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ORIGINAL ARTICLE

# *Trichoderma atroviride* as a promising biocontrol agent in seed coating for reducing *Fusarium* damping-off on maize

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## Abstract

**Aims:** The objective of this work was to identify a fungal strain showing potential biocontrol abilities against two *Fusarium* damping-off agents and to test it as a Biological Control Agent (BCA) in maize seed coating under field conditions.

**Methods and Results:** A collection of native fungal strains associated with maize in Belgium was screened for antagonistic potential against *Fusarium avenaceum* and *Fusarium culmorum*. The strain with highest biocontrol potential was identified as an endophytic *Trichoderma atroviride* BC0584. In greenhouse, it significantly improves the emergence of seedlings infected by *F. avenaceum* or *F. culmorum* pathogens. In most field trials carried out during the season 2017, it significantly increased the emergence rate of infected seedlings compared to untreated seeds. One slurriable powder formulation allows BCA conidia to survive over a 6-month storage period at 4°C.

**Conclusions:** The fungal BC0584 strain is a promising BCA that could be an alternative to synthetic fungicides. It is adapted to local environmental conditions, is easily and cheaply produced and can be stored in a low-cost formulation.

**Significance and Impact of the Study:** In Belgium, this is the first study to use a *T. atroviride* native strain against *Fusarium* damping-off on maize crop. Modes of action and required conditions for ensuring high biocontrol activity in the field have still to be investigated.

## Introduction

In Belgium, maize is the second most important crop after wheat in terms of surface area and is mainly used for animal feed. Eight to nine million tonnes of maize are harvested each year for both forage and grain maize (Belgian Federal Government 2019).

During pre- and post-emergence, maize seeds and seedlings are subjected to external stresses including pests (game, crows or insects) and soil-borne pathogenic fungi. Many *Fusarium* spp. are present in Belgian maize fields, several of these are among maize's main damping-off agents (Kluth and Varrelmann 2010; Maron *et al.* 2011; Scauflaire *et al.* 2011). They may cause huge crop losses if there is no seed treatment. Maize seeds are therefore coated with synthetic fungicides.

Chemical plant protection products can have side effects on living organisms and human health (Saunders and Watkins 2001; Lopez-Antia *et al.* 2015) and a lot of them have been removed from the market. The main actions for the sustainable use of pesticides include reducing their level of application, their amount and finding low-risk substances to use instead (European Directive 2009/128/EC). Developing new efficient and safe alternatives for crop protection is thus necessary, especially for crops of such an extent.

The use of beneficial micro-organisms (notably fungi) seems to be a promising and environmentally sound

method for biological control of phytopathogens. Among fungal Biological Control Agents (BCAs), *Trichoderma* species, in particular *T. viride*, *T. harzianum*, *T. asperellum*, *T. atroviride* and *T. gamsii* are among the most studied (Harman *et al.* 2004; Kakvan *et al.* 2013; Chen *et al.* 2016; Daryaei *et al.* 2016). They control fungal pathogens by different modes of action such as mycoparasitism, competition for space and nutrients, production of inhibitory compounds (antibiosis) and/or indirect stimulation of plant defences (Benítez *et al.* 2004; Verma *et al.* 2007). These BCAs can be used as biofungicides to replace synthetic fungicides in organic farming or even be combined with other pesticides, as the so-called Integrated Disease Management (IDM) strategy prescribes. However, applications are still limited due to the lack of consistency in BCAs when applied in the field (Gerbore *et al.* 2014). In Belgium, only one biofungicide called Trianum-P® (made with *T. harzianum* T-22, Koppert®) is currently available for maize seed protection against damping-off agents (Phytoweb 2019). In order to broaden and benefit from useful strategies, there is a pressing need to identify and develop new BCAs for field crops that are efficient, safe and available at competitive prices for farmers (Butt *et al.* 2001; Keswani *et al.* 2016).

The process of biological seed coating also requires further studies that address the survival of spores related to desiccation conditions; the variable bioactivity in the field; its possible influence on seed germination and the feasibility of production and application (Keswani *et al.* 2016); O'Callaghan 2016; Swaminathan *et al.* 2016).

In this context, this paper focuses on the development of a novel and native fungal BCA, which is able to control maize *Fusarium* damping-off. The aims of the research were (i) to isolate antagonistic fungi from soils located in Belgium and to screen those fungi against Belgian *Fusarium* soil-borne pathogens by *in vitro* antagonistic experiments, (ii) to select and characterize the best strain for further tests, (iii) to check its bioactivity in greenhouse, and (iv) under field conditions and finally (v) to develop a seed coating formulation including this BCA, which can be produced in mass, is applicable in fields, stable enough for the spore to endure shelf life of a 6-month storage period, and is completely adapted to local environmental conditions.

## Materials and methods

### Pathogenic fungi

Five strains of *F. avenaceum* (T001 (MUCL53460), P061 (MUCL57070), R013 (MUCL53458), S111 (MUCL 53459), P002 (MUCL53456)) and five strains of *F. culmorum* (Q004 (MUCL53467), T037 (MUCL53470), P027 (MUCL53466), R064 (MUCL53468), S139 (MUCL53469)) deposited in the BCCM/MUCL Agro-food & Environmental Fungal Collection (UCLouvain, Belgium) were used in the assays. They were all isolated from maize rhizosphere in 2007 in Belgium by Scauflaire *et al.* (2011). The T001 and Q004 strains were used as benchmark as they showed ability to cause damping-off of maize under *in vitro* conditions.

### Fungal culture conditions

Fungal cultures are stored in tubes on potato dextrose agar (PDA, Oxoid®) at ambient temperature in the dark and filled with paraffin. For assays, they were subcultured on PDA plates at room temperature (20–21°C) in the dark. For mass production and seed treatment, the BCA fungus was grown in Erlenmeyer on solid substrate made with sterile humid wheat bran during at least 6 days in a natural day/night cycle at room temperature (protocol adapted from Cavalcante *et al.* 2008).

### Isolation, screening and selection of the BCA

In this study, more than 900 fungi were isolated from 1800 samples of soil, and of maize collar and roots in the early stages of the crop (in 2012 and 2013). Isolates came from 10 various maize fields located in the Southern part of Belgium. More than 200 other fungal strains previously collected in Belgian maize fields were added to the collection. Isolates were subsequently (i) identified at the genus level, (ii) tested against *F. avenaceum* or *F. culmorum* in direct confrontations and (iii) tested for their potential to produce lytic enzymes (cellulases, chitinases and proteases) at 25°C under *in vitro* conditions (Supporting Information). The inhibition rate of the pathogen growth by the antagonist at 25°C (Hmouni *et al.* 1996), the interaction type between the potential BCA and the pathogen, as well as the ability of the potential BCA to produce enzymes were used to determine a total estimated value of the antagonistic activity of each strain (Supporting Information). A ranking of the 40 best strains was subsequently established (Supporting Information). The abilities of these strains to grow or to conidiate and to control pathogens at lower temperatures (15, 10 and 5°C) were also taken into consideration when selecting the BCA (Supporting Information). The best strain was then selected for further experiments.

