Research

Role and In Vivo Localization of *Fusarium oxysporum* f. sp. *strigae* and *Bacillus subtilis* in an Integrated *Striga hermonthica* Biocontrol System

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Abstract

To improve the efficiency of *Striga hermonthica* biological control in the context of an integrated biocontrol system, the role/impact of coinoculating the mycoherbicide *Fusarium oxysporum* f. sp. *strigae* (*Fos*) with a plant growth promoting rhizobacterium, *Bacillus subtilis* isolate GB03, on *S. hermonthica* attachment and sorghum biomass was investigated. *Fos* isolates (Foxy-2, FK3) and GB03, including *Trichoderma viride* (IMB12098 strain) as reference, were applied as single and combined treatments to *S. hermonthica*-infested rhizoboxes with sorghum as the host crop. In vivo localization/interaction of *Fos* and GB03 in *S. hermonthica* was monitored by fluorescent gene expression of transformed *Fos* and transformed GB03. Combined treatments of FK3 + GB03 and Foxy-2 + GB03 increased sorghum aboveground dry biomass ($P < 0.05$), but not IMB12098 + GB03. None of the combined treatments suppressed *S. hermonthica* attachment. Single treatments of FK3 and GB03 increased sorghum aboveground dry biomass ($P < 0.05$), but Foxy-2 and IMB12098 did not. Only FK3, of all single treatments, suppressed *S. hermonthica* attachment ($P < 0.05$). GB03 promoted sorghum yield when applied alone or combined with *Fos* ($P < 0.05$). *Fos* penetration of *S. hermonthica* through trichome entry was revealed. Although *Fos*, either as sporal (conidia) or vegetative form (mycelium), co-occupied common ecological niches with GB03 (they colocalize) in diseased *S. hermonthica* shoots, mainly in flavonoid-rich regions. Nevertheless, GB03 thwarted *Fos* suppressive activity against *S. hermonthica* attachment. In the given setup, coinoculation of *Fos* with GB03 presented no added advantage in suppressing the incidence of the sampled *S. hermonthica*.

Keywords: *Striga hermonthica*, *Fusarium oxysporum* f. sp. *strigae*, Foxy-2, FK3, *Bacillus subtilis* GB03, ecological niche, plant/microbe-microbe interactions

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The noxious, hemiparasitic witchweed *Striga hermonthica* (Delile) Benth. constitutes one of the most serious threats to cereal production, especially in tropical agroecosystems (Atera et al. 2011; Dawud 2017; Mounde et al. 2020; Teka 2014). Generally, *S. hermonthica* incidence is managed through four main approaches, which include traditional/cultural practices such as hand pulling, crop rotation, intercropping, trap/catch crop planting, and soil fertility improvement (nitrogen fertilization). The other *S. hermonthica* management strategies rely on chemical (weedicide, e.g., imazapyr), genetic (*S. hermonthica*-resistant crop varieties), and biological (biological control agents [BCAs], e.g., fungi, bacteria, and certain insects) approaches. Each of these approaches alone is not completely efficient in combating *S. hermonthica*; thus, an integrated *S. hermonthica* management strategy is often recommended (Baiyegunhi et al. 2018; Berner et al. 1996; Mounde et al. 2020; Sibhatu 2016).

The mycoherbicidal effectiveness of the most renowned fungal BCA against *S. hermonthica*, which is *Fusarium oxysporum* f. sp. *strigae* (*Fos*), under both controlled and field conditions, has been well documented (Ciotola et al. 1995; Nzioki et al. 2016; Schaub et al. 2006). *Fos* has proven to inhibit *S. hermonthica* germination when applied pre-*Striga* seed conditioning period (Kroschel et al. 1996; Yonli et al. 2010) and/or to suppress *S. hermonthica* when applied at post-*Striga* attachment period (Abbasher et al. 1998; Marley et al. 1999). *Fos* attacks *S. hermonthica* plants by its hyphal colonization of xylem vessels at inception, followed by total vessel obstruction in mature (emerged) plants or complete tissue digestion of younger plantlets belowground, thereby killing *S. hermonthica* from wilting and tissue degradation (Ndambi et al. 2011). The *Fos* isolate Foxy-2 from Ghana has been widely reported for successful *Striga* spp. biocontrol management in West Africa (Abbasher et al. 1998; Elzein and Kroschel 2004). Similarly, FK3 is a Kenyan *Fos* isolate with proven antagonism against *S. hermonthica* in the East African region (Beed et al. 2013; Kangethe et al. 2016). A major drawback of *Fos* is the inconsistent effectiveness of its isolates, especially (but not limited to) M12-4, Foxy-2, and FK3, against diverse *S. hermonthica* populations of various origins (Avedi et al. 2014; Mounde et al. 2020; Venne et al. 2009). With respect to *S. hermonthica*, it has recently been shown that genomic variation in *S. hermonthica* is a superior determinant of the inconsistent effectiveness of *Fos* isolates (Foxy-2, FK3) against *S. hermonthica*, rather than the geographic origin of *S. hermonthica* populations (Anteyi and Rasche 2020). The inconsistency of *Fos* isolates in effectively controlling genetically diverse *S. hermonthica* populations lowers *Fos* reliability as an efficient mycoherbicide against *S. hermonthica* in various agro-ecological zones. Consequently, it hinders widespread acceptability of *Fos* as a BCA against *S. hermonthica* (Massart et al. 2015; Velivelli et al. 2014).

Comparably, plant growth promoting rhizobacteria (PGPR), particularly of the genera *Pseudomonas* sp., *Azospirillum* sp., and *Bacillus* sp., have been reported to ameliorate the menace of *S. hermonthica*, by reducing the seed germination, halting haustorial development/attachment, and delaying aboveground emergence (Ahonsi et al. 2002; Hassan et al. 2009; Mounde et al. 2015). PGPR are a heterogeneous group of rhizosphere-colonizing beneficial bacteria that improve plant health and/or promote plant growth directly (biofertilization, phyto-stimulation, rhizoremediation) or indirectly (phytopathogen elimination, e.g., antibiosis, systemic resistance induction, competitive colonization for nutrients, and niche) (Backer et al. 2018; Lugtenberg and Kamilova 2009; Mariutto and Ongena 2015). In assessing the activity of four common PGPR strains in a *S. hermonthica*–sorghum pathosystem, Mounde et al. (2015) verified *Bacillus*

subtilis isolate GB03 as the most promising, compared with *B. subtilis* isolate Bsn5, *B. amyloliquefaciens* isolate FZB42, and *Burkholderia phytofirmans* isolate PsJN, in terms of inhibiting *S. hermonthica* germination with haustorial attachment and promoting the biomass of *S. hermonthica*-free sorghum plants.

