Fusarium oxysporum Strains as Potential *Striga* Mycoherbicides: Molecular Characterization and Evidence for a new *forma specialis*

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Abstract: *Fusarium oxysporum* isolates (Foxy 2 and PSM197) are potential, highly host specific mycoherbicides for the control of the parasitic weeds *Striga hermonthica* and *S. asiatica*. Their target weeds, *Striga* spp., are major biotic constraints in cereal and legume production in semi-arid tropical Africa, where they adversely affect livelihood of millions of subsistence farmers. The aim of this study was to characterize and sequence the *Striga* mycoherbicides Foxy 2 & PSM197 in order to more clearly distinguish them from other morphologically similar pathogenic *Fusarium oxysporum* strains. The fungal isolates were cultivated on PDA medium and characterized based on the analysis of partial DNA sequence of the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene. Both isolates were identical in their ITS-sequence. The unique and identical ITS-sequence of the two isolates obtained, compared to the sequences of *Fusarium oxysporum forma speciales* deposited in GenBank along with the host specificity to *Striga* demonstrated in previous studies, provides strong evidence to propose these pathogens of *Striga* as a new *forma specialis* (f. sp. *strigae*). The possibility to clearly distinguish between the new *forma specialis* and all pathogenic *Fusarium oxysporum* strains sequenced so far will facilitate and encourage the acceptance and introduction of *Striga*-mycoherbicides for practical field application by regulatory authorities and farmers.

Keywords: Weed biocontrol, mycoherbicides, host specificity, bio-safety, ITS sequence, *Striga hermonthica*.

INTRODUCTION

Globally, root parasitic weeds of the genus *Striga*, particularly *S. hermonthica* (Del.) Benth., have a greater impact on human welfare than any other parasitic angiosperm, because their hosts are cereal crops of subsistence farmers in areas marginal for agriculture in the Sahelian and the Savannah zones of Africa. In infested areas, yield losses associated with *S. hermonthica* infestation on sorghum (*Sorghum bicolour* (L.) Moench) and maize (*Zea mays* L.) are often significant, ranging from 40 to 100% [1-3], and aggravate hunger and poverty. Control of *Striga* is particularly difficult due to its special biology and intimate physiological interaction with its hosts. In addition, significant damage is done to the host before parasite shoots emerge from soil. So far, no economically feasible single method can solve the problem, and therefore an integrated approach, in which biocontrol could be a crucial component, appears to be the most promising strategy for reducing *Striga* infestations.

Mycoherbicides are particularly attractive, since they can be weed-specific, have low environmental impact and are often cost-effective [4]. Biological control of *S. hermonthica* by soil application of a mycoherbicide containing *Fusarium oxysporum* Schlecht., has been reported to have several advantages. It attacks the target weed before emergence [5-8], just before most of the damage to the host occurs. This reduces the *Striga* seed bank in the soil, prevents production of new seeds and increases the grain yield of the crop in the same cropping season. Additionally, it is assumed to be cost-effective, requiring no changes in crop rotation and, if applied as a seed treatment, no additional labour is needed [9]. Two fungal stains, Foxy 2 and PSM197 of *F. oxysporum*, isolated from diseased *S. hermonthica* plants from Ghana and Nigeria, respectively, are specific towards their hosts, highly aggressive against all developmental stages of *S. hermonthica* including seeds and can be mass-produced using agricultural by-products [7, 8, 10, 11]. Thus, these fungal isolates are well suited to be developed into a specific mycoherbicide, to support and enhance the existing *Striga* control measures. Both isolates exhibited potential efficacy in controlling *S. hermonthica* and improving crop performance under controlled and field conditions of West

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Africa when developed into Pesta granular formulations or delivered as seed treatment on crops [9, 12-14]. Further, both mycoherbicides maintained excellent viability on Pesta products and treated seeds after one year of storage, sufficient for their use under practical conditions of storage, handling and delivery [9, 15].

