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CHAPTER 6

From Lab to Field: applying the Fo47 biocontrol strain in potato fields

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*Authors contributed equally to the research presented within this chapter

Abstract

Endophytic microbes conferring biocontrol are an eco-friendly alternative to control diseases in crops. Unfortunately, the use of endophytes to control diseases is not yet widespread as their application in agricultural settings is challenging and the outcome variable. Translating strains that perform good under lab conditions to the field poses several challenges. One is the scaling up inoculum production in a cost-effective manner. Here, we developed a framework to scale up inoculum production of Fo47 and assessed inoculum viability, performance in the field and beneficial effects for potato. The *Fusarium oxysporum* endophyte Fo47 is a well-described biocontrol agent, isolated from disease suppressive soils in the 80's. Using mung bean medium, we could routine produce ≈$7x10^8$ spores/mL. Using 60 ml of inoculum we could re-isolate the fungus 79 days after application from 60-70% of the potato plants in a potato field trial performed in Clenze (Germany). The presence of the fungus did not negatively affect plant yield or starch production, and did not result in increased sensitivity to endogenous pathogens. This protocol can be used to assess Fo47 biocontrol potential under field conditions.

Introduction

Many phylogenetically diverse soil-inhabiting fungi are harmless endophytic colonizers of plant roots. Over the last four decades, endophytes have drawn the attention of the scientific community as some enhance plant fitness and increase resilience to pathogens and abiotic stresses, thereby potentially reducing pesticide dependency (Alabouvette, 1986; Busby et al., 2016; Ghorbanpour et al., 2018; Latz et al., 2018).

Among the diverse microbiota, *Fusarium oxysporum* (Fo) is ubiquitously present in soils. Fo is infamous for causing vascular wilt diseases in over 100 different crops (Edel-Hermann and Lecomte, 2019). In fact, Fusarium wilt diseases rank among the most devastating diseases, constituting a significant agricultural threat (Dean et al., 2012; Fisher et al., 2012). However, most Fo strains are saprotrophs and not able to cause disease. Notably, wilt-disease suppressive soils carry Fo endophytes able to colonise plant roots that confer biocontrol against pathogenic Fo strains (Alabouvette, 1986). Upon sterilization of these soils their biocontrol capacity is lost, but this can be reconstituted by supplementing Fo strains (Tamietti et al., 1993). Biocontrol to Fo pathogens has been reported to be a universal feature of non-pathogenic Fo strains (Bao et al., 2004) and even avirulent Fo pathogens can reduce susceptibility of the host to virulent Fo pathogens (Biles and Martyn, 1989; Huertas-Gonzalez et al., 1998). Fo47 is the best studied biocontrol-conferring Fo strain and has originally been isolated from wilt-disease suppressive soils in Châteaurenard (Alabouvette, 1986). Fo47 does not promote plant growth, but can reduce susceptibility to vascular fungal pathogens like Fo (Alabouvette et al., 2009; Aimé et al., 2013; de Lamo and Takken, 2020) and *Verticillium dahliae* (Veloso and Díaz, 2012; Veloso et al., 2016) and to non-vascular pathogens such as root-infecting oomycetes *Pythium ultimum* in cucumber (Benhamou et al., 2002) and *Phytophthora capsici* in pepper (Veloso and Díaz, 2012). The mechanisms employed by Fo47 and other Fo's to confer biocontrol are proposed to consist of two components; a direct activity against the root pathogen through mycoparasitism, antibiosis and competition for nutrients and root niches (Benhamou et al., 2002; Alabouvette et al., 2009; Le Floch et al., 2009), and an indirect activity by inducing a root-specific plant-mediated resistance response termed endophyte-mediated resistance (EMR) (de Lamo and Takken, 2020).

Fo47 has been found to reduce susceptibility to root pathogens in various Solanaceae such as tomato (Aimé et al., 2013; de Lamo et al., 2018; Constantin et al., 2019), pepper (Veloso and Díaz, 2012; Veloso et al., 2016) and eggplant (Pantelides et al., 2009; Zhang et al., 2018). The Solanaceae family embraces plant species of striking relevance to humans as food source (pepper, tomato, eggplant or potato), ornamentals (petunia) or drugs (tobacco) (Kimura and Sinha, 2008). Within Solanaceae, potato is one of the few crops that can be cultivated in open fields in northern Europe. Therefore, a potato field trial was performed in Clenze (Germany) with the ultimate goal of developing a reproducible methodology to test the marketable potential of Fo47 as a biocontrol agent. Traits like cost-effective spore production, durability and stability upon storage or tolerance to the changing environment of fields are important for biocontrol agents (Spadaro and Gullino, 2005). Here, we set out to develop cost-effective large scale Fo47 spore formulations and to assess resilience of the fungus during storage and in the field. We assessed its ability to colonize potato

plants under agricultural conditions in absence or presence of fertilizer and we monitored the impact of endophytic colonisation on crop performance.