Notwithstanding the advancements in *S. hermonthica* biocontrol research, related to the ecology and biocontrol potential of *Fos* and PGPR against *Striga* spp., it is still unknown if the combined application of these *S. hermonthica*-attacking agents will optimize *S. hermonthica* suppression. The goal is that technically a synergistic action of *Fos* attacking *S. hermonthica* and PGPR improving the host cereal growth can be achieved. It is also uncertain if the coinoculation of *Fos* and PGPR would be equally effective when applied either as pre-*Striga* seed conditioning or post-*Striga* attachment treatment. Furthermore, the localization/interaction of *Fos* and PGPR, in affected *S. hermonthica*, is unclear. Hence, the main research questions of this study were as follows: Will the coinoculation of *Fos* and PGPR improve the efficiency of combating *S. hermonthica*, while simultaneously promoting sorghum yield in a *S. hermonthica*–sorghum pathosystem? Does the response pattern of *S. hermonthica* and sorghum differ, suggesting a preference for an application period (pre-*Striga* seed conditioning or post-*Striga* attachment) of the coinoculating *Fos*-PGPR treatments? How do *Fos* and PGPR localize/interact in *S. hermonthica*, when coinoculated in an integrated *S. hermonthica* biocontrol system? Answering these questions will foster the understanding of the functional role of the fungal–bacterial coinoculation in a *S. hermonthica*–sorghum pathosystem, the impact of the microbial treatments at different *Striga* stages/lifecycles (i.e., germination inhibition or attachment/emergence suppression), and the physiology of the host (i.e., *S. hermonthica*)–microbe (i.e., *Fos*, PGPR) interaction in an integrated *S. hermonthica* biocontrol system. This study hypothesized that the coinoculation of *Fos* and *B. subtilis* will jointly infect and co-occupy (colocalize) the diseased *S. hermonthica*, thereby optimizing *S. hermonthica* suppression by circumventing the limitations from both the inconsistent effectiveness of *Fos* isolates against *S. hermonthica* (Anteyi and Rasche 2020) and the instability of *S. hermonthica* germination inhibitor(s) produced by PGPR (Miché et al. 2000). It was expected that this would result to an improved growth promotion (biomass) of *S. hermonthica*-infected sorghum, because PGPR alone, as exemplified by *B. subtilis*, *B. amyloliquefaciens*, and *B. phytofirmans*, were shown not to improve the biomass of *S. hermonthica*-infected sorghum (Mounde et al. 2015). Our expectation for a synergistic interaction between *Fos* and PGPR was based on the compatibility of *Fos* with indigenous maize rhizosphere microbiota (Musyoki et al. 2016; Zimmermann et al. 2016a). Thus, in the framework of a *S. hermonthica*–sorghum pathosystem, the study aimed at examining: (i) the role/impact of *Fos* isolates (Foxy-2, FK3) with *B. subtilis* isolate GB03 coinoculation on *S. hermonthica* attachment and sorghum biomass, when applied as *Striga* preconditioning and *Striga* postattachment treatments, and (ii) the localization/interaction of the *Fos*-GB03 coinoculants in affected *S. hermonthica* plants.

MATERIALS AND METHODS

Preparatory work

Plant materials, microbial isolates/strains, and plasmids. *S. hermonthica* seeds, sampled from Sirinka, Ethiopia, were used as parasitic (model) plant in this study. *S. hermonthica*-susceptible sorghum (*Sorghum bicolor* L. Moench) cultivar PI563294 from

Niger was used as host (model) crop. From our previous study (Anteyi and Rasche 2020), the sampled *S. hermonthica* population displayed selective susceptibility to FK3 but not to Foxy-2. Also, the utilized sorghum cultivar exhibited susceptibility to diverse *S. hermonthica* populations from East and West Africa. *B. subtilis* isolate GB03, designated as 3A37 strain, was obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio. It is noteworthy to state that the GB03 isolate was first identified as *B. subtilis* and later as *B. amyloliquefaciens*; however, due to its genome sequence, the strain 3A37 has been reclassified as *B. velezensis* (D. R. Zeigler, *personal communication*). *B. subtilis* isolate GB03 was selected as a representative PGPR for this study, following its outstanding ability to inhibit *S. hermonthica* germination with haustorial attachment and promote the biomass of *S. hermonthica*-free sorghum plants in a *S. hermonthica*–sorghum pathosystem, compared with other PGPR (i.e., *B. subtilis* isolate Bsn5, *B. amyloliquefaciens* isolate FZB42, and *B. phytofirmans* isolate PsJN) (Mounde et al. 2015). *Trichoderma viride* strain IMB12098, DSM number 63065, was procured from the Leibniz-Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) GmbH, Braunschweig, Germany. *Fos* isolates Foxy-2 and FK3 were obtained as 20% glycerol stocks from a -80°C microbial cell bank at the Institute of Agricultural Sciences in the Tropics, University of Hohenheim, Germany.

pFPL-Ch (61647) and pFPL-Rh (61649) plasmids were procured from Addgene (2019) repository. Development of these plasmids were described by Gong et al. (2015). Both plasmids contain antibiotic resistance genes for chloramphenicol, kanamycin, and hygromycin (as selectable markers). pFPL-Ch contained a 720-bp gene insert, enhanced cyan fluorescent protein, for cyan fluorescence expression. A 678-bp gene insert, monomeric red fluorescent protein in pFPL-Rh, is responsible for red fluorescence expression (Fig. 1).

