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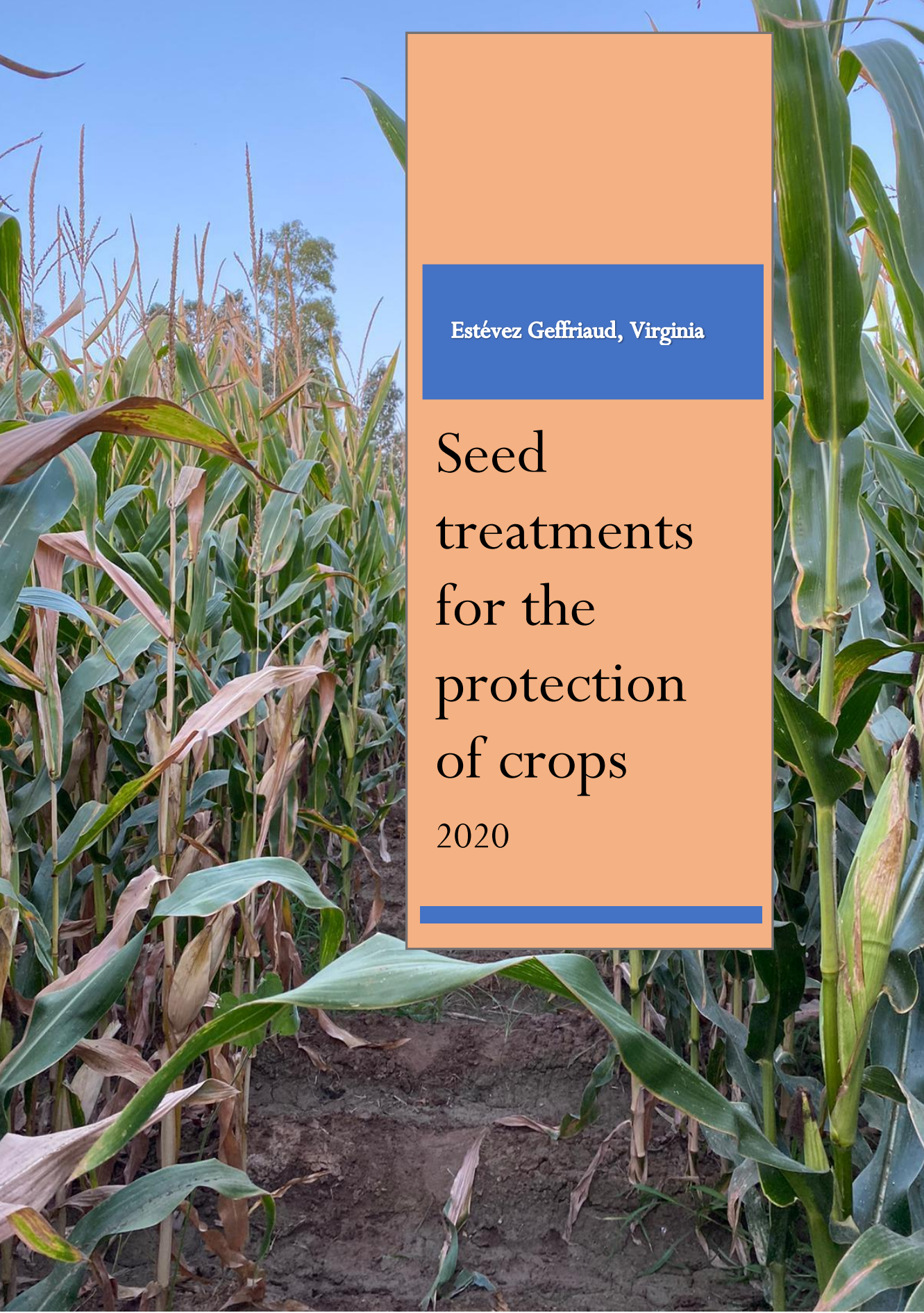
Seed treatments for the protection of crops