As a result, we developed a robust method to (1) cost-effectively mass-produce Fo spores for potato field experiments, (2) to assess the viability of the generated spores, (3) to inoculate potato mother tubers in the field, (4) to monitor successful Fo endophytism under field conditions and (5) to measure tuber yield, starch content and diseases. We propose this method to validate the suitability of Fo endophytes in crops such as potato as in our hands, Fo47 successfully colonized potato without negatively affecting crop performance.

Results

Mung bean medium yields high concentration of viable Fo microconidia

To identify an easy-to-produce and cost-effective medium that allows high Fo47 microconidia yields three different media were tested (**Figure 1A**). As reference NO_3 medium (0.17% YNB without amino acids or $(NH_4)_2SO_4$, 3% sucrose and 100 mM KNO_3) was used, as this is the standard medium for Fo propagation in our lab (Gawehns et al., 2014). However, its relatively high cost is prohibitive for mass-scale spore production and field application. As alternative media to grow the fungus, brown rice, mung beans and polenta were tested as these products are relatively cheap and readily available in supermarkets. Brown rice retains the bran layer that contains micronutrients like manganese and iron, which favour sporulation of some fungi (Michal Johnson et al., 2011; Li et al., 2012). Mung beans are well-known as a suitable media for growing Fo such as banana-infecting Fo f.sp. *cubense* strains (Bai and Shaner, 1996; Garcia-Bastidas et al., 2019). Polenta was taken along as it has been used to propagate microbes (Kocic-Tanackov et al., 2019).

Six days after inoculation, all 1% w/v media were observed to produce a higher number of Fo spores than those containing 0.4% w/v (**Figure 1A**). Mung bean broth was found to be the most efficient medium yielding ≈$7x10^8$ spores/mL, followed by NO_3 (≈$4.8x10^8$ spores/mL), 1% brown rice (≈$1.6x10^8$ spores/mL) and 1% polenta (≈$5.3x10^7$ spores/mL). This result led to selection of mung bean medium for large-scale spore production. Next, viability and infectivity of spores produced in mung bean medium was assessed. Since plant colonisation by an endophyte is laborious to asses and quantify, the tomato pathogen Fol007 was used instead and wilt disease symptoms were assessed (Gawehns et al., 2014). Thereto, soil containing 10-days-old tomato seedlings was inoculated with spores from mung bean medium by pouring 10, 25 or 50mL of 10^7 spores/mL on 12 cm diameter pots. Fusarium wilt disease symptoms were assessed three weeks post inoculation (**Figure 1B**). Except for the mock, wilt disease was observed for all inoculations showing that Fo inoculum is viable and able to infect tomato (**Figure 1B)**. In summary, mung bean medium is a cost-effective and high spore yielding medium allowing large scale production of viable Fo inoculum.