Seed sterilization. Surface sterilization of *S. hermonthica* and sorghum seeds was performed according to the method of Anteyi and Rasche (2020). For *S. hermonthica*, the seeds were first submerged in a mixture of 5% (v/v) sodium hypochlorite (containing 12% chlorine) (Carl Roth GmbH, Karlsruhe, Germany) and 0.02% (v/v) Tween 20 (Carl Roth GmbH). Then, sonication for 3 min (Bandelin Sonopuls GmbH, Berlin,

Germany) was followed by five to six rinses with double-distilled water (ddH_2O). Surface sterilization of sorghum seeds was by immersing in 70% ethanol for 2 min, followed by rinsing thrice with ddH_2O . Then, seeds were submerged in a blend of 1% sodium hypochlorite and 0.02% (v/v) Tween 20 for 3 min; finally, they were rinsed five to six times with ddH_2O . Glass fiber filter papers (90 mm, Macherey-Nagel GmbH, Düren, Germany) were oven sterilized for 48 h at 65°C and then placed under a laminar flow hood with UV for 2 h. Surface-sterilized *S. hermonthica* and sorghum seeds were separately air dried on the sterilized glass fiber filter papers under a laminar flow hood without UV for 48 h.

Germination percentage of surface-sterilized *S. hermonthica* seeds. Approximately 200 surface-sterilized *S. hermonthica* seeds were spread on an 8-mm punched disc of sterilized glass fiber filter papers. Six discs (replicates) carrying the *S. hermonthica* seeds were placed on two layers of 90-mm sterilized glass fiber filter papers, within a 90-mm Petri dish. Six milliliters of ddH_2O was added to the Petri dish. Then, the Petri dish was covered, sealed with Parafilm, and incubated in a dark chamber at 30°C for 14 days, for preconditioning. Thereafter, the discs (with preconditioned seeds) were transferred onto a fresh glass fiber filter paper, within a Petri dish, and 3 ml of a 0.1-ppm synthetic strigolactone analog, rac-GR24 (Chiralix B.V., Nijmegen, Netherlands) was applied to the Petri dish. The setup was reincubated at 30°C for 24 h for germination. Afterward, germinated seeds were counted under a binocular microscope (Zeiss Stemi 2000-C Stereomicroscope, Carl Zeiss Microscopy GmbH, Jena, Germany), coupled with the Zeiss AxioCam HRC (Carl Zeiss Light Microscopy, Göttingen, Germany). This germination assay was done in two experimental repetitions.

Fos-GB03 coinoculation study

Pre-Striga seed conditioning treatment application. Microbial culturing and inocula concentrations. Mycelial suspensions of Foxy-2 and FK3, including IMB12098, were separately prepared by flooding the surface of their 2-week-old actively growing potato dextrose agar (PDA) (Carl Roth GmbH) cultures in 90-mm Petri dishes, each with 10 ml of 25% potato dextrose broth (PDB) (Carl Roth GmbH). Then, they were left to stand for

FIGURE 1

Schematic plasmid maps of pFPL-Rh and pFPL-Ch with fluorescent proteins, specific primers used, and antibiotic resistance. CmR = chloramphenicol; KanR = kanamycin; HygR = hygromycin; eCFP = enhanced cyan fluorescent protein; and mRFP = monomeric red fluorescent protein. Plasmid maps were created with SnapGene Viewer 5.0.7.

30 min, before gently scraping the mycelial mass into sterile flasks containing 2 liters of 25% PDB. For colony growth, the flasks were loosely covered with their lids and placed in a dark incubating shaker for 15 days at 65 rpm and 28°C. A colony of GB03 was separately transferred from overnight Luria-Bertani (LB) agar cultures into sterile flasks containing 2 liters of 15% LB broth. The loosely covered flasks were placed in a dark incubating shaker for 4 days at 80 rpm and 35°C.

Inocula concentrations in colony forming units per milliliter (CFU/ml) were determined from six replicates of each microbial inoculum: Foxy-2 and FK3, 10^4 to 10^5 ; GB03, 10^6 to 10^7 ; and IMB12098, 10^7 . The prepared fungal and bacterial inocula were subsequently used as pre-*Striga* seed conditioning planting treatments.

Planting trials. Sixty grams of sterilized modular seed substrate (Klasmann-Deilmann GmbH, Geeste, Germany) was filled into polyvinyl chloride rhizoboxes of dimensions 20 × 5 × 2 cm. Then, 60 mg of surface-sterilized *S. hermonthica* seeds were sown 5 cm below the substrate surface, and the front side of each rhizobox was covered with Plexiglas. Sixty milliliters of ddH₂O or the prepared microbial inocula (as single or combined fungi + bacteria coinoculation) were applied to each rhizobox as treatments. For rhizoboxes containing combined fungi + bacteria treatments, 30 ml of each microbial inoculum was added. Also, *T. viride* (IMB12098 strain) was introduced among the planting treatments to serve as a check BCA treatment, because *T. viride* was reported to completely inhibit *S. hermonthica* seed germination at 10^6 spores/ml inoculum concentration (Hassan et al. 2013). Hence, treatments included (i) ddH₂O without *S. hermonthica* (negative control); (ii) *S. hermonthica* with ddH₂O (positive control); (iii) *S. hermonthica* with FK3 and GB03 (*Striga* + FK3 + GB03); (iv) *S. hermonthica* with Foxy-2 and GB03 (*Striga* + Foxy-2 + GB03); (v) *S. hermonthica* with IMB12098 and GB03 (*Striga* + IMB12098 + GB03); (vi) *S. hermonthica* with GB03 (*Striga* + GB03); (vii) *S. hermonthica* with FK3 (*Striga* + FK3); (viii) *S. hermonthica* with Foxy-2 (*Striga* + Foxy-2); (ix) *S. hermonthica* with IMB12098 (*Striga* + IMB12098). The setup was incubated at 30°C for 10 days in a dark climate chamber (Percival Intellus Environmental Controller, EA-75HIL, Perry, IA) for *S. hermonthica* seed preconditioning. The experiment was set up in a randomized complete block design, with three replications for each treatment.