### Characterization of the selected BCA

*Molecular identification*

DNA of the BCA was extracted according to the manufacturer's protocol recommended by the InnuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). Universal ITS5

(5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers were used for the amplification of ITS regions in the ribosomal DNA (White *et al.* 1990). The extracted DNA was diluted 10 times in the PCR mix which was made with forward and reverse primers (2% each), deoxyribonucleotides (3%), *Go Taq* enzyme (0·5%), *Go Taq* buffer (20%), magnesium chloride (10%) and diethyl pyrocarbonate (DEPC) water (53%). The DNA was then amplified by PCR through an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1·5 min at 56°C, 2 min at 72°C and ended at 72°C during 10 min, using MJ Mini™ thermal cycler. Products were highlighted using a 1·2% agarose gel. Sequencing of purified PCR products was undertaken by the Macrogen Company (Amsterdam, The Netherlands). The fungal sequence was subsequently aligned and corrected by the Sequencher 4.8® software (Gene Codes Corporation, Ann Arbor, MI) before being identified with the standard nucleotide Basic Local Alignment Search Tool (BLAST®) on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (U.S. National Library of Medicine, 2016).

*Antagonism activity against pathogenic strains*

The selected BCA was directly confronted with five strains of *F. avenaceum* (T001, P061, R013, S111 and P002) and with five strains of *F. culmorum* (Q004, T037, P027, R064 and S139) in order to highlight the ability of the BCA to control the mycelial development of 10 *Fusarium* pathogenic strains. Agar discs of 6 mm diameter were cut from actively growing colonies of antagonists or pathogens. For each pathogen–BCA combination, the agar discs were put mycelium side down at a distance of 45 mm on Petri dishes (85 mm diameter) containing PDA medium (three replications). They were incubated at 15°C in a day/night cycle (12 h/12 h). Controls consisted in growing the pathogen only in one side of the Petri dish. Radius of the pathogen was measured when the mycelial growth was stabilized. The inhibition rate of the pathogenic growth by the BCA (I%) was determined as (Hmouni *et al.* (1996)) (1):

$$I\% = \left(1 - \frac{\mathrm{Rp}}{\mathrm{Rc}}\right) \times 100 \qquad (1)$$

where *Rp* is the radius of the pathogen in confrontation with the BCA and *Rc* the radius of the pathogen growing alone (control).

### *Production of enzymatic compounds under* in vitro *conditions*

Three specific media were used to reveal the production of proteases, cellulases and chitinases. These were, respectively, skimmed milk agar (skimmed milk powder 30 g $l^{-1}$ and agar 20 g $l^{-1}$), carboxymethylcellulose agar (carboxymethylcellulose-Na 10 g $l^{-1}$, NaCl 2 g $l^{-1}$, peptone 0·2 g $l^{-1}$, $KH_2PO_4$ 1 g $l^{-1}$ and agar 17 g $l^{-1}$) and colloidal chitin agar (chitin 10 g $l^{-1}$ previously digested in HCl, $NH_4H_2PO_4$ 1 g $l^{-1}$, KCl 0·2 g $l^{-1}$, $MgSO_4.7H_2O$ 0·2 g $l^{-1}$ and agar 20 g $l^{-1}$). Active colonies of the BCA were inoculated in the centre of each specific medium at 20°C in the dark. There were three replicates per treatment. The ratio between the size of the halo around the colony and the colony diameter was measured after 3 days (or 1 month for the colloidal chitin agar). Diluted lugol (KI 6·66 g $l^{-1}$ and $I_2$ 3·33 g $l^{-1}$) was used to reveal the halo caused by cellulose degradation. The experiment was carried out twice.

### *Biocontrol activity of volatile compounds under* in vitro *conditions*

The BCA was indirectly confronted with *F. avenaceum* T001 and *F. culmorum* Q004 using a protocol based upon the work of Castillo *et al.* (2011). Two Petri dishes filled with PDA medium were inoculated with agar disc (6 mm diameter) of the BCA growing colonies at the centre of the lower dish and with agar disc of one *Fusarium* strain at the centre of the upper one. Petri dishes were joined together, sealed several rounds with Parafilm and incubated at 15°C. Control consisted in growing the pathogen alone. The diameter of the pathogenic colonies growing in contact with the BCA was measured and compared to the diameter of the control in order to set the inhibition rate of pathogen mycelial growth by the biocontrol strain (Formula 1). Diameters were measured when the control covered the edge of the plate. Each BCA–pathogen combination was replicated four times. The experiment was carried out twice.

### *Biocontrol activity of soluble compounds under* in vitro *conditions*

Two Erlenmeyers filled with 50 ml of potato dextrose broth (PDB, Oxoid®) were each inoculated with three agar discs (6 mm diameter) of the BCA active colonies. They were incubated during 12 days in the dark at 22°C with a regular agitation of 125 rev $min^{-1}$. The fungal culture of each Erlenmeyer was centrifuged for 4 min at 4000 rev $min^{-1}$, and the obtained supernatant was then filtered through a syringe filter of cellulose acetate (0·20 $\mu$m, VWR®) and four times concentrated. Afterwards, 100 $\mu$l of the filtrate was homogenously spread on a Petri dish filled with PDA and chloramphenicol (0·2 g $l^{-1}$) with a glass rod. One plug of the pathogenic fungal strain (*F. avenaceum* T001 or *F. culmorum* Q004)

was put in the centre of the dish. Dishes were incubated at 20°C in the dark. Controls consisted in spreading pure sterile PDB and inoculating the pathogenic strain. Each culture medium replicate was spread twice on Petri dishes in order to have four replicates per treatment. The inhibition percentage of pathogenic fungal growth was established when the controls covered the whole dish (Formula 1). The experiment was repeated twice.

### Biocontrol activity of the BCA under greenhouse and endophytic behaviour

*In planta bioassays*

The inoculum of *F. culmorum* Q004 was produced by growing the fungus on polenta agar medium (polenta 40 g $l^{-1}$ and agar 15 g $l^{-1}$) in Petri dishes during 15 days at 20°C in the dark. The inoculum of *F. avenaceum* T001 was produced on sterile humidified wheat bran (1 : 2 w/v) in an Erlenmeyer during 10 days at 22°C in the dark. Spores of the BCA were also produced on sterile humidified wheat bran in an Erlenmeyer during 15 days at ambient temperature before coating the seed. On the day before sowing maize seeds, spores were harvested with water amended with Tween® 20. The suspension concentration was set by using a haemocytometer (Thoma cell®). On the same day, the suspension was subsequently added to a basic seed coating formulation (F1 in the Table 1) made with methylcellulose 2% (Merck®) in order to have $10^4$ viable spores on each seed. Coated seeds were dried overnight in a horizontal laminar flow.

The following day, the pots were inoculated and the seeds sown. Sterile perlite and vermiculite (1 : 1 v/v) were mixed with water and the pathogen (either with agar medium colonized by *F. culmorum* Q004, or with wheat bran colonized by *F. avenaceum* T001 at 1% w/w). At the same time, 10 maize seeds (Troizi variety from Caussade®), which were previously surface-disinfected, were sown per pot (3–4 cm deep). Negative control consisted in sowing non-coated seeds in non-inoculated pots. Pots were incubated at 15°C (in a 12 h day/12 h night cycle). They were watered with a 10-fold diluted Hoagland solution ($Ca(NO_3)_2.4H_2O$ 0·25 l, $KNO_3$ 0·25 l, micro-elements 0.1 l, Fe EDTA 0.1 l, $MgSO_4.7H_2O$ 0.1 l, $KH_2PO_4$ 0·05 l and deionized water 9·15 l). Each treatment was repeated in four pots. The emergence rate and the height of seedlings were recorded for each pot 20 days after sowing at the three- or four-leaf stage.

*Endophytic behaviour*

The roots of the maize seedlings coming from the experiment described above were studied (protocol adapted from Amkraz *et al.* 2010). For non-inoculated pots (without any pathogen) sown with untreated seeds and BCA-coated seeds, three seedlings (out of 10) were selected from each pot replicate (four replicates). For each treatment and for each seedling (coming from untreated seeds and from BCA-coated seeds), portions of root were surface-disinfected and placed on water agar medium amended with chloramphenicol (0·2 g $l^{-1}$). For each treatment, 12 Petri dishes inoculated with root portions were incubated at ambient temperature.