Virginia Estévez Geffriaud



Estévez Geffriaud, Virginia

Seed treatments for the protection of crops

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Evolutive Biology, Ecology and Environmental Sciences Department

Plant Physiology Section

University of Barcelona

Barcelona, 2020

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Seed treatment for the protection of crops

Memorandum presented by Virginia Estévez Geffriaud to opt for the title of Doctor by the University of Barcelona (UB). This work was sustained inside the doctoral programme in Plant Biology corresponding to 2015/2020 of the Evolutive Biology, Ecology and Environmental Sciences Department in the Plant Physiology Section of the Faculty of Biology of the University of Barcelona, Spain. This work has been developed under the direction and mentorship of Dr. Mª Isabel Trillas Gay and co-directed by Dr. Juan Jesús Narváez Reinaldo (Fitó Seeds).

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Barcelona, December 2020

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“A winning strategy must include losing”

Rich Dad, Poor Dad. Robert T. Kiyosaki (1997)

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- *Maman, merci pour m'avoir élevé et pour m'aimer comme tu le fais. Je t'aime.*
- *Papa, gracias por enseñarme (casi) todo lo que sabes. Esta tesis es tuya. Te quiero.*
- *A mi melliza, le persona más lista que conozco. Te quiero bubu.*

So that the future does not bring the hunger, emigration and wars that my grandparents had to endure.

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Summary

This thesis focuses on the development and subsequent application of a seed treatment protocol using a phytosanitary product named T34 Biocontrol (Biocontrol Technologies S.L., Spain) containing the active ingredient *Trichoderma asperellum* strain T34, which is registered for use as a plant protection agent in the EU. Greenhouse and field assays were conducted in order to observe the effects of this product on *Zea mays* (maize) under a) abiotic stress (drought) with or without a commercial fungicide; b) biotic stress in a field naturally infected with the soil pathogen *Harpophora maydis* (previously known as *Cephalosporium maydis*). The seed treatment was also applied to seeds of two parental (male and female) lines of cucumber (*Cucumis sativus*) and their hybrid in order to observe its effects on yield under intensive greenhouse production. In summary, we obtained a viable seed treatment with a T34 load of up to $1 \cdot 10^7$ CFU/seed at baseline. T34 seed treatment was viable for up to two years with a load of $8 \cdot 10^2 – 2 \cdot 10^5$ CFU/seed or up to at least 310 days with $3 \cdot 10^4 – 1 \cdot 10^6$ CFU/seed, with or without fungicide, respectively, depending on storage and dose. In maize, regardless of water regime, T34 improved kernel P and C content, kernel dry weight and number. Under drought, T34 treatment improved leaf relative water content, water use efficiency, PSII maximum efficiency and photosynthesis. However, the seed treatment did not prevent a decline in yield in the maize genotype and stress conditions used. On the other hand, T34 reduced the percentage disease incidence by up to 13% and enhanced the final yield per plant in some maize genotypes with different levels of disease tolerance. In cucumber, T34 load in the rhizosphere was 10^6 CFU/g DW at the end of the crop cycle and it increased the early flower count and early fruit yield in male and female parental lines of cucumber in comparison to untreated plants. Moreover, in the hybrid, T34 increased total fruit yield, mean fruit weight and diameter. Finally, seeds from T34-treated plants showed a modified bacteriome, with fewer Proteobacteria and more Bacteroidetes and Firmicutes than seeds from control plants.

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1. Introduction

Introduction

Importance of seeds and seed industry

Seeds are the result of the sexual reproduction of plants. Seeds develop from the fertilized ovules of female floral parts, following fertilization by pollen from male floral parts. When ovules and pollen come from different plants, the resulting seed is a mixture of both. Sexual reproduction avoids the loss of genetic load and promotes the genetic variability needed to evolve in a changing environment. Throughout evolution, plants have evolved to create mechanisms that facilitate cross-pollination (Freedman, 2004).