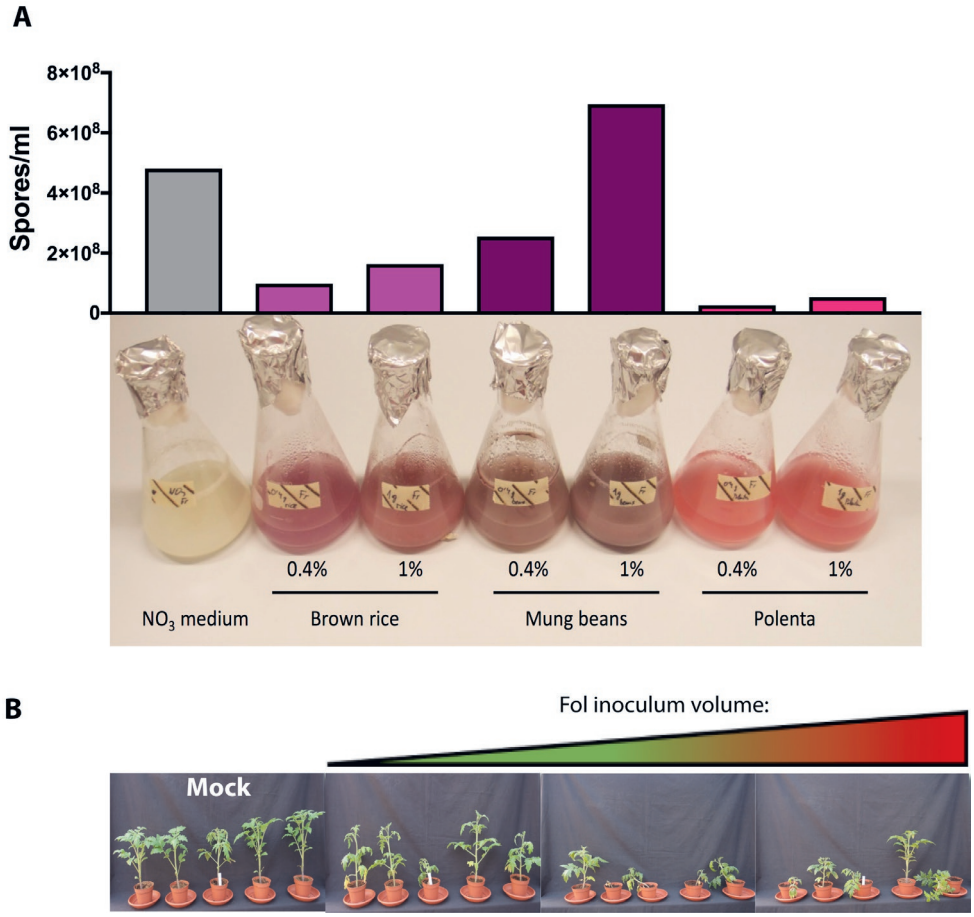

Figure 1. Mung bean medium yields high concentration of Fo spores. (A) Microconidia concentration of Fo47 inoculated in different media was measured with a counting chamber. Six-days-post-inoculation 1% mung bean medium was found to contain the highest microconidial concentration followed by the commonly used NO_3 medium, brown rice and polenta. **(B)** Viability and infectivity of Fol spores produced using mung bean medium was assessed using a tomato bioassay. Different volumes (0, 10, 25 and 50 mL of 10^7 spores/ml from left to right) were added to the soil containing 10-day-old tomato plants.

Fo47 colonizes potato plants under green house and field conditions

To determine whether Fo47 spores produced in mung bean medium can colonize potato plants and tubers, a small-scale experiment was performed in the greenhouse. Tubers were planted and inoculated by pouring 60 ml of water (mock) or Fo47 spores (10^7 spores/ml) in the planting hole. The tuber was subsequently covered with soil. After 42 days, samples from the thickest stem (up to the second leaf), mother tuber and root were harvested from each plant. Samples were surfaced-sterilized and placed on PDA plates containing antibiotics. Four days after incubation, the mycelia emerging from the plant material that resembled Fusarium was harvested and gDNA was isolated followed by PCR with Fo-specific *FEM* primers, and Fo47-specific *SCAR* primers. Four out of

seven plants treated with Fo47 spores showed fungal outgrows that were both *FEM*- and *SCAR*-positive, showing that they are effectively colonized by Fo47 (**Figure 2A**). These data show that pouring Fo47 microconidia (harvested from mung bean medium) is an effective method to inoculated potato tubers.

Based on this observation, the viability of applying an endophyte in large scale was tested in field experiment. Common agriculture practices in the region where the field was located include applying fertilization to increase tuber yield. Therefore, to test the influence of fertilization on endophytic performance, part of the field remained unfertilized while the other was treated by applying N and K fertilization (**Figure S1A, B**). Two treatments, which consisted of pouring 60 mL of water (Mock) or Fo47 spores (10^7 spores/ml) were applied in both parts of the field. Each treatment was performed in nine randomly selected plots, where one plot consisted of 40 potato tubers (**Figure S1A, B**). From each plot, one potato plant was harvested 79 days after inoculation from the same location in the plot (**Figure S1C**). The main root below the thickest stem and one potato tuber per plant were surface-sterilised to determine the presence of the Fo47 inside the plant (**Figure 2B**). Unlike in the green-house experiment, mycelium resembling Fo grew out of five the mock-treated tubers. PCR analysis showed that it was indeed Fo (*FEM*-positive) but not Fo47 (*SCAR*-negative). Fo47 could be re-isolated from seven and six plants from non-fertilized and fertilized plots, respectively (**Figure 2B**). These data show that fertilization does not affect endophytic colonization of potato plants by Fo47 and that our lab protocol can be efficiently scaled up to field conditions.