The acceptance and implementation of inundate biological control by regulatory authorities are based on safety issues which include avoidance of any non-target adverse effects associated with the use of biological control agents whether the agent be indigenous or non-indigenous, naturally occurring or genetically modified. It is very important that host specificity testing and risk assessment methodologies should both lead to prevention of the release of any organism that is likely to have detrimental impacts on non-target plants or on environment. Several approaches have been used to provide the required information for proper risk assessment including: quantifying the relative susceptibility of the target and non-target plant species [16]; microscopic and histological examination of infection events [17]; measuring relative plant damage [18]; morphological and molecular comparisons between foreign and indigenous organisms [19]; and epidemiology [20]. In two studies [10, 21] it was shown that the *Striga* pathogenic strains Foxy 2 and PSM197 are non-pathogenic to all sorghum varieties tested and also to all other crops tested. Among these were 25 (for Foxy 2) and 17 (for PSM197) non-target plant species including cereals, legumes, fruits, vegetables, oilseeds and fibrous crops. To further investigate the possibility that the two strains might be a new *forma specialis*, a molecular phylogenetic approach was used to characterize the two potential mycoherbicide strains.

Molecular markers have proven to be powerful tools for the characterization and identification of several plant pathogenic fungi. With the advent of polymerase chain reaction (PCR), inexpensive DNA sequencing, and a relatively large databank of ribosomal DNA sequences, it is now possible to more objectively characterize and identify fungal species on the basis of sequence stretches commonly used for calculating molecular phylogenies or for identifying pathogens. Among these sequences are different regions of the nuclear ribosomal DNA (nrDNA) cistron, in particular the internal transcribed spacers [22], of which numerous sequences from *F. oxysporum* isolates are deposited in GenBank. The objective of this study was to characterize and sequence the potential *Striga* mycoherbicides Foxy 2 and PSM197 in order to test, if they can be clearly distinguished from other morphologically similar pathogenic *F. oxysporum* strains.

MATERIALS AND METHODS

Origin of Fungal Isolates

The isolates Foxy 2 and PSM197 used for this study were obtained from severely diseased *S. hermonthica* collected in North Ghana [5] and in Samaru, Nigeria [8], respectively. Taxonomic identification of the isolates was confirmed by the Federal Biological Research Centre for Agriculture and Forestry, Berlin, Germany, for Foxy 2, where the isolate was deposited under accession number BBA-67547-Ghana, and the International Mycological Institute (IMI), Egham, UK, for PSM197 which is deposited at Medical Research Council, Tygerberg, South Africa under accession number MRC 8537. Since then the isolates were preserved on Special Nutrient poor Agar (SNA) medium [23] with 5% (v/v) glycerol at -40^{o}C in the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Stuttgart, Germany. All investigations were performed with a single-spore isolate of either Foxy 2 or PSM197.

Fungal Cultures

Mycelial and conidial cultures of Foxy 2 and PSM197 were prepared on Potato Dextrose Agar (PDA) medium. Four PDA Petri-dishes (i.e. 4 replicates) were aseptically inoculated each with one agar disc (Ø 0.6 cm) of active growing fungal colony of Foxy 2 or PSM197. Additionally, four Petri-dishes (i.e. 4 replicates) were aseptically inoculated each with one sorghum seed coated with dried chlamydospores of Foxy 2 or PSM197 using Arabic Gum (40%) as an adhesive [9] and placed in the centre of the Petri-dish. Thereafter, the inoculated Petri dishes were incubated in the dark at 25 ^{o}C for 7 days.

DNA-Extraction and PCR

For DNA extraction, 5 mg of hyphae of each of the samples were disrupted in a mixer mill (Reetsch, Germany) using two magnetic balls of 3 mm in diameter. DNA-extraction was done using the QIAquick Plant DNA extraction kit (Qiagen, Germany), according to the manufacturer's instructions. PCR was done on an Eppendorf Mastercycler (Eppendorf, Germany) using the universal primers ITS1 and ITS4 [24], with the conditions described there. The amplicons obtained were separated on 1 % agarose gels, stained with ethidium bromide and cut from the gel using sterile scalpels. The PCR products were cleaned using the QIAquick Gel-Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Sequencing was done by a commercial sequencing company (GATC, Germany) with the primers used for PCR amplification.