Ten days after inoculation, fungal mycelium and spores which had colonized the medium were harvested. DNA was extracted according to the CTAB (cetyl trimethylammonium bromide) method. Extracted DNA was quantified (from 100 to 500 ng $\mu l^{-1}$). Specific primers (forward: 5′-CTT GAT GAA ATC ACG GTG ACC-3′ and reverse: 5′-CAA TTG CAT CGT CTT CTC CAT C-3′) which were designed for *T. atroviride* species were used to amplify the translation elongation factor 1-alpha. Extracted DNA was 10 times diluted in the PCR mix which was made with forward and reverse primers (2% each), deoxyribonucleotides (3%), *Go Taq* enzyme (0·5%), *Go Taq* buffer (20%), magnesium chloride (10%) and diethyl pyrocarbonate (DEPC) water (53%). DNA was amplified by PCR through an initial denaturation

**Table 1** Four seed coating formulations (F1, F2, F3 and F4) with their composition (matrix, additives, spore type, spore quantity) and the respective ratio of coating matrix volume (ml) on seed weight (g)

| Formulation | Coating type | Matrix | Additive(s) | Spore type and quantity | Ratio matrix/seeds |
|---|---|---|---|---|---|
| F1 | Film coating | Methylcellulose 2% w/v | – | Fresh spores<br>$>10^4$ CFU* per seed | 3 : 10 v/w |
| F2 | Film coating | Methylcellulose 1% w/v | Skimmed milk 10% w/v | Fresh spores<br>$>10^4$ CFU per seed | 3 : 10 v/w |
| F3 | Film coating | Methylcellulose 2% w/v | Sucrose 2% w/v<br>Calcium carbonate 1% w/v | Fresh spores<br>$>10^4$ CFU per seed | 3 : 10 v/w |
| F4 | Slurry coating | Deionized water | Talc 80% w/w<br>Wheat bran 18% w/w<br>Calcium carbonate 2% w/w | Dry spores<br>$>10^5$ CFU per seed | 1 : 25 v/w |

*Colony forming units corresponding to germinated spores.

step of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C and 40 s at 72°C, and ended at 72°C during 10 min, using MJ Mini™ thermal cycler. The products were highlighted using a 1·2% agarose gel. The percentage of seedlings that had been colonized by the fungal BCA inside of roots was established. The experiment was performed twice.

### Seed coating formulations with the BCA and spore shelf life during storage

Four seed coating formulations (F1, F2, F3 and F4) were tested for their ability to maintain high viability of BCA spores at three storage temperatures (Table 1). On the day coating was performed, fresh spores intended for formulations F1, F2 and F3 were collected, using sterile water amended with Tween® 20, from wheat bran culture. For formulation F4, the culture medium containing spores was air-dried overnight under laminar flow hood at room temperature on the day before coating. On the coating day, those dry spores were sifted (0·5 mm) to obtain a homogeneous dry powder.

After surface disinfection and drying, maize seeds (Troizi variety) were coated by hand with each formulation, among which the formulation F1 has been used as a reference (gelling matrix of methylcellulose (MC) 2% without any additive) (Table 1). Formulations F2 and F3 were made with matrix of MC 1 or 2% and additives (skimmed milk 10%, sucrose 2% or calcium carbonate 1%). Formulation F4 consisted of talc (80%), calcium carbonate (2%) and the homogeneous dry spores with wheat bran (18%). This slurriable powder was added at a rate of 0·4 g $ml^{-1}$ to deionized water (formulation adapted from Singh *et al.* 2014 and Sabaratnam and Traquair 2002). The matrix and additives of formulations F2, F3 and F4 were chosen based on preliminary assays showing spore ability to survive in seed coating during a 6-month storage period at 4°C (data not shown). After the mixture of matrix and seeds, all coated seeds were left to dry overnight in a horizontal laminar flow. Each formulation was stored at 4°C, 15°C and ambient temperature (20–21°C) in the dark in Petri plates. There were 12 treatments.

Spore viability in seed coating was assessed several times over a 6-month storage period. For each treatment and for each assessment time, two sets of three coated seeds were diluted in 9 ml of deionized water added with Tween® 20. These solutions were subsequently $10^2$- to $10^3$-fold diluted. For each set, 100 $\mu$l of diluted solution was spread twice on PDA plates with a glass rod. There were four replicates for each treatment. PDA dishes were then incubated at ambient temperature for 2 days until germinated spores were visible. Germinated conidia were counted. They correspond to viable spores or colony forming units (CFU). At each trial period (24, 49, 66, 94, 122, 153 and 179 days after coating), the relative rate of CFU ($CFU\%_t$) was determined as (2):

$$CFU\%_t = \frac{CFU_t}{CFU_i} \times 100 \quad (2)$$

where $CFU_t$ is the amount of CFU per seed at the trial day and $CFU_i$ the initial amount of CFU per seed at the day after coating. The assessment of spore viability in each treatment was ended when it decreased below 1%.

### BCA biocontrol activity under field conditions

Field trials were carried out early in the maize growing season (mid-April 2017). Four fields were selected for being damping-off disease prone. They were located in the Southern part of Belgium in Vieusart, Saint-Symphorien, Marneffe and Neufchâteau. Two maize varieties were used for their sensitivity to fungal damping-off: Troizi (Caussade®) and Torres (KWS®). All seeds were previously coated with the crow repellent Mesurol FS 500® (500 g $l^{-1}$ methiocarb; Bayer CropScience). This first methiocarb coating did not show toxicity on BCA growth under *in vitro* and *in vivo* conditions in our preliminary experimentations (data not shown). Seeds coated with one unique repellent coat were used as negative control. After the first repellent coating, seeds were secondly coated with BCA spores by hand: the formulation F1 on Troizi and Torres varieties, and formulations F2, F3 and F4 on the Troizi variety solely. For all formulations, each coated seed had at least $10^5$ CFU per seed. To prove the fungal damping-off pressure in the field, a treatment with chemical fungicide Flowsan FS® fungicide (533 g $l^{-1}$ thiram, Taminco B.V.B.A.) was also used as a second coating in the four tested fields. Seeds were air-dried overnight. Coated seeds were kept in a fridge (4°C) between 1 and 9 days before sowing.

Four replicates of nine plots (corresponding to the 9 treatments) were set up in complete randomized block design in each field. Each plot was made up of four rows (48 seeds per row). The number of plants emerged in the two inner rows of each plot were counted at the six- or seven-leaf stage. Biocontrol efficiency of the BCA (BCE%) was determined as (3):

$$BCE\% = \left(\frac{E}{E_0} - 1\right) \times 100 \quad (3)$$

where $E$ is the number of emerged seedlings in BCA-inoculated plots and $E_0$ the average number of emerged seedlings in untreated plots. At the end of the growing season, the average dry yield (tonnes per hectare) per treatment was also assessed.

### Statistical analysis

The inhibition rates, the ratio of halo/colony diameter, the emergence rate of seedlings, the seedling height, the BCA biocontrol efficiency and the dry yield were analysed using ANOVA with the statistical software JMP®. The analysis used fungal strain, enzyme and maize seed conditions (variety, formulation, field) as main factors. The means of all data were compared through the Student's *t* test with a probability threshold of 5% ($\alpha = 0{\cdot}05$). For the assessment of biocontrol efficiency in field trials, the means of data were compared to the negative control (untreated plots) through the Dunnett test with a probability threshold of 5% ($\alpha = 0{\cdot}05$).