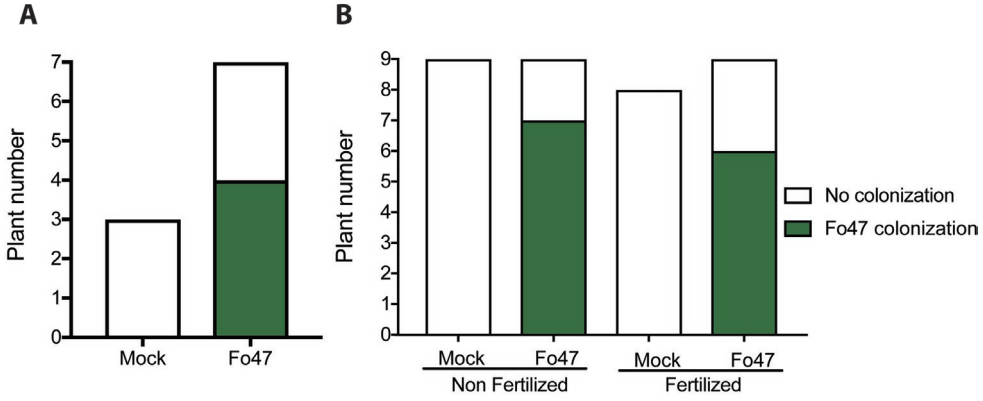

Figure 2. Fo47 is able to colonize green house- and field-grown potato plants. Each potato tuber was treated with 60 mL inoculum that contained either water (Mock) or Fo47 (10^7 spores/mL). **(A)** Stem, roots and tuber were collected 42 days after inoculation of green house-grown potato plants or **(B)** roots and tubers of field-grown potato were harvested 79 days after inoculation. Samples were surfaced-sterilized for 3 minutes in 70% ethanol, washed and arranged on PDA plates with antibiotics. Four days after plating, gDNA was isolated from the emerging mycelia and used for PCR reactions with *SCAR* and *FEM* primers. Nine plants per treatment were analysed except for the mock-fertilized treatment where only eight plants were analysed. Fo47 was considered as an endophytic colonizer when mycelia emerging from surface-sterilized root, stem or tuber from one biological sample could be confirmed by PCR with *FEM* and *SCAR* primers.

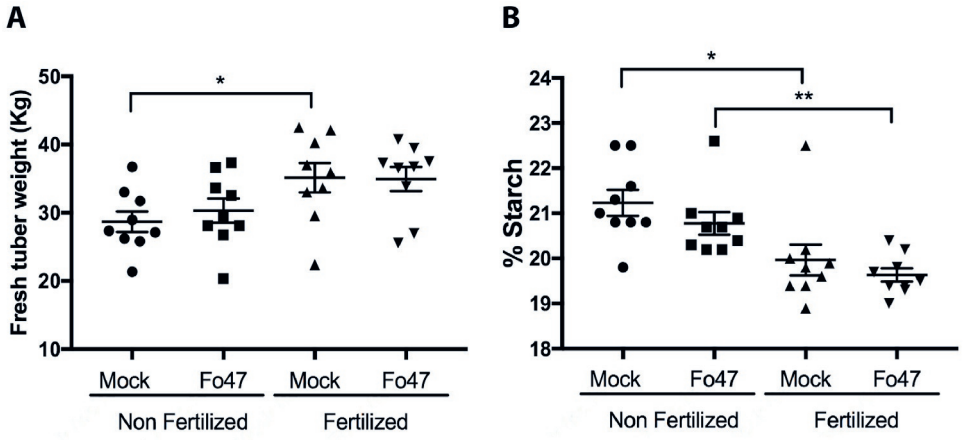

Figure 3. Potato yield and starch content is affected by fertilization but not by Fo47 application. **(A)** Average tuber yield per plot in each treatment. Every dot represents the yield of a single plot (19 plants each) **(B)** Average percentages of starch accumulated in 5 kg of tubers (Ø > 6.5 cm) per treatment. Each dot represents a plot. Data was analysed using a Mann-Whitney test where $*P < 0.05$ and $**P < 0.01$.