Thereafter, a single sorghum seed was sown in every rhizobox, and climate chambers were set at 31 and 27°C, for day and night temperatures, respectively. Light of mean illuminance 46,000 lx was available for 12 h (as day), but without light as night. Plants were irrigated twice per week, first with ddH₂O and then with nutrient solution comprising a blend of 0.2% (v/v) Wuxal

universal liquid fertilizer (Aglukon Spezialdünger GmbH, Düsseldorf, Germany) and Yoshida nutrient solution (Yoshida et al. 1976) in a ratio of three parts Wuxal to two parts Yoshida. The planting trials were done in two experimental repetitions, with a 5-week growing period for each trial.

Post-*Striga* attachment treatment application and localization study. Antimycotic and antibiotic sensitivity determination.

Freshly prepared, molten PDA was supplemented with hygromycin B (Carl Roth GmbH) at 0, 25, 50, 75, 100, 125, 150, 175, and 200 µg/ml. An 8-mm agar plug from actively growing Foxy-2 and FK3 mycelia was each transferred to 90-mm Petri dishes containing the solidified media. The experiment was set up in three replications. The cultures were incubated in the dark for 7 days at 28°C, followed by determination of mycelial radial growth. Hygromycin B concentration of 125 and 150 µg/ml completely inhibited mycelial growth of wild-type Foxy-2 and FK3, respectively (Fig. 2). Sensitivity assay revealed that 200 µg/ml of hygromycin B completely inhibited wild-type GB03. For kanamycin sensitivity, 50 µg/ml was used (<http://www.addgene.org/mol-bio-reference/>). Hence, growth medium supplemented with 200 µg/ml of hygromycin B and 50 µg/ml of kanamycin sulfate (Carl Roth GmbH), hereafter abbreviated as Hyg-B/Kan, was adopted for selectively screening transformed *Fos* or transformed GB03.

Plasmid DNA isolation. Using the Invisorb Spin Plasmid Mini Two (Stratag Molecular GmbH, Berlin, Germany), plasmid DNA (pDNA) was isolated from the bacteria containing the plasmids (i.e., pFPL-Ch and pFPL-Rh) after culturing a single colony of the bacteria in 15-ml tubes containing 2 ml of 15% LB broth (Carl Roth GmbH) with Hyg-B/Kan for 15 h at 100 rpm and 37°C in a dark shaker. Diagnostic restriction digestion with *Hind*III, *Fsp*I, *Kpn*I, and *Afl*II (New England Biolabs GmbH, Frankfurt am Main, Germany) was performed to verify the plasmids. Digested DNA products confirmed the plasmids, as shown by 1.5% agarose gel electrophoresis (see Supplementary File S1).

Fos transformation mediated by polyethylene glycol (PEG). The procedure for PEG-mediated transformation of *Fos* was modified from the protocol for PEG-mediated transformation of *F. oxysporum* f. sp. *conglutinans* (Zhang et al. 2014). The detailed methodology is given in Supplementary File S2.

GB03 transformation (“mixed salts” mediated). The protocol for GB03 transformation was adapted from the International Genetically Engineered Machine (iGEM 2014) (Ludwig Maximilian University of Munich, Germany), but with minor adjustments. The detailed methodology is provided in Supplementary File S3.

Screening and verification of *Fos* and GB03 transformants. *Fos* and GB03 transformants were screened by their ability to grow on antibiotic-supplemented selective medium, in which wild-type *Fos* and GB03 were unable to grow (Fig. 3). The mycelia of transformed *Fos* and a colony of transformed GB03 were also screened for fluorescence expression using the Zeiss Axioskop-2 Plus Upright Fluorescence Microscope (Carl Zeiss Microscopy GmbH), and AxioVision SE64 Rel. 4.8 software (Jülich GmbH, Bonn, Germany). Red fluorescence expression in transformed *Fos* was detected at 546 nm (excitation) and 580 nm (emission), whereas cyan fluorescence expression in transformed GB03 was detected at 475 nm (excitation) and 500 nm (emission) (Fig. 4).

To verify successful fluorescence gene integration in *Fos* and GB03 transformants, total genomic DNA (gDNA) was extracted from a 100-mg biomass of *Fos* hyphae and GB03 cellular pellets transformants (Fast DNA spin kit, MP Biomedicals, LLC). Primer sequences in the fluorescent gene inserts of the plasmids

FIGURE 2 Hygromycin B sensitivity test to determine mycelial growth inhibition in wild-type FK3 and wild-type Foxy-2. Error bars indicate standard deviation. PDA = potato dextrose agar.

were identified with SnapGene Viewer 5.0.7 (GSL Biotech, <https://www.snapgene.com/>): mCherry primers for pFPL-Rh (mCherry-F, 5'-CCCCGTAATGCAGAAAGA-3'; mCherry-R, 3'-TTGGTCACCTTCAGCTTGG-5') and EGFP primers for pFPL-Ch (EGFP-F, 5'-CATGGTCCTGCTGGAGTTCGTG-3'; EGFP-R, 3'-CGTCGCCGTCAGCTCGACCAG-5'). Polymerase chain reaction (PCR) was done by a 50- μ l reaction system, consisting of template DNA (pDNA, 5 ng; gDNA, 200 ng), 10 $\times$ PCR buffer, 50 mM MgCl₂, 10 mM dNTPs, 20 μ M forward and reverse primers for both mCherry and EGFP primers (biomers.net GmbH, Ulm, Germany), and 2.5 U of Accuzyme DNA Polymerase (Bioline GmbH, Luckenwalde, Germany). PCR conditions with mCherry primers included initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Reaction was completed with a final extension at 72°C for 4 min. With EGFP primers, PCR conditions were initial denaturation at 95°C for 3 min, then 30 cycles at 95°C for 15 s, 60°C for 15 s, 72°C for 45 s, and a final elongation at 72°C for 4 min.

Microbial transformants culturing and inocula concentrations.