## Results

### Isolation, screening and selection of the BCA

All rhizospheric fungal isolates were morphologically identified at the genus level. More than 26 fungal genera were detected. *Trichoderma*, *Fusarium* and *Bionectria* were the three most common genera found in the maize rhizosphere and they represented more than 75% of all isolated strains (data not shown). In direct confrontation tests under *in vitro* conditions, both *Trichoderma* and *Penicillium* genera showed high antagonistic activities at 25°C (data not shown). Many isolates from the genera *Bionectria*, *Coniothyrium*, *Eutypella*, *Mortierella*, *Mucor*, *Torrubiella* and *Trichoderma* produced chitinases (data not shown). Many isolates from the genera *Aureobasidium*, *Bionectria*, *Eutypella*, *Hypholoma*, *Pseudozyma* and *Torrubiella* produced cellulases (data not shown). Finally, many isolates from the genera *Aureobasidium*, *Hypholoma*, *Pseudozyma* and *Torrubiella* produced proteases (data not shown).

A ranking of the 40 strains showing highest biocontrol activities under *in vitro* conditions was established (Table S1). *Trichoderma* is the most frequent genus presented in this ranking with a high rate of pathogen inhibition rising to over 90% at 25°C due to fast mycelial growth of the fungus. Direct confrontations at lower temperatures (15, 10 and 5°C) have brought additional information about the ability of such strains to control *Fusarium* development in suboptimal conditions. Some strains listed in the ranking are not even able to inhibit pathogen development at lower temperatures while others can.

The first strain in the ranking has the highest total estimated value of antagonistic activity and corresponds to the BC0584 strain (Table S1). It was isolated in 2012 from maize roots in a field located in Naast, Wallonia, Belgium. The inhibition rate of *F. avenaceum* development by BC0584 is 97, 74, 67 and 63% at 25, 15, 10 and 5°C, respectively (Table S1). When confronted to *F. culmorum*, the BC0584 inhibited the pathogenic growth by 97, 71, 72 and 56% at 25, 15, 10 and 5°C, respectively. The antagonistic fungus invades pathogenic mycelium and produces chitinases in a moderate way at 25°C. In addition, this strain grows and produces conidia easily, quickly and in high quantities. Therefore, BC0584 is considered as a potential BCA against both *Fusarium* species and has been used for further experiments in this paper.

### Characterization of the BCA

Based on the sequence of nuclear ribosome regions ITS1-5.8S-ITS2, the BC0584 strain was identified as a *Trichoderma atroviride* P. Karst. 1892 species (MBC8718) (synonym *Hypocrea atroviridis* Dodd, Lieckf. & Samuels 2003) with 100% homology. At 15°C, the mycelial growth of four out of five *F. avenaceum* strains decreased by at least 70% by the confrontation of BC0584 (Fig. 1a). Concerning the five *F. culmorum* strains, BC0584 slowed down their growth at a rate higher than 75% (Fig. 1b). BC0584 produced a lysis halo in the three tested media, revealing the production of cellulases, proteases and chitinases (Fig. 1c). In the three cases, the halo size was smaller than the colony (ratio < 1). Through the production of BC0584 compounds, the mycelial growth of *F. avenaceum* T001 significantly decreased when the pathogen was exposed to BC0584 volatile (at 15°C) and soluble (at 20°C) compounds ($P < 0{\cdot}001$) (Fig. 1d). Conversely, soluble compounds of BC0584 did not significantly affect the mycelial growth of *F. culmorum* Q004 (Fig. 1d). The growth of *F. culmorum* Q004 is barely slowed down when confronted to BC0584 volatile compounds (Fig. 1d). The thickness of the mycelial mat was thinner for pathogens having indirect contact with BCA volatile compounds than that of pathogens growing alone (Fig. 1e,f).

### Biocontrol activity of the BCA under greenhouse and endophytic behaviour

Maize seeds were coated with the BCA or non-coated. They were sown in pots inoculated or not with the pathogen. Twenty days after sowing, the emergence rate of *Fusarium*-infected seedlings was significantly higher when the seeds were coated with BC0584 for pots infected by *F. avenaceum* or by *F. culmorum* ($P < 0{\cdot}001$) (Fig. 2a). Seedlings coming from seeds coated with BC0584 and growing in *F. culmorum*-infested pots had also a significant higher height compared to non-coated seeds ($P < 0{\cdot}001$) (Fig. 2b). For *F. avenaceum*-infected

plants, the height of seedlings coming from coated and non-coated seeds was not significantly different (Fig. 2b). Unlike *Fusarium* infection, coating seeds with BC0584 alone did not cause any symptoms of necrosis, neither on roots nor on the collar.

After cultivating some root fragments coming from pots without pathogens *in vitro*, no fungus has grown in dishes incubated with roots coming from non-coated seeds (Fig. 2c). Conversely, 100% (12 out of 12) of the roots coming from BC0584-coated seeds were visually infected by *Trichoderma*