Data Analysis

Because a high sequence similarity was observed, alignments were done with clustalX, version 1.8 using the factory settings. From the alignment obtained, all gaps present in more than half the samples were removed. For molecular phylogenetic reconstruction, Mega 3.1 [25] was used. Gaps were treated as pairwise deletion. Minimum Evolution analysis was performed using the Tamura-Nei substitution model [26] and a starting tree obtained by Neighbor-joining [27], keeping only one of the best trees obtained. Maximum Parsimony analysis was done using the applicable parameters mentioned above. In both cases, all parameters not mentioned equalled the factory settings of the program. In both cases, 1000 bootstrap replicates [28] were conducted.

RESULTS

PCR and Sequencing

PCR resulted in bright, single fragments of about 680 bp in length. Partial ITS sequence obtained from these fragments was 596 bp. The ITS sequences obtained were deposited in GenBank under accession numbers EU264073 and EU264074, for Foxy 2 and PSM197, respectively. A blast search [29] revealed a unique ITS-sequence of the two isolates compared to any other *F. oxysporum* sequence of com-

parable length. Sequence similarity among *F. oxysporum* isolates was generally high (above 99 %).

Molecular Phylogenetic Reconstructions

The single best tree obtained by Minimum Evolution (ME) analysis is presented in Fig. (**1**). The topology of the consensus tree of the 435 most parsimonious trees with a tree length of 40 is in concordance with the major groups shown in the ME tree. The first and the second number above the branches indicate bootstrap support in Maximum Parsimony (MP) and ME analysis, respectively. Bootstrap values below 33 are not shown.

Sister-group relationship of *F. oxysporum* and *F. subglutinans* is well supported in both analyses performed. Within the *Fusarium oxysporum*, resolution was generally low and the support for the different lineages weak. However, the clade containing the new *forma specialis* and *F. oxysporum* f. sp. *cubense* as well as f. sp. *radicis-lycopersici* was found in 89 % of the most parsimonious trees and was supported by a support value of 74 in interior branch tests for ME (data not shown). The other clades present in the majority consensus tree of the MP analysis were also present in the ME tree, although with lower frequency. Only the partition that received a bootstrap support of 53 in the ME analysis was not resolved in the MP consensus tree.

DISCUSSION

The mycoherbicides *F. oxysporum* Foxy 2 and PSM197 are highly pathogenic and host specific to *S. hermonthica* and non-pathogenic to a wide range of crops tested [10, 21]. In addition, these strains do not produce any toxic compounds that present health risks [30]. Hence, these isolates are of great interest as promising potential mycoherbicide candidates for the control of *Striga* species. However, the safety of non-target cultivated and wild plants must be ensured prior to release of the agents in the field, irrespective of potential benefits of the biological control agents. Host specificity is an important part of risk assessments for plant pathogens in weed biocontrol, since its assessment is the best way of predicting both direct and indirect effects on non-targets [31]. Our recent results showed that the host range of Foxy 2 and PSM197 is restricted to the genus *Striga*, and none of the tested non-target plant species showed any symptoms of infection [10, 21]. The tested species comprised some selected poaceous crops related to sorghum, crop species reported to be highly susceptible to *Fusarium* diseases in tropical and subtropical regions, as well as other economically important crops cultivated in the regions of *Striga* infestation. The category of the highly susceptible species to *F. oxysporum* diseases tested included: banana (*Musa textilis* Née), chickpea (*Cicer arietinum* L.), cotton (*Gossypium barbadense* Mill.), cucumber (*Cucumis sativus* L.), egg plant (*Solanum melongena* L.), faba bean (*Vicia faba* L.), okra (*Abelmoschus esculentus* (L.) Moench), pea (*Pisum sativum* L.), soybean (*Glycine max* (L.) Merr.), roselle (*Hibiscus sabdariffa* L.) and tomato (*Lycopersicon lycopersicum* (L.) Karsten ex Farw.). In other host-range studies, the indigenous *F. oxysporum* isolates from Burkina Faso, Mali and Nigeria were also found to infect only *Striga* spp. and none of the crops and vegetables tested [6, 32]. Those results confirm the restriction of pathogenicity of *F. oxysporum* isolated from *Striga* spp. to the target species.