Preparation of transformed *Fos* and GB03 microbial cultures and determination of their inocula concentrations in CFU/ml were similar to the procedures described above for their corresponding wild-type microbial treatment cultures. Inocula concentrations of the transformed microbial cultures in CFU/ml were 10⁵ to 10⁶ for transformed Foxy-2 and transformed FK3 and 10⁶ for transformed GB03. The prepared transformed microbial inocula, together with their wild types, were used as post-*Striga* attachment planting treatments.

Planting trials and localization study. The planting trials made use of wild-type microbial treatments to study the impact of the post-*Striga* attachment treatment application on the sorghum-*S. hermonthica* pathosystem. It utilized the transformed microbial treatments to examine the localization/interaction of *Fos* and GB03 in affected *S. hermonthica*, to give further insight into the post-*Striga* attachment treatment application of *Fos*-GB03. Planting for the post-*Striga* attachment treatment application study followed similar technical steps, experimental

conditions, setup, and iterations as described for the pre-*Striga* seed conditioning treatment application study (see above). However, here, treatments were applied following *S. hermonthica* attachment to sorghum root, precisely 3 weeks after sowing sorghum. This timing was also ascertained from the average period for visible *S. hermonthica* attachment.

Sixty milliliters of the treatments, that is, ddH₂O or the prepared microbial inocula (as single or combined fungi + bacteria coinoculation, in separate wild-type and transformed setups) was directly applied on the substrate surface and/or on emerged (aboveground) *S. hermonthica* in each rhizobox. Similarly, in rhizoboxes for combined fungi + bacteria treatments, 30 ml of each microbial inoculum was added. Thus, treatments were (i) ddH₂O without *S. hermonthica* (negative control); (ii) ddH₂O with *S. hermonthica* (positive control); (iii) *S. hermonthica* with wild-type FK3 and wild-type GB03 (*Striga* + FK3 + GB03); (iv) *S. hermonthica* with wild-type Foxy-2 and wild-type GB03 (*Striga* + Foxy-2 + GB03); (v) *S. hermonthica* with wild-type GB03 (*Striga* + GB03); (vi) *S. hermonthica* with wild-type FK3 (*Striga* + FK3); (vii) *S. hermonthica* with wild-type Foxy-2 (*Striga* + Foxy-2); (viii) *S. hermonthica* with transformed FK3 and transformed GB03 (*Striga* + FK3[T] + GB03[T]); (ix) *S. hermonthica* with transformed Foxy-2 and transformed GB03 (*Striga* + Foxy-2[T] + GB03[T]); (x) *S. hermonthica* with transformed GB03 (*Striga* + GB03[T]); (xi) *S. hermonthica* with transformed FK3 (*Striga* + FK3[T]); and (xii) *S. hermonthica* with transformed Foxy-2 (*Striga* + Foxy-2[T]). Planting period was for 7 weeks to allow for adequate microbial colonization of sorghum for microscopic analysis.

In vivo localization analysis (microscopy and PCR). Localization of *Fos* and GB03 in infected and diseased *S. hermonthica* was studied through fluorescence expression by transformed *Fos* and transformed GB03 in infected *S. hermonthica* plants. Histological sections of *S. hermonthica* belowground and aboveground shoots and leaves were analyzed by digital imaging, using Zen software (version 2010) with a Zeiss laser scanning microscope (Carl Zeiss Microscopy GmbH). The presence of *Fos* and GB03 transformants in *S. hermonthica* was further confirmed by PCR, using primers specific to the fluorescence gene regions in the plasmids (see above).

Plant data collection and biostatistical analysis

The total counts of attached *S. hermonthica*, belowground and aboveground, in each rhizobox were recorded. Sorghum aboveground biomass was harvested and oven dried at 65°C for 10 days, and dry mass was determined. Quantitative data of the

FIGURE 3

Wild-type GB03 growing on Luria-Bertani (LB) agar (top left). Wild-type GB03 unable to grow on LB agar supplemented with 200 μ g/ml of hygromycin B and 50 μ g/ml of kanamycin sulfate (Hyg-B/Kan) (top center). Transformed GB03 growing on LB agar supplemented with Hyg-B/Kan (top right). Wild-type FK3 growing on potato dextrose agar (PDA) (bottom left). Wild-type FK3 unable to grow on PDA supplemented with Hyg-B/Kan (bottom center). Transformed FK3 growing on PDA supplemented with Hyg-B/Kan (bottom right).

FIGURE 4

Mycelia of transformed FK3 from potato dextrose agar supplemented with 200 μ g/ml of hygromycin B and 50 μ g/ml of kanamycin sulfate (Hyg-B/Kan) expressing red fluorescence (left). Short chains of transformed GB03 from Luria-Bertani agar supplemented with Hyg-B/Kan expressing cyan fluorescence (right). Scale bars = 50 μ m.

attached *S. hermonthica* and the sorghum aboveground dry biomass were tested by analysis of variance (ANOVA) and post hoc analysis by Tukey's range test. For purposes of clarity and easy comparison between the *S. hermonthica* attachment and sorghum aboveground dry biomass, graphical presentation of Tukey's range test for the mean data values was standardized (as percentage), relative to the positive control (without altering the output of the analyses).

Calculation by Pearson correlation coefficient was used to corroborate the negative correlation between the sampled *S. hermonthica* attachment and sorghum aboveground dry biomass (of the adopted sorghum cultivar) in the presence of microbial treatments. This was because from repeated preliminary studies, in the absence of microbial treatments, a strong negative correlation ($r = -0.98$, $P < 0.0001$) was calculated between the sampled *S. hermonthica* attachment and the aboveground dry biomass of the same sorghum cultivar (see Supplementary File S4). Evaluation of the impact of *Fos* and GB03 on *S. hermonthica*, when the microbial treatments were applied at post-*Striga* attachment, was indirectly determined from the sorghum aboveground dry biomass. This was a reliable estimator for the microbial treatments' effects on the already attached *S. hermonthica* plants. This indirect estimation was also supported by the stable and negative correlation between the *S. hermonthica* attachment and the sorghum aboveground dry biomass. All statistical analyses were carried out using SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Role/impact of *Fos*-GB03 coinoculation on *S. hermonthica* attachment and sorghum biomass