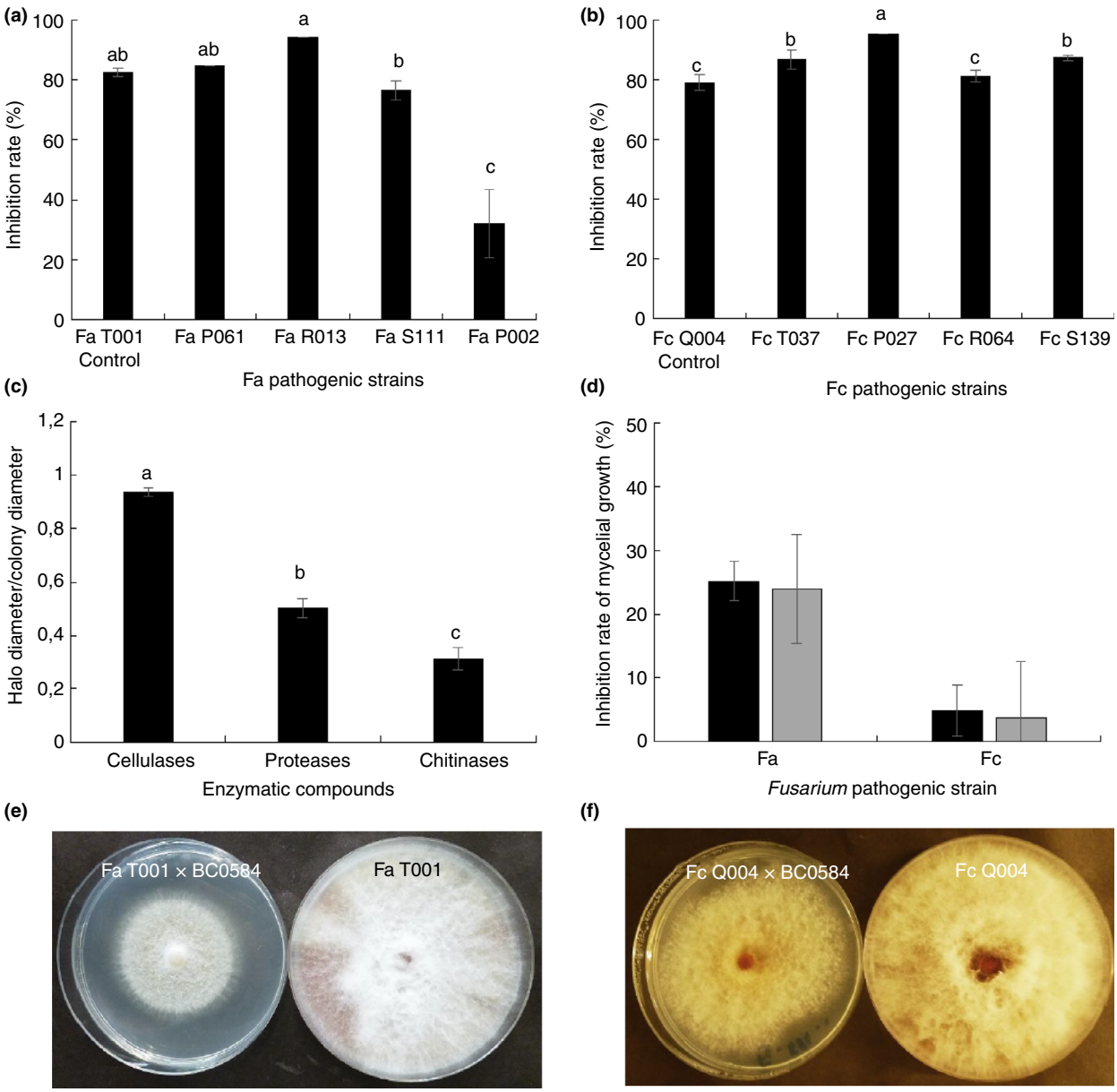


**Figure 1** Characteristics of the BC0584 strain. Inhibition rate of mycelial growth of (a) five *Fusarium avenaceum* (Fa) strains (T001, P061, R013, S111 and P002) or (b) five *Fusarium culmorum* (Fc) strains (Q004, T037, P027, R064 and S139) by direct confrontation with BC0584 at 15°C on potato dextrose agar medium. (c) Ratio between the degradation halo size and the fungal colony diameter caused by the production of enzymes (cellulases, proteases and chitinases) by BC0584 on specific media at 20°C. (d) Inhibition rate of mycelial growth of *F. avenaceum* T001 and *F. culmorum* Q004 by volatile (■) and soluble (□) compounds produced by BC0584 on potato dextrose agar medium. Vertical bars represent SD. Sticks with different letters are significantly different (Student's *t* test, $\alpha = 0{\cdot}05$). Mycelial mat of (e) *F. avenaceum* T001 and (f) *F. culmorum* Q004 growing alone (right) and indirectly growing with volatile compounds of BC0584 (left).

fungus. The specific detection of *T. atroviride* was confirmed by PCR for all replicates of collected fungal materials.

### Seed coating formulations with the BCA and spore shelf life during storage

The viability of spores was assessed for four formulations F1, F2, F3 and F4 (Table 1) that were stored at 4°C, at 15°C and at ambient temperature (20–21°C). At ambient temperature, the rate of viable spores of formulations F1, F2 and F3 rapidly decreased from 100% to 4·5 ± 2·8%, 5·3 ± 1·0% and 26·3 ± 6·8%, respectively, 24 days after coating (Fig. 3). The viability of these three formulations has then continued to fall rapidly until the death of all spores, 3 months later. Conversely, at ambient temperature, spore viability of F4 increased from 100% to 221 ± 28·0% 24 days after coating. Then, F4 spore viability fell to 1·9 ± 0·3% 49 days after coating and still decreased thereafter. When the spores were stored at 15°C, spore viability of F1, F2 and F3 moderately slowed down from 100% to 25·9 ± 4·1%, 32·4 ± 3·9% and 39·1 ± 5·6%, respectively, 24 days after coating. In these conditions, spore viability in F1 and F2 still continued to decrease to 8·0 ± 6·3% and to 12·2 ± 4·0%, respectively, 2 months after coating. Thereafter, the viability decreased until a very low level. At 15°C, spores in the formulation F3 had a slower viability decrease and reached 8·9 ± 2·2% 6 months after coating. As for the ambient storage temperature, spore viability of F4 stored at 15°C has increased from 100% to 121·4 ± 15·6%, 24 days after coating, and was still higher than 100% 2 months after coating. Subsequently, it slowly decreased to 5·1 ± 1·6% over the next 4 months. Spore survival was generally higher at 4°C than at 15°C or at ambient temperature. At 4°C, with formulation F1, F2 and F3, viability decreased from 100% to 34·3 ± 16·1%, 41·2 ± 13·2% and 53·1 ± 27·8%, respectively, 24 days after coating. After 6 months, the viability was still at 15% minimum for formulations F1 and F2. 51·7 ± 4·0% of the spores from the formulation F3 were still viable six months after coating. At that temperature, spore viability in formulation F4 increased during 4 months (above 100%) after coating. This formulation was rather stable over this 6-month period and 84·1 ± 14·3% of coated spores were still viable at the end of the trial.

### Biocontrol activity of the BCA under field conditions

In Vieusart and in Marneffe fields, the number of emerged seedlings inoculated with the BC0584 fungus alone (F1) was not significantly different from untreated plots for both maize varieties Troizi and Torres (Fig. 4 a–c,d). In these conditions, the BCA had no significant effect on raising the emergence rate of infected seedlings.

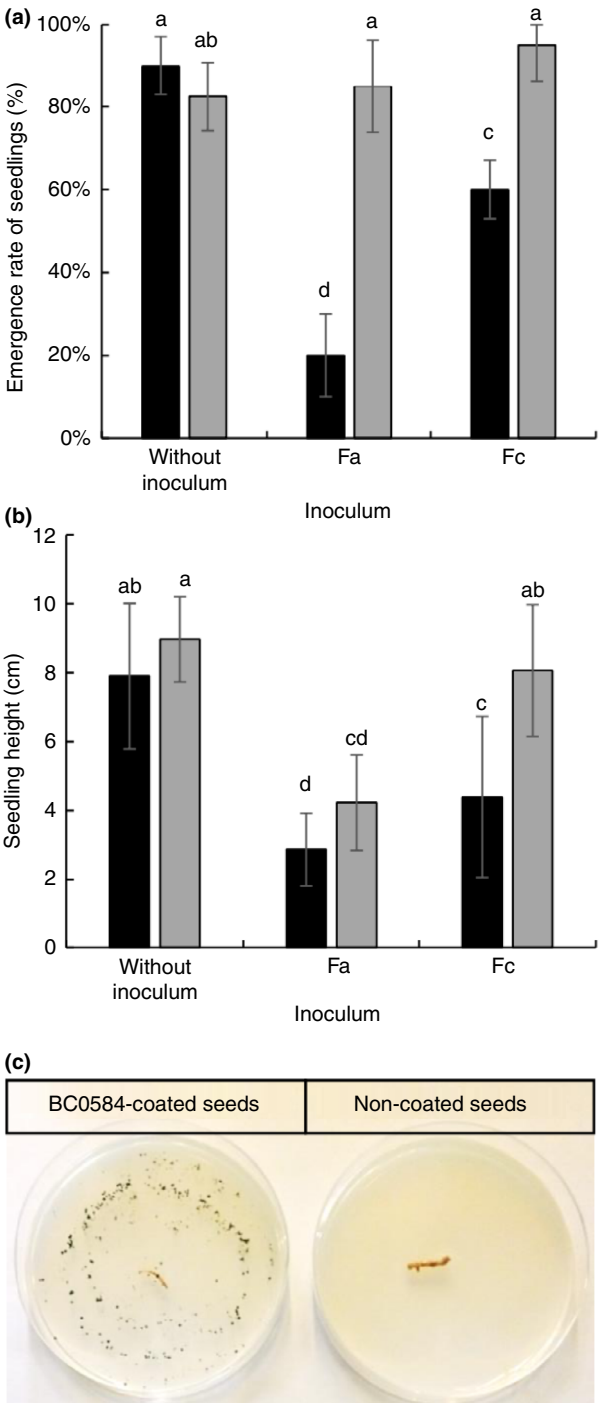


**Figure 2** *In planta* biocontrol activity of maize Troizi seeds that have not been coated (■) or have been coated with BC0584 (▭), and grown in non-inoculated pots (without inoculum, ▭) or in pots inoculated with *Fusarium avenaceum* T001 (Fa) or *Fusarium culmorum* Q004 (Fc) (◈) at 15°C in a 12h day/12h night cycle. The (a) emergence rate of seedlings and (b) their height were established for each treatment 20 days after sowing. Vertical bars represent SD. Sticks with different letters are significantly different (Student's *t* test, $\alpha = 0{\cdot}05$). (c) Root pieces of seedlings emerged from BC0584-inoculated seeds (left) and from non-inoculated seeds (right).

