter was done with 100 pmol *Eco*RI-ad forward, 100 pmol *Eco*RI-ad reverse and 1× Buffer C

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