Pre-*Striga* seed conditioning treatment application. The surface-sterilized *S. hermonthica* seeds had a mean percentage germination of 55%. ANOVA revealed that pre-*Striga* seed conditioning treatment application had different effects on *S. hermonthica* attachment ($P < 0.01$) and sorghum aboveground dry biomass ($P < 0.01$). Combined treatments of FK3 + GB03 and Foxy-2 + GB03 increased sorghum aboveground dry biomass ($P < 0.05$), but IMB12098 + GB03 did not. Neither FK3 + GB03, Foxy-2 + GB03, nor IMB12098 + GB03 reduced *S.*

FIGURE 5

Tukey's range test for relative mean values of sorghum aboveground dry biomass (sorghum ABM) and *Striga hermonthica* attachment responses to pre-*Striga* seed conditioning treatment application. Negative control = ddH₂O without *S. hermonthica*. Positive control = *S. hermonthica* with ddH₂O. Bars having at least a letter in common represent treatments means that are not significantly different ($\alpha = 0.05$). Error bars indicate standard error.

hermonthica attachment ($P > 0.05$). FK3 and GB03, applied as single treatments, increased sorghum aboveground dry biomass ($P < 0.05$). Single treatments of Foxy-2 and IMB12098 did not increase sorghum aboveground dry biomass ($P > 0.05$). FK3 suppressed *S. hermonthica* attachment ($P < 0.05$), an effect not revealed for GB03, Foxy-2, and IMB12098. (Fig. 5). GB03 applied as a single treatment was ineffective in suppressing the attachment of the sampled *S. hermonthica*, and it thwarted *Fos* suppressive activity against *S. hermonthica* attachment. Nevertheless, GB03 promoted sorghum yield, either when applied alone or in combination with *Fos* ($P < 0.05$). This indicated that *Fos* (FK3) was primarily responsible for causing disease and death to *S. hermonthica*, whereas *B. subtilis* (GB03) was responsible for relieving the menace of *S. hermonthica* on sorghum productivity by promoting sorghum growth. In the presence of the microbial treatments, sorghum aboveground dry biomass showed a strong negative correlation to *S. hermonthica* attachment ($P < 0.05$) (Fig. 6).

Post-*Striga* attachment treatment application. Similarly, post-*Striga* attachment microbial treatment application had a different effect on sorghum aboveground dry biomass ($P < 0.0001$). In both wild-type or transformed microbial treatments, all *Fos*-GB03 combined treatments and single treatments of FK3 and GB03 improved sorghum aboveground dry biomass ($P < 0.05$). However, with single treatments of Foxy-2, the sorghum aboveground dry biomass was not higher than the positive control ($P > 0.05$) (Fig. 7).

Localization/interaction of *Fos* and *B. subtilis* in emerged *S. hermonthica*

Morphologically, *S. hermonthica* from the positive control (i.e., treated with ddH₂O) showed no infection signs (Fig. 8A). Also, microscopic examination of the positive control showed healthy *S. hermonthica* leaf, phloem, and xylem tissues (Fig. 8B to D). For *S. hermonthica* treated with *Fos* (particularly FK3), infection of *S. hermonthica* through trichome entry was revealed (Fig. 8E). *Fos* infection and pathological damage of emerged (aboveground) *S. hermonthica* originated (and was more intense) on *S. hermonthica* shoots, before spreading throughout the entire plant structure (Fig. 8F and G). Following *Fos* (FK3) colonization of *S. hermonthica*, *Fos* degraded *S. hermonthica* tissues until plant death (Fig. 8H). Then, it thrived on the dead and decaying *S. hermonthica* matter (this was also substantiated by the persistent fluorescence expression) (Fig. 8I). This necrotrophic attribute of FK3 was not displayed by GB03. Cyan fluorescence detection at 475 to 500 nm indicated high GB03

FIGURE 6

Correlation and regression between relative mean values of sorghum aboveground dry biomass (sorghum ABM) and *Striga hermonthica* attachment in presence of microbial treatments.

localization in flavonoid-rich spots of *S. hermonthica* shoot tissue (Fig. 8J). *Fos*, either as sporal (conidia) or vegetative form (mycelium), infected and co-occupied common ecological niches with GB03 in diseased *S. hermonthica* shoots (i.e., both *Fos* and GB03 colocalized degrading shoot tissue of infected *S. hermonthica*) (Fig. 8K and L). The interaction of *Fos* and GB03 (colocalization) was not distinctly observed in *S. hermonthica* leaves. Colocalization of *Fos* and GB03 in the infected *S. hermonthica* was further confirmed by PCR of fluorescent genes carried by the transformed microbial inoculants, from planting trials of the post-*Striga* attachment treatment application (see Supplementary File S5).

DISCUSSION

FK3 controlled *S. hermonthica* attachment, and it infected *S. hermonthica* through trichome entry when applied post-*Striga* attachment

Among the microbial treatments, only FK3 effectively controlled the attachment of the sampled *S. hermonthica*. In assessing the variable susceptibility pattern of genetically diverse *S. hermonthica* populations from East and West Africa to *Fos* isolates, similarly sampled *S. hermonthica* from Sirinka, Ethiopia, displayed significant susceptibility to FK3 but not to Foxy-2 (Anteyi and Rasche 2020). This implied that the susceptibility pattern of a *S. hermonthica* population to *Fos* isolates, thus far, is a stable feature that is primarily explained by the innate attributes (genetic) of *S. hermonthica*. It therefore explains a finding from this study, as to why the effectiveness of a *Fos* isolate against *S. hermonthica* was not altered by the fungal treatment application period during planting. This was shown by the significant susceptibility of the sampled *S. hermonthica* to FK3 but not to Foxy-2, either when the microbial treatments were applied pre-*Striga* seed conditioning or post-*Striga* attachment. Hence, this corroborated the narratives that *Fos* mycoherbicidal action against *S. hermonthica* may occur through chemical (i.e., release of certain metabolites that inhibit *S. hermonthica* germination and/or attack young *S. hermonthica* plantlets below-ground, before haustorial attachment) as well as mechanical (i.e., epidermal penetration of emerged or mature *S. hermonthica* shoot, especially through natural opening or wounds) modes (Abbasher and Sauerborn 1992; Yonli et al. 2010). For instance, *Fusarium* spp. have been reported to produce chemical substances (e.g., fusaric acid, 9,10-dehydrofusaric acid, trichothecenes, and β -lactones) that inhibit *S. hermonthica* seed germination (Sugimoto et al. 2002; Xiang et al. 2017; Zonno et al. 1996). Likewise, the successful post-*Striga* attachment foliar application of *Fos* isolate PSM-197 against *S. hermonthica* has been earlier noted (Marley et al. 1999).