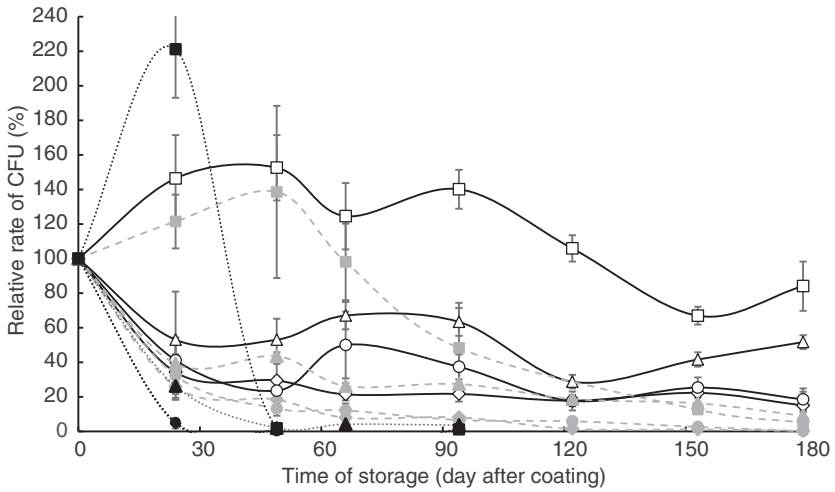


**Figure 3** Evolution of the rate of BC0584 conidia viability (colony forming units) on coated seeds during a 6-month storage period for four seed coating formulations that have been stored at 4, 15 and 20–21°C: F1 at 4°C (◇), F1 at 15°C (△), F1 at ambiant temperature (♦); F2 at 4°C (○), F2 at 15°C (◍), F2 at ambiant temperature (●); F3 at 4°C (Δ), F3 at 15°C (▢), F3 at ambiant temperature (▲); F4 at 4°C (□), F4 at 15°C (▣), F4 at ambiant temperature (■). Vertical bars represent SD.

In Saint-Symphorien, the treatment with BC0584 (F1) did not increase the emergence of Troizi seedlings, while this treatment had a significant positive impact on Torres seedlings ($P < 0{\cdot}001$). In Neufchâteau, the BCA allowed a significant increase in the emergence rate of both Troizi and Torres seedlings ($P = 0{\cdot}045$ and $P = 0{\cdot}032$, respectively).

Regarding the BCE of coating formulations on Troizi variety, there was no significant difference of BC0584 BCE between the four formulations (Fig. 4b). The effects of formulation and maize variety on the dry yield were assessed for all fields except for Neufchâteau due to technical reasons (Fig. 5). Dry yield was not significantly impacted by the coating treatment (negative control, positive control or maize inoculated with BC0584) on the Troizi variety in all fields. However, yield varied depending on the field and on the harvest type (grain or forage maize) from 10 to 20 T $ha^{-1}$. In contrast, yield of the Torres variety, which is known to be more susceptible to damping-off fungal agents, was affected differently depending on the coating treatments. Dry yield of the fungicide treatment with thiram of Torres variety was significantly higher than untreated seeds and BCA-treated seeds in Vieusart. The same scenario was observed in Marneffe for the chemical treatment on Torres variety compared to untreated seeds and BCA-treated seeds. In Saint-Symphorien, chemical treatment and the BCA-treated plants significantly increased dry yield compared to untreated plots on Torres variety.

## Discussion

Biocontrolling soil-borne plant pathogens is becoming an important strategy to support sustainable agriculture. Those pathogens are notoriously difficult to manage but particular strains, species or genera of micro-organisms such as rhizobacteria, pseudomonads or soil-borne fungi can considerably mitigate infectious root diseases notably by producing antifungal antibiotics, by inducing systemic resistance into the plant or by interfering with pathogenicity factors (Cliquet and Scheffer, 1996; Haas and Défago, 2005).

This paper illustrates how we carried out the screening and the subsequent selection of one native biocontrol agent—*T. atroviride* BC0584—among more than 1100 soil-borne fungi isolated from maize fields in Belgium. This strain is therefore expected to be fully adapted to the local pedoclimatic conditions, to develop in the maize rhizosphere and to control *Fusarium* soil-borne pathogens causing damping-off of maize. Ensuring a high production of inoculum, a long survival period for conidia during storage and a sufficient biocontrol activity for a wide-scale use of BCA were also among the purposes of this research.

Results obtained from tests of various soil-borne fungal isolates indicated that BC0584 had the greatest antagonistic activity against one *F. avenaceum* and one *F. culmorum* strain under *in vitro* conditions. In addition, it was a fast-growing and a highly sporulating strain, which gives it advantages as a potential BCA. *T. atroviride*, a very common species found in European soils, is already well known for its biocontrol abilities in the scientific literature and on the market of plant protection products (Hebbar and Samuels 2015). Specific *in vitro* tests revealed that it produces some cell-wall-degrading enzymes (cellulases, proteases and chitinases), some