The use of nrDNA sequences often allows unequivocal determination of fungal species [33]. In this study it was shown that it was possible to distinguish Foxy 2 and PSM197 from other morphologically similar, pathogenic *F. oxysporum* strains by ITS-sequencing, which is applicable for their routine identification. The ITS-sequence obtained from the two strains was not identical to any ITS-sequence deposited in GenBank, a fact that, in combination with their host specificity (so far infectiveness could only be demonstrated to species of *Striga*), clearly indicates that the two strains belong to a new *forma specialis*. The high similarity between the two isolates can be linked to their high specificity towards *Striga* and maybe their geographic origin [34].

Although the resolution of the phylogenetic reconstruction within *F. oxysporum* was generally low, it should be noted that the clade consisting of the two strains pathogenic to *Striga* and *F. oxysporum* f. sp. *radicis-lycopersici* as well as *F. oxysporum* f. sp. *cubense* was the only clade consistently supported, although with weak support, in both Minimum Evolution and Maximum Parsimony analyses. It is noteworthy that the highly susceptible target hosts of the most closely related *formae specialis*, *F. oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker (tomato) and *F. oxysporum* f. sp. c*ubense* (E. F. Sm.) W. C. Snyder & H. N. Hansen (banana) showed no symptoms after inoculation with Foxy 2 and PSM197, even at high levels of pathogen pressure [10, 21]. Both hosts showed immunity to the new forma specialis and none developed any symptoms of disease infestation (e.g. wilting, dieback, necrosis and chlorosis normally caused by *F. oxysporum*). In addition, no direct or indirect negative effects on their vegetative growth parameters, including number of leaves, plant height, photosynthetic rate, and root and shoot biomass, were recorded after inoculation with Foxy 2 and PSM197. On the contrary, some positive effects on vegetative growth of tomato as a result of inoculation were observed. This demonstrated convincingly that tomato and banana are not hosts of the new *forma specialis* of *F. oxysporum* [10, 21].

Gerlach and Nirenberg [35] have reported that *Fusarium* spp. are mostly specific at the host family or genus level, and such pathogens are taxonomically classified as *formae speciales*. Thus, the high specificity of the two isolates Foxy 2 and PSM197 to the genus *Striga* and their unique ITS-sequence, which allows their molecular characterization, provides convincing evidence to propose these pathogens of *Striga* as a new *forma specialis*. This new *forma specialis* is named ***Fusarium oxysporum*** f. sp. ***strigae*** Elzein et Thines, ***f. sp. nova***. The strain of Foxy 2, deposited at the Federal Biological Research Centre for Agriculture and Forestry, Berlin, Germany, under accession number BBA-67547-Ghana, is designated here as the type culture of *Fusarium oxysporum* f. sp. *strigae*.

The possibility to characterize *F. oxysporum* f. sp. *strigae* by its host range and, perhaps even more important, by its unique ITS-sequence, will greatly improve the acceptance of its use as a mycoherbicide by farmers and officials, because it allows its unequivocal identification and differentiation compared with other *F. oxysporum* isolates so far sequenced.