Fluorescence microscopic analysis revealed that when the microbial treatments were applied post-*Striga* attachment, trichome entry was among the mechanical means by which *Fos* (FK3) penetrated *S. hermonthica*. This finding contrasted the acknowledged indirect mode by which *Fos* infects *S. hermonthica*, whereby *Fos* hyphae penetrate *S. hermonthica* through the “sorghum central cylinder-*S. hermonthica* haustorial” connecting tissues within sorghum root, without causing any known pathological damage to sorghum (Elzein et al. 2010; Ndambi et al. 2011). *Fos* infection of *S. hermonthica* by trichome entry is possible, because *S. hermonthica* shoots and leaves are largely covered with unique trichomes (CABI 2019). Apart from the primary roles of trichomes, which include protection, water/moisture absorption, storage or secretion of specialized metabolic compounds, and elimination of excess toxic substances

(Huchelmann et al. 2017; Werker 2000), they are also host sites for plant relationships with endophytic fungi. Injured trichomes give rise to open bases or stalks, which can serve as favorable entry points for phytopathogens, thereby encouraging fungal infection (penetration and colonization). Thus, trichomes serve as an uncommon fungal niche (Andargie and Li 2016; Calo et al. 2006; Kim 2019).

FK3 colonized, degraded, killed, and thrived on the dead tissue of *S. hermonthica*. This is a typical necrotrophic lifestyle of *F. oxysporum* (Moore et al. 2018). The necrotrophic feature of *Fos*, together with its chlamydospore-forming ability, facilitates its existence as a soil-borne phytopathogen (Elzein et al. 2008, 2010). This supports its ability to persist and proliferate in substrate or soil for long periods, even beyond the completion of a particular *S. hermonthica* biocontrol season (Ciotola et al. 1995; Sauerborn et al. 1996), although without underrating the importance of site-specific factors (i.e., soil physicochemical properties and climatic conditions) (Zimmermann et al. 2016b).

B. subtilis counteracted the fungal activity against *S. hermonthica* attachment

B. subtilis (GB03) did not prevent the attachment of the sampled *S. hermonthica*. This outcome was not in agreement with the observations of Mounde et al. (2015), in which they reported GB03 suppressive activity toward *S. hermonthica* attachment, through reduction of *Striga* germination and haustorial attachment, including tubercle death. It is uncertain if this discord was also due to a possible inconsistency of GB03 effectiveness against differing *S. hermonthica* populations (as existent with *Fos* isolates), especially going by GB03 effectiveness against a Malian (West African) *S. hermonthica* population used by Mounde et al. (2015), whereas our study was based on an Ethiopian (East African) *S. hermonthica* population.

Furthermore, integration of GB03 with fungal treatments (FK3, Foxy-2, IMB12098) counteracted their activity against *S. hermonthica* attachment. This resulted in an undesirable promotion of *S. hermonthica* attachment, in the order of the suppressive tendency of the applied fungal treatments toward *S. hermonthica*: FK3 < Foxy-2 < IMB12098. This fungal counteractivity by GB03 could be explained by potential fungal

FIGURE 7

Tukey's range test for sorghum aboveground dry biomass (sorghum ABM [g]) response to post-*Striga* attachment application of wild-type and transformed (indicated with [T]) microbial treatments. Negative control = sorghum + ddH₂O without *Striga hermonthica*. Positive control = sorghum + ddH₂O with *S. hermonthica*. Bars having at least a letter in common represent treatments means that are not significantly different ($\alpha = 0.05$). Error bars indicate standard error.

antagonizing substances released by *B. subtilis*. Generally, PGPR secrete extracellular metabolites in the form of lytic enzymes, lipopeptides, and hydrogen cyanide, which can antagonize a variety of fungi and other phytopathogens (Beneduzi et al. 2012; Goswami et al. 2016). For instance, *B. subtilis* produces a bioactive metabolite, bacillaene, a polyene polyketide that confers antifungal activity against the genera *Fusarium* sp. and *Trichoderma* sp. (Um et al. 2013). Other *B. subtilis* metabolites, such as bacillomycin D, macrolactins, iturin, and fengycin, are also important antagonists to *F. oxysporum* (Caulier et al. 2019; Cawoy et al. 2015; Yuan et al. 2012). This, however, does not imply that in field conditions or natural ecologies *Fos* will be easily subdued (and restrained from controlling *S. hermonthica*) by *B. subtilis* or other comparable members of the soil microbiota. It was recently shown that apart from *Fos* being a naturally occurring, soil-borne phytopathogen with a successful biocontrol record against *S. hermonthica* in various agroecosystems, it is

also compatible with indigenous maize rhizosphere microbiota, which include prokaryotic and fungal communities (Musyoki et al. 2016; Zimmermann et al. 2016a).