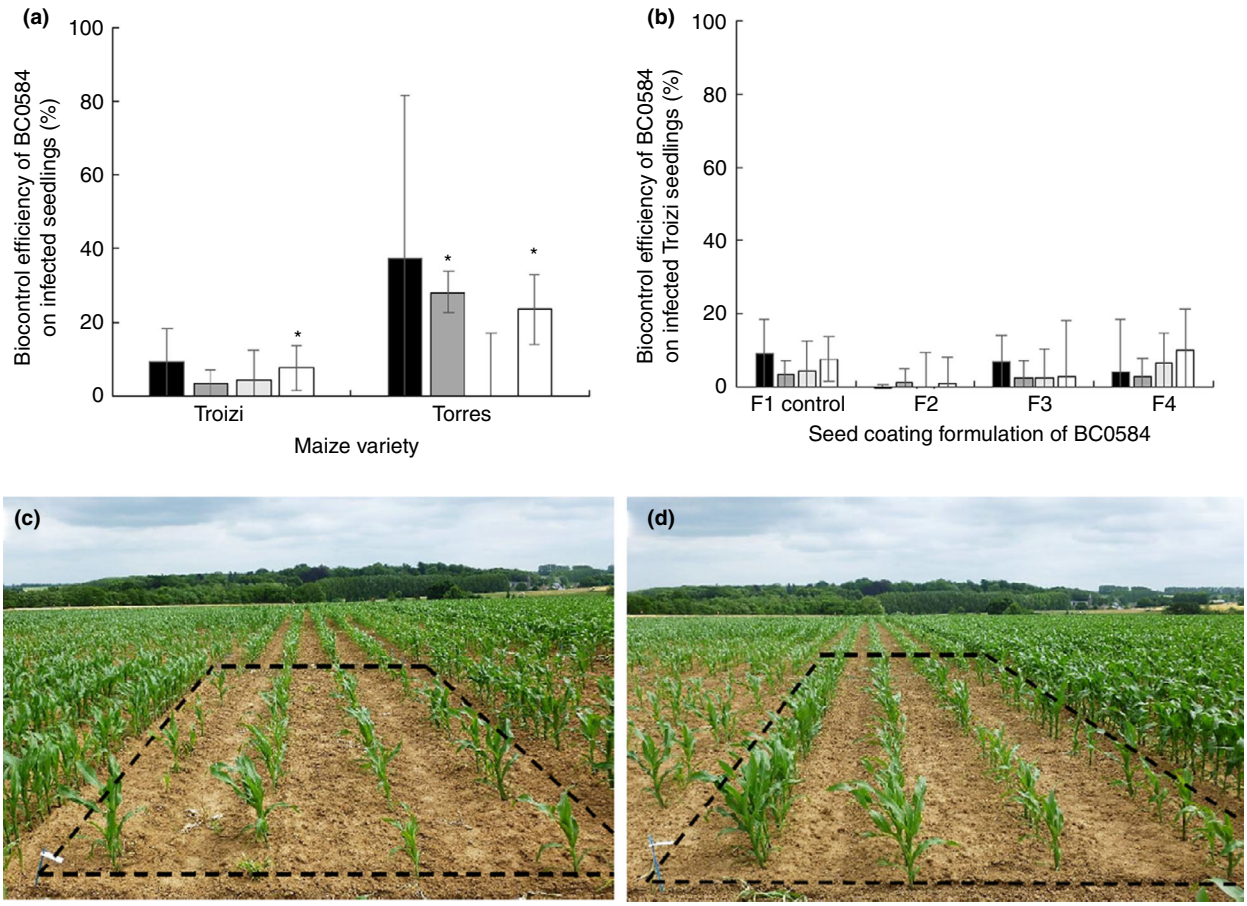


**Figure 4** Emergence of seeds and first developments of maize seedlings under field conditions in 2017. (a) Biocontrol efficiency of the coating formulation F1 with BC0584 compared to untreated treatment in four Belgian fields (Vieusart (■), Saint-Symphorien (▨), Marneffe (◈) and Neufchâteau (□)) for two maize varieties (Troizi and Torres). (b) Biocontrol efficiency of the coating formulations F1 (control), F2, F3 and F4 with BC0584 compared to untreated treatment in four Belgian fields (Vieusart (■), Saint-Symphorien (▨), Marneffe (◈) and Neufchâteau (□)) for the Troizi maize variety solely. Vertical bars represent SD. Sticks with stars are significantly different from the untreated control (Dunnett test, $\alpha = 0{\cdot}05$). Infected plot located in Vieusart with Torres maize variety at the 11-leaf stage with (c) only Mesurol® (methiocarb) treatment and (d) with both Mesurol® (methiocarb) and F1 (BC0584 spores) treatments.

volatile compounds and some soluble growth inhibitors that limit the mycelial growth of *F. avenaceum* T001. This production could be correlated to modes of action against pathogenic *Fusarium* species such as mycoparasitism and antibiosis. Depending on the culture medium, *T. atroviride* is effectively able to produce endochitinases and endo-β-1,3-glucanases detectable by specific enzyme activity assays (Thrane *et al.* 2000; Rocha-Ramirez *et al.* 2002). Oh *et al.* (2000) have identified the production of three peptaibols—atroviridins A, B and C—by *T. atroviride* that could be implied in biocontrol mechanisms. Lehner *et al.* (2013) highlighted the production of 14 siderophores such as coprogen, fusigen, ferricrocin, fusarinin and dimerum acid by three *T. atroviride* strains. This shows the ability to withdraw important elements from the environment that are not readily available, such as iron. Another strain of *T. atroviride*—*T. atroviride* P1—is also known to produce 6-n-pentyl-6H-pyran-2-one volatile compound that shows stimulation of plant defences against *Botrytis cinerea* on pea (Vinale *et al.* 2008). Other secondary metabolites are also probably produced by BC0584, as scientific literature refers to a multitude of compounds for different strains of *T. atroviride*. For further investigation, gas or liquid chromatography coupled to mass spectrometry would help the identification of volatile and soluble organic compounds, respectively (Vinale *et al.* 2008; Chen *et al.* 2016; Saravanakumar *et al.* 2016). Our tests also revealed the systematic endophytic behaviour of the strain towards the maize roots. Hosting a fungal endophyte could help the plant withstand abiotic stresses as well as biotic stresses (such as pathogens) by the production of secondary metabolites

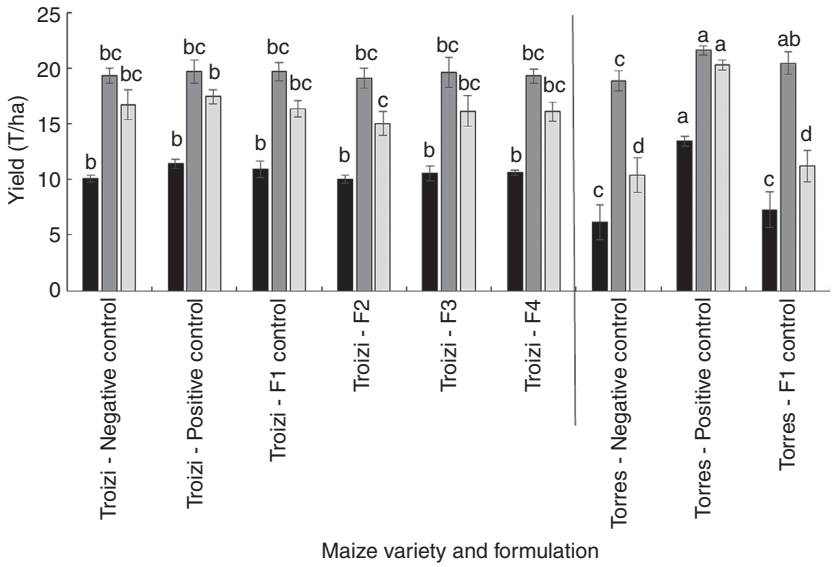


**Figure 5** Dry yield (in tons per hectare) of grain maize (Vieusart field (■)) and forage maize (Saint-Symphorien (▩) and Marneffe (◈) fields) at harvest for two maize varieties (Troizi and Torres) and for untreated seeds (negative control), seeds treated with fungicide thiram (positive control), seeds treated with BC0584 spores in the formulation F1, F2, F3 and F4 under field conditions in 2017. Vertical bars represent SD. Sticks with different letters are significantly different (Student's *t* test, $\alpha = 0{\cdot}05$).

(O'Callaghan 2016). It suggests specific interactions between the fungus and the plant. Guzmán-Guzmán *et al.* (2017) highlighted that some *T. atroviride* strains expressed genes coding for effector-like proteins during the interaction between *Rhizoctonia solani* and *Arabidopsis thaliana*. Studying and understanding the specific modes of action involved between the BCA, the pathogen and the plant are therefore essential as they could optimize bioactivity conditions and avoid the emergence of resistant pathogens (Butt *et al.* 2001).

Greenhouse experiments of this study clearly showed the positive effect of BC0584 on the emergence rate of *Fusarium*-infected seedlings (Troizi variety) and to a lesser extent, on their height. BC0584 is therefore a biocontrol agent of one *F. avenaceum* strain and one *F. culmorum* strain under *in planta* conditions when it is coated and present in the maize rhizosphere. This supports the fact that this BCA produces antagonistic metabolites and/or that specific mechanisms are involved in the stimulation of plant defences and/or antagonism. For example, Cliquet and Scheffer (1996) demonstrated the correlation between the production of volatile antibiotics by some *Trichoderma* strains that were coated on seeds and the biocontrol effect on the soil-borne *Pythium ultimum* pathogen in greenhouse.