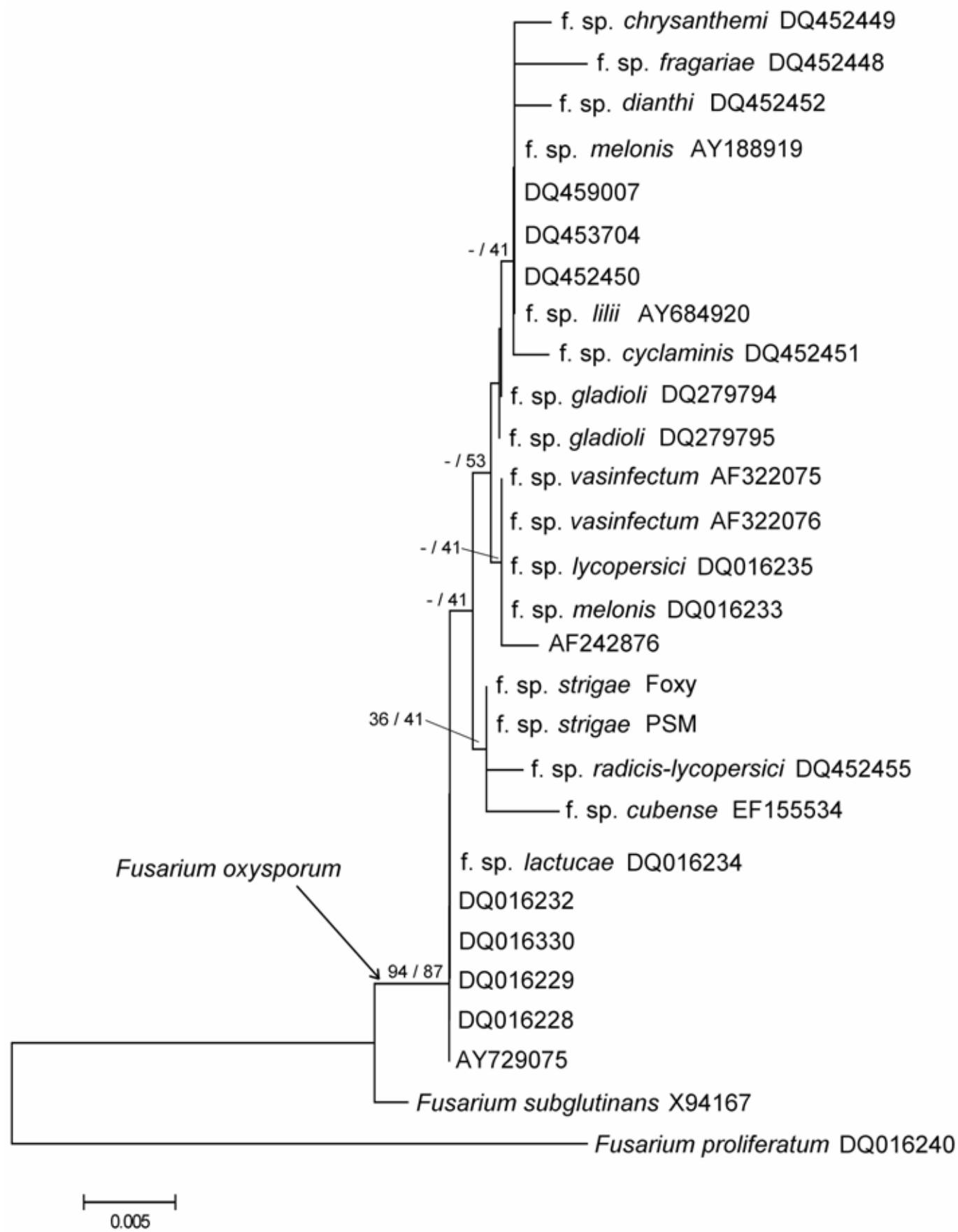

Fig. (1). Single best tree obtained by Minimum Evolution (ME) analyses of the nrITS of several *Fusarium oxysporum* isolates. The first and the second number above the branches indicate bootstrap support in Maximum Parsimony and ME analysis, respectively. Bootstrap values below 33 not shown.

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