Cyan fluorescence detection revealed high GB03 localization in flavonoid-abundant spots of *S. hermonthica* shoot tissue. Typically, *Striga* spp. are rich in a variety of flavones (Hiremath et al. 1997; Kim et al. 2010; Nakanishi et al. 1985), and at 470 to 525 nm, flavonoids emit in the blue or cyan region of the visible spectrum (Müller et al. 2013; Roshchina 2012). Flavonoids are important signaling compounds for initiating plant-microbe interactions (Straney et al. 2002). For instance, it stimulates *Fusarium solani* macroconidia germination (Ruan et al. 1995), it is a carbon source for some bacteria (Hassan and Mathesius 2012), and it acts as a chemo-attractant for symbiotic rhizobacterial interaction with plants (Steinkellner and Mammerler 2007). Microbial recognition of *S. hermonthica* characteristic flavonoids (and/or other specific metabolites) may explain the

FIGURE 8

A, Healthy *Striga hermonthica* plants (yellow arrows) parasitizing sorghum (green arrow) in the positive control rhizobox. **B**, Healthy *S. hermonthica* leaf from the positive control showing chloroplasts (numerous dark spots). **C**, Sieve plate of sieve tube element (yellow arrow), chloroplasts (green arrows), and a companion cell nucleus (brown arrow) of healthy *S. hermonthica* phloem from the positive control. **D**, Reticulate vessel of noninfected *S. hermonthica* xylem from the positive control. **E**, A germinating FK3 chlamydospore (green arrow), with its germ tube penetrating the *S. hermonthica* trichome (yellow arrow). **F**, FK3-infected *S. hermonthica* (yellow arrow), parasitizing sorghum (green arrow). **G**, FK3 colonizing diseased *S. hermonthica* shoot (yellow arrow). **H**, Dead and degrading *S. hermonthica* plant completely colonized by FK3. **I**, Transformed FK3 expressing red fluorescence in diseased, necrotic, and decaying *S. hermonthica* tissue. **J**, Transformed GB03 expressing cyan fluorescence within shoot tissue of diseased *S. hermonthica* (ubiquitous autofluorescence is due to flavonoids). **K**, Consortia of FK3 macroconidia (yellow arrows), FK3 microconidia (green arrows), and short chains of GB03 (brown arrows) colocalizing in a diseased *S. hermonthica* shoot. **L**, Transformed FK3 hyphae and transformed GB03 occupying a common ecological niche of degrading *S. hermonthica* shoot tissue. Scale bars are 20 μ m.

attraction/infection of FK3 and GB03, in colocalizing *S. hermonthica* shoot tissue.

T. viride* was not effective against the sampled *S. hermonthica

T. viride (IMB12098) was initially included among the microbial treatments of the pre-*Striga* seed conditioning treatment application study, to serve as a reference to the investigated treatments. The decision was taken based on reports of the *S. hermonthica* seed germination inhibition activity of *Trichoderma* spp. For instance, Hassan et al. (2013) had reported *T. viride* of 10^6 spore/ml concentration to completely inhibit *S. hermonthica* germination (0%), but it enhanced *S. hermonthica* germination (90%) compared with their control (85%) at 10^1 spore/ml. In our study, a slightly higher *T. viride* concentration of 10^7 CFU/ml was used; nonetheless, it was not effective in controlling *S. hermonthica* germination and attachment. It could be speculated that this nonconformity of *T. viride* suppressive activity against *S. hermonthica*, as revealed by Hassan et al. (2013), was due to a different strain of *T. viride* used in their study. Alternatively, it may be ascribable to the different *S. hermonthica* population from Sinnar State (Sudan) used by Hassan et al. (2013), which was susceptible to *T. viride*, whereas our *S. hermonthica* from Sirinka (Ethiopia) was resistant to *T. viride*. The reason for the nonconformity remained unclear.

CONCLUSIONS

Despite the colocalization of *Fos* and GB03 in infected *S. hermonthica* shoots, the combined BCA treatment did not result in a stronger *S. hermonthica* control compared with the single FK3 treatment. The sampled *S. hermonthica* maintained its selective susceptibility to FK3 but not to Foxy-2. This variable susceptibility of *S. hermonthica* was perhaps also responsible for the inconsistent effectiveness of other acclaimed *S. hermonthica* BCAs (e.g., *B. subtilis* and *T. viride*) against our differing *S. hermonthica* population. Hence, prior to any *S. hermonthica* biocontrol program, a BCA strain/isolate confirmed for its effectiveness against a particular *S. hermonthica* population should be adhered to. This is clearly preferable, rather than applying a combination of contrasting BCAs, for which knowledge of their interaction or awareness on the susceptibility pattern of the *S. hermonthica* population toward the BCA is unknown or insufficient.

The effectiveness of a specific *Fos* isolate (i.e., FK3) against the sampled *S. hermonthica* was shown to be unaffected with changing *Fos* treatment application period during planting (i.e., pre-*Striga* seed conditioning or post-*Striga* attachment). Nevertheless, it is suggested to apply the BCA pre-*Striga* seed conditioning, because this would prevent *S. hermonthica* from infecting the host crop in the first place. Most times, the physiological damage initiated to the host crop by *S. hermonthica* could be irreversible (Beed et al. 2007; Rumsey 2012). Thus, exometabolites that specifically inhibit germination of *S. hermonthica* seeds, and/or haustoria development with attachment, shall be identified and validated from potent BCAs of *S. hermonthica* through tracer-assisted metabolomic techniques such as stable isotopy, positron emission tomography, nuclear magnetic resonance, and liquid/gas chromatography-mass spectrometry (Jang et al. 2018; Lin and Chung 2014). This could lead to the discovery of ecologically safe, specific metabolites, with stronger and broader efficacy range against diverse *S. hermonthica* populations.

Lastly, the discovery from this study, involving a phyllosphere-transmitting, direct *S. hermonthica* infection by *Fos* through

trichome entry, offers an alternative entry point. This is an addition to the recognized, rhizosphere-transmitting, indirect infection mechanism through the “sorghum central cylinder-*S. hermonthica* haustorial” connecting tissues within sorghum root (Elzein et al. 2010; Ndambi et al. 2011). This revelation paves the way for further research possibilities that center on exploiting this newly identified anatomical pathway for better *S. hermonthica* biocontrol. For instance, the induction of *Fos* spore germination by particular *S. hermonthica* secondary metabolites can be further investigated through molecular identification and characterization of transcription factors with regulatory constituents by which a potent, *S. hermonthica*-attacking microbial strain specifically recognizes its host.

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Author contributions: W. O. Anteyi designed and performed the experiments, collected, and analyzed the data, and prepared the manuscript. F. Rasche conceived of the study, supervised the work, critically reviewed the manuscript draft, and gave final approval of the published version.

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