In field conditions, this effect was confirmed on the sensitive Torres maize variety in two fields. The biocontrol efficiency of the BCA differed depending on the field and on the variety rather than on the formulation. However, this maize emergence improvement by BC0584 was strongly mitigated, which shows variability and limitation of the BC0584 bioactivity in the field. The absence of effect of the BCA on Troizi variety emergence notably suggests a moderate natural resistance to damping-off agents of this variety in field conditions.

In the same way, many researchers encountered difficulties in proving the bioactivity of a strain in field conditions while they had interesting findings in the laboratory (Butt *et al.* 2001; O'Callaghan 2016). A limited BCA bioactivity against fungal damping-off agents could be due to a strong competition with other micro-organisms within the maize rhizosphere (O'Callaghan 2016). McLean *et al.* (2012) reported that a disease-prone field also strongly influences the bioactivity of *T. atroviride* against the onion white rot pathogens. Indeed, complex interactions between pathogens and antagonists can influence the balance of plant disease and health (Haas and Défago 2005). As exposed in this paper, disease pressure could be linked to a negative effect on yield for more sensitive maize varieties (like Torres). To avoid variability of the bioactivity, combination of complementary BCAs is an interesting way to encompass a broader range of biocontrol activity (Hebbar and Samuels 2015; O'Callaghan 2016). Guetsky *et al.* (2001) proved that mixing two BCAs (the yeast *Pichia guilermondii* and the bacterium *Bacillus mycoides*) significantly reduced the variability of the biocontrol on *B. cinerea*.

Nevertheless, our *T. atroviride* strain was able to develop in the maize rhizosphere from the early emergence stages until the harvest (data not shown). But we know that using living organisms such as BCA generates constraints that do not exist with chemical fungicides (Butt *et al.* 2001). This gap between BCA fungal development and bioactivity can be due to non-optimal conditions for biocontrol activity and for the release of inhibitory compounds (e.g. the drought at the emergence stage in April 2017). Indeed, several papers reported the importance of temperature, humidity, microflora and soil type for the biocontrol activity of *Trichoderma* spp. (Mao *et al.* 1997; Butt *et al.* 2001). At the BCA mass production stage, other studies showed how initial growth conditions of conidia like pH, water activity, temperature and incubation time impact further biocontrol activity and conidia germination of *T. atroviride* LU132 against *R. solani* in culture media (Daryaei *et al.* 2015; Daryaei *et al.* 2016). Besides higher spore quality, the activity of BC0584 in the field could also be enhanced with a higher conidia concentration on each seed (Keswani *et al.* 2016) than the $10^5$ viable spores coated on each seed of our field plots. Mao *et al.* (1997) studied some biocontrol *Trichoderma* species against *F. graminearum* and *Pythium* spp. in seed coating of maize. According to them, seeds coated with at least $10^6$ CFU per seed were more efficient (same level as for a fungicide treatment) than a concentration of $10^4$ CFU per seed.

Ensuring spore survival and spore quality during storage is another concern since spores have to resist low water activity in the seed coating and then rapidly germinate just after maize sowing. Our results showed that the evolution of spore viability (corresponding to the number of spores that germinate) is dynamic over time during storage and varies with formulations and temperatures. In the current study, the slurriable talc-based powder formulation (formulation F4) has been the only one to ensure more than 80% of viable spores in seed coating at 4°C over a 6-month storage period. These results confirm the ones from our preliminary experimentations. Talc and calcium carbonate are carriers, and they help in stabilizing the formulation (Keswani *et al.* 2016). They are also crucial factors for maintaining conidia survival in the long term. Dry spores present in the slurriable powder (F4) are also suspected to have a positive effect on the spore shelf life compared to the spores freshly put in the coating formulation (formulations F1, F2 and F3). Indeed, Sabaratnam and Traquair (2002) already pointed out the efficiency of talcum powder formulations to keep *Streptomyces* propagules alive in the same storage conditions. The 'increase' in the number of viable spores in the F4 is supposed to be linked to the reactivation of dormant spores in the initial inoculum of seed. Jin *et al.* (1991) effectively demonstrated that reducing water activity of *T. harzianum* spores increased CFU number on culture medium amended with polyethylene glycol 200. On the contrary, as showed in results obtained for formulations made with methylcellulose (F1, F2 and F3), Cliquet and Scheffer (1996) provoked a rapid collapse of spore viability (in a period from one to several months) in methylcellulose coating (2%) at 15°C and even at 4°C depending on the *Trichoderma* strain used. Our results are consistent with the findings of Swaminathan *et al.* (2016) showing that conidia shelf life of *T. atroviride* reduced as the storage temperature and relative humidity increased, and that the choice of additives had a strong influence in wheat seed formulations.

Regarding applicability of this BCA, our field trials showed that BC0584 spores can be massively produced (between $10^9$ and $10^{10}$ CFU per g of medium). The formulation F4 showed highest conidia survival during storage is a low-cost seed coating (by-product of wheat transformation and basic contents), which could be a competitive alternative to chemicals and commercially viable for a registration application. Seed coating formulations made of BC0584 spores could therefore be used in crop field for large areas. On top of ensuring spore survival, the formulation F4 dried quickly (2 h), coated seeds were not sticky at all, and they can be sown using mechanical machinery. These are qualities that would allow for commercial use. These trials also indicated that the biological coating was applicable with conventional machinery and compatible with pesticides use (herbicides and double coating with crow repellent), suggesting an IDM strategy. Regarding storage temperature, cold storage at 4°C is considerable in Europe (Swaminathan *et al.* 2016). Keeping coated seeds in the fridge takes up space compared to small package boxes. A more relevant option for commercialization of this BCA would be to make a water-dispersible powder allowing farmers to carry out the seed coating themselves.

In conclusion, this is the first study that suggests a specific seed coating for field crop such as maize, with the action of *T. atroviride* BC0584 strain against *F. avenaceum* and *F. culmorum* soil-borne pathogens in Belgium. Results of this research highlighted one promising native fungal BCA for the protection of maize seeds and seedlings in Belgium via an applicable formulation that can be stored at low temperatures for at least 6 months. In practice, *Fusarium*-sensitive maize varieties, such as Torres, could be cropped in an IDM strategy or even in organic farming with such a biofungicide coating, if disease pressure is moderate. In Belgium, the surface area of land dedicated to organic farming increased by more than 40% between 2010 and 2017, which clearly justifies the demand for biopesticides (Belgian Federal Government, 2019). However, BC0584 modes of

action and required biological coating conditions still need further investigation to allow for a better bioactivity in the field. Safety of the product also has to be deepened, especially regarding the environment and non-target species (Butt *et al.* 2001; Keswani *et al.* 2016). In the future, the use of this BCA could be extended to neighbouring countries whose geographical proximity entails similar pedoclimatic conditions. The spectrum of action could also be studied for other important crops such as sugar beet and wheat, and crop pathogens such as *Phytophthora infestans*, which has shown promising results under *in vitro* conditions (data not shown). Molecules of interest that could be extracted from the fungus should be investigated to get biopesticides, as these could be used alongside the micro-organism itself. This study should enable a new potential BCA strain to be found that could be developed for the biopesticides market in order to promote a more sustainable agricultural production.

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## Conflict of Interest

No conflict of interest declared.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Ranking of the 40 best antagonistic fungal strains selected from maize rhizosphere and sorted according to their ability both to inhibit *Fusarium avenaceum* T001 and *F. culmorum* Q004 at 25°C and to produce lytic enzymes (chitinases, cellulases and proteases). Grey boxes correspond to values higher than the mean result obtained for a parameter.