Tuber weight and starch content are not affected by Fo47 colonization unlike fertilization treatment

To assess whether Fo47 has an impact on potato production, the yield and starch content of the tubers from the two central rows of every plot were harvested five months after inoculation (**Figure S1C**). The middle rows were selected to avoid edge effects. In total, 19 plants/plot were harvested as one was collected during the mid-term sampling to assess Fo47 colonization. Non-fertilized water (mock)- and Fo47-inoculated plots yielded 28.7 kg and 30.3 kg on average, while applying fertilizer increased the yield significantly to 35.1 kg in the mock plots and 35.0 kg in the Fo47-inoculated plots (**Figure 3A**). In contrast, the starch content was significantly reduced in fertilized plots as mock and Fo47-inoculated plots without fertilization yielded 21.2% and 20.8% starch while fertilized plots yielded 20.0% and 19.6%, respectively (**Figure 3B**).

Altogether, Fo47 did not negatively affect tuber yield nor starch content, despite being a potato endophyte (**Figure 3A, B**), and regardless of the application of fertilizer. As expected, fertilization significantly increased the tuber yield regardless of a Fo47 treatment (**Figure 3A**). Moreover, fertilized plots exhibited a reduced starch content of the tubers.

Black scurf disease symptoms on potato tubers are reduced by fertilization but not by Fo47 treatment

To assess whether Fo47 colonisation of roots affects the susceptibility of potato plants to endogenous pathogens, 20 tubers were selected randomly per plot. Visual inspection of the tubers revealed symptoms of scab disease caused by *Streptomyces spp.* and black scurf caused by *Rhizoctonia solani*. Disease symptoms associated with Fusarium spp. where not observed on the tubers. Scab symptoms were omitted for scoring due to the difficulty of consistently assessing the disease level

– only black scurf symptoms were assessed. For scoring black scurf disease symptoms, tubers were washed to remove dirt and enable detection of sclerotia. A disease index from 0-4 was established based on the area covered by sclerotia relative to the total tuber area (**Figure 4A**). Fo47 treatment did not cause a significant reduction in black scurf disease symptoms compared to mock treatment in either fertilized or non-fertilized plants (**Figure 4B**). However, the application of fertilizer reduced black scurf disease symptoms in both mock and Fo47-treated plants (**Figure 4B**).

Discussion

Presently, public perspective and legislation are encouraging the use of alternatives for chemical pesticides and fertilizers in agriculture. Fungal endophytes have been successfully implemented as biocontrol or biostimulant agents in small scale set-ups such as greenhouses (Gill et al., 2016; de Lamo and Takken, 2020). However, one stumbling block in field implementation of endophytes as biocontrol agents is a cost-effective protocol for inoculum production. Here, we established a cheap, large-scale production method to obtain viable Fo47 microconidia and we confirmed plant colonisation of the endophyte under field conditions.

All media tested (NO_3, mung beans, brown rice and polenta) were suitable for producing Fo47 microconidia, with mung bean being the most effective, yielding $\approx 7x10^8$ spores/mL. This yield is comparable to the $\approx 9x10^7$ Fo spores/mL previously described using this medium, (Garcia-Bastidas et al., 2019), showing that this method is reproducible across different labs. Moreover, this medium is not limited to Fo but can also be used to obtain large amounts of spores from other fungi such as *Rhizopus* (Nout et al., 1987). Additionally, we could show that mung bean medium-produced microconidia proved to "infective" in bioassays by scoring disease symptoms (**Figure 1A**), and by being able to re-isolate the endophytic strain from inoculated potato plants (**Figure 2A, B**). When comparing NO_3 medium with mung bean medium, the latter is not only faster to make but also is approximately 180 times cheaper (1 L of NO_3 costs 8.5 euros while 1 L of mung bean costs 0.0478 euros). Therefore, mung bean medium is good candidate for scaling-up effective spore production of fungal endophytes with a low production cost.

Another important aspect in our protocol consisted of determining the effectiveness of Fo47 as an endophyte. This is an important step to predict efficiency of a biocontrol agent and pin-point possible negative outcomes (e.g. no protection due to low endophytic colonization levels). In our field experiment, single Fo47 application at planting time resulted in 60-70% of the plants scoring Fo47 positive at mid-harvest. Fertilization treatment which consisted of N and K, did not affect Fo47 colonization of potato plants, showing that, unlike arbuscular mycorrhizal fungi (Ortas, 2012), common management practices are compatible with Fo47 field application. Notably, Fo47 could not be re-isolated from newly formed tubers at the final harvest, despite being detectable in 20% of the tubers during mid-harvest (data not shown). This shows that the fungus is not transmitted to the final product and probably has to be re-applied every season.

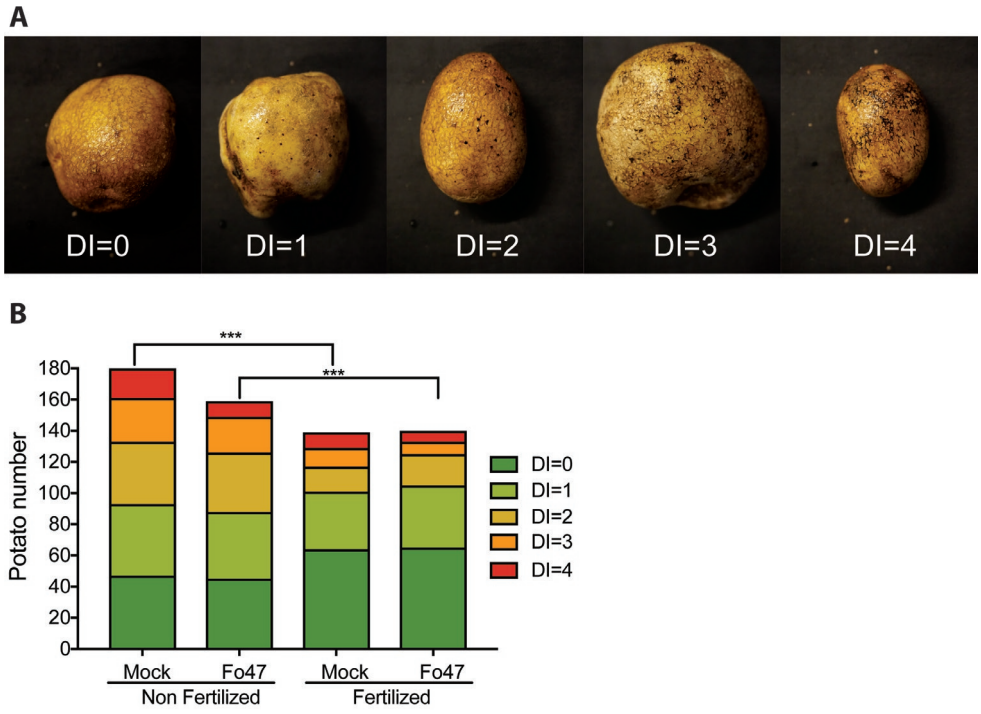

Figure 4. Fertilization, but not Fo47 treatment, reduces black scurf disease symptoms on potato tubers. (A) Representative pictures of potato tubers showing black scurf disease symptoms from scale 0-4. Based on the percentage of the tuber covered by sclerotia, the following scale was used: DI=0, less that 1%; DI=1, 1% of the tuber covered by sclerotia, DI=2, up to 5% of the tuber covered, DI=3, up to 10%; DI=4, sclerotia covers more than 15% of the tuber area. **(B)** Black scurf disease symptoms on newly formed potato tubers. From each plot 20 tubers were selected randomly. Data was analysed using a Mann-Whitney test where ****$P < 0.001$. DI= disease index.

Three other important parameters for potato tuber marketability were assessed: yield, starch and disease susceptibility in this study. Fo47 treatment did not affect potato tuber yield and starch content showing that there was no penalty of endophytic colonization. When assessing disease symptoms, however, only scab and black scurf disease symptoms could be observed. This low disease incidence is most likely due to the unusually hot and dry conditions during the growing season of the field trial (2018). Therefore, we could only show that Fo47 did not affect disease susceptibility to black scurf disease.

Fertilization treatment which consisted of addition of N and K, increased potato yield but negatively affected the starch content. Sole nitrogen fertilization has been shown to influence potato yield, while the effect of K is dependent on pre-existing concentration in the soil (Westermann et al., 1994b). Moreover, K and N treatment has been reported to decrease the starch content at both ends of the tuber (Westermann et al., 1994a). Our data indicate that the yield increase by K and N fertilization is not related to an increase of total starch content, but likely due to an increase of the water content in the tuber as also proposed by others (Schippers, 1968).

Additionally, fertilization reduced black scurf disease symptoms on the assessed tubers. This is line with previous observations that reported an inverse correlation between black scurf disease symptoms and N concentration (Rêbarz and Borowczak, 2007; Klikocka, 2009). Three mechanisms are proposed to explain this finding: 1) Black scurf symptoms are scored as a percentage of the potato surface area. As fertilization could increase the size of the tubers, a similar amount of sclerotia would be scored as a lower disease severity. 2) Fertilization can have a direct impact on plant defence responses and thereby suppress pathogen colonization (Mur et al., 2017). 3) Fertilization could impact the pathogen directly by for example altering the pH or by chelating minerals.

In summary, we developed and validated an easily scalable method to produce and apply fungal endophyte spores to crops. As a proof of concept, Fo47 spores were applied to potato plants, without affecting yield or starch content of the treated plants. Colonisation of the plants by Fo47 was unaffected by common fertilization practices in the region and did not result in increased susceptibility to endogenous pathogens, such as black scurf disease. This method is of great relevance in order to explore the capacity of fungal biocontrol agents in the field. For example, it could be used to test the ability of Fo47 (or other Fo endophytes) to suppress Fusarium wilt disease in crops such as tomato or asparagus where Fo endophytes were shown to be efficient under lab conditions (de Lamo and Takken, 2020). For further commercialization of fungal endophytes, parameters such as shelf-life stability should be tested in future research.

Materials and Methods

Inoculum preparation

Fo47 (Alabouvette, 1986) was grown on potato dextrose agar (PDA) plates for at least five days. From these plates, agar plugs from the edge of the colony containing the youngest mycelium were used to inoculate ten 250mL-flasks containing 100 ml of mung bean medium, two 1 L-flasks containing 0.5 L, two 2 L-flasks containing 1 L and three 5 L-flasks containing 2.5 L for a total of 11,5 L of 1% mung bean medium (www.mahuna.eu, autoclaved 10 g of intact mung beans/L at 120^0C for at least 20 minutes and over 220kPa). After 6 days of shake-incubation at 150 rpm at 25 ^{0}C the cultures were poured through sterile filter made with a layer of Miracloth (Millipore) without applying removing the mycelia and keeping the microconidia generated. The microconidia solution was spun down at 700 *g* for 10 min in a Beckman centrifuge with a JA-10 fixed-angle rotor. The pellet containing Fo47 spores was washed with sterile MilliQ water, and after a second centrifugation step was re-suspended in 1 L.

Biological materials and inoculation procedure

The above-mentioned concentrated Fo47 spore solution was quantified by a counting chamber, subsequently gently diluted to 10^9 spores/ml by adding MilliQ water, and stored at 4^0C until field application. Pathogen-free seed potatoes of the starch potato cv. Jasia (www.saatzucht-niehoff.de/) were planted in agricultural soil in Clenze (Germany). Inoculum was prepared in the field by adding 50ml of 10^9 spores/ml to 5 L of tap water which was gently shaked resulting in 10^7

spores/mL inoculum. Each seed potato was inoculated with 60 mL of the latter inoculum by using a ladle.

Field fertilization and pesticide application

Some plots were fertilized by adding 120 N kg/ha (7% nitrate, 7% ammonium, 14% urea) and 160 K kg/ha Korn-Kali 40, 40% K_2O, 6% MgO once at planting time. The following pesticides were applied: Monceren against *Rhizoctonia*, Boxer against insects such as Colorado Beetles and Infinito, Banjo forte and Shirlan against *Phytophthora*.

Fungal re-isolation

To determine Fo47 presence on the surface of a tuber, a peel fragment from the mother tuber was placed on PDA plates supplemented with 200 mg/l streptomycin and 100 mg/l penicillin. These antibiotics were added to prevent bacterial growth without affecting fungal outgrowth. To check whether Fo47 was an endophyte, therefore colonizing inner tissues of potato, mother tubers and roots were surface-sterilized as described (Constantin et al., 2019). In short, tubers were submerged in 70% ethanol for 3 min and washed with sterile water. Mother tuber sections and root slices were placed on the above-mentioned PDA plates. All plates were scanned after four days of incubation at 25^0C. To confirm that mycelium outgrowth corresponded to Fo47, gDNA was isolated from the mycelium using phenol:chloroform extraction method as described (van Dam et al., 2018) and PCR was performed using Fo-specific *FEM* and Fo47-specific *SCAR* primers (Edel-Hermann et al., 2011). When PCR reaction was positive for *FEM* and *SCAR* it was considered as Fo47. At harvest time, when endophytic and epiphytic colonization of Fo47 on potato tubers was assessed the same procedure was followed.

Mid-term sampling

Seventy-nine days after tuber inoculation with Fo47, one plant per plot was collected to assess Fo47 colonization. From each plot, the plant from the second column and second row was sampled and stored in cool conditions until further analysis (**Figure S1C**). Fo47 colonization in the mother tuber, the lateral root and a stem emerging from the mother tuber was monitored. The thickest stem emerging from mother tuber was surface sterilized as described above and two cross-sections of stem sections from the basal (region of contact with the tuber) and crown level were placed on PDA plates to assess Fo47 outgrowth. Additionally, one piece of a lateral root was sterilised and incubated on PDA plates to assess fungal outgrowth. Confirmation of Fo47 identity was done by PCR as described above.

Harvest

The field was divided into 646 plots (**Figure S1A, B**). One part of the field was non-fertilized (plots 1-405) while the other was fertilized by applying N and K (plots 406-646) (**Figure S1A, B**). Each plot contained 4 rows of 10 potato plants each (40 plants in total) (**Figure S1C**). A treatment consisted of inoculating nine randomly distributed plots (**Figure S1B**) with 60 ml of water (mock) or Fo47 spores (10^7 spores/ml) to each potato tuber. Only the two central rows of each plot were

harvested. The peripheric two rows were not harvested to avoid the edge effects (**Figure S1C**). The tubers from the two middle rows were used to measure yield, and a subset of those were used to analysed starch content and *Rhizoctonia* disease incidence (**Figure 4A**). Starch content was measured according to the commission regulation (EC) No 2235/2003. All the data was analysed by performing a Mann-Whitney test through the software PRISM 7.0 (GraphPad).

Acknowledgements

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Supplementary material

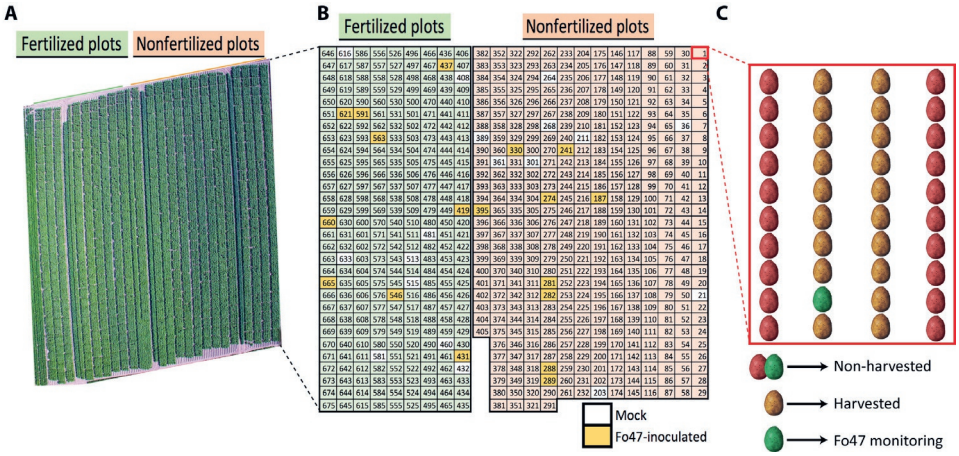

Figure S1. Layout of the field trial depicting mock and Fo47 treatments. (A) Picture of the potato field taken from a drone two months after planting. **(B)** Schematic representation of the field depicting the plots utilized in this study. Water-inoculated (mock) plots are depicted in white while Fo47-inoculated plots are shown in orange. The green plots were fertilized while the brown coloured were not. **(C)** Each plot consisted of 40 potato plants. Biological replicates shown in red were not harvested to avoid edge effects in scoring. The yellow-coloured plants were harvested and yield, starch content and disease were determined. The green-coloured plants were used to monitor Fo47 colonization.

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