In structure, a seed is a fertilized dormant ovule that encompasses the embryo but also primitive forms of root, shoot and leaves that are surrounded (and protected) by a nutrient reserve like starch and/or oil that will nourish the embryo while creating a plantlet. This process will stop when the plant can photosynthesize. Seeds need to wait for the optimal conditions to grow and reproduce. In terms of use, seeds are mainly important as human and animal food, while some seeds are used as raw materials for other industries (Freedman, 2004).

The importance of maintaining seed genetic material reached a point in recent decades where a project was needed to protect this material for future generations on a global scale. Following the International Treaty on Plant Genetic Resources, in 2014 the Norwegian Government committed to fund and establish the Svalbard Global Seed Vault. Currently the Seed Vault contains more than a million samples and holds the most diverse plant collection of food crop seeds in the world (Crop Trust: Svalbard Seed Vault Web).

Market research studies on the global market for seeds estimated that it is worth 59 billion USD (2020) and is expected to reach 81 billion by 2025 with a CAGR

(Compound Annual Growth Rate) of 6.4% for the forecast period 2020–2025 (Global Seed Market Forecast 2020-2028, Inkwood Research, 2020).

Among the factors boosting this growth are: a) advances in seed technology (Seed Market, Allied Market Research, 2017), b) the increasing demand for food production, c) the rising use of biofuels and animal feed and d) favourable government support (Global Seed Market Forecast 2020–2028, Inkwood Research, 2020).

The US is considered the leading customer and manufacturer of seeds in the North American region. It also accounted for the largest market share in the global agri-business market in 2016 (Global Seed Market Forecast 2020–2028, Inkwood Research, 2020).

A current list of most important seed companies is displayed in **Table 1**.

Company name	**Country**
BASF SE	Germany
Bayer AG	Germany
KWS SAAT SE	Germany
Syngenta Group	Switzerland
Groupe Limagrain	France
DLF	Denmark
Enza Zaden	The Netherlands
Barenbrug Holding BV	The Netherlands
Rijk Zwaan Seed Cultivation and Seed Trading BV	The Netherlands
Corteva Agriscience	US
Land O'Lakes	US
United Phosphorus Limited	India
Rallis India Limited	India
Longping Hi-Tech	China
Sakata Seed Corporation	Japan
Takii & Co, Ltd	Japan

Table 1. Important companies in the global seed market industry (Global Seed Market Forecast 2020-2028, Inkwood Research, 2020; Seed Market Report, Marketsandmarkets.com, 2020)

Fito Seeds (*Semillas Fitó*) is a family seed business founded in 1880 and currently led by the fifth generation of the Fitó family. It was the first seed company in Spain and in 2016 its annual revenue was 90 million EUR. Exports represent more than 55% of the sales in more than 70 countries. It has over 500 employees in 15 countries and more than 15% of sales are invested in Research and Development (R&D). It has seven centres of R&D. It is a pioneer in the use of plant biotechnology in Spain and since 2014 has been carrying out Seed Technology activities (Semillas Fitó Web Page).

Seed Technology

Seed Technology comprises a series of techniques and protocols that allow the improvement of the seed after it is created (Araújo et al., 2016; Ma, 2019; Pedrini et al., 2017).

We can break this down into four major parts:

- Seed viability prediction and seed sorting: encompasses a series of seed measurements (direct or indirect) to predict different traits such as germination and/or vigour in order to ensure the highest quality seeds. An example of viability prediction would be electrical conductivity measurements in sunflower to predict vigour (Szemruch et al., 2019).
- Seed disinfection: This procedure consists of the imbibition of the seed in several products like fungicides for a short period of time with the aim of external disinfection of the seed from potential pathogens (Kubota et al., 2012).
- Priming of seeds: This procedure consists of the imbibition of the seed in certain temperature, oxygenation and light conditions for a short period of time in order to accelerate germination processes in the seed (Jisha et al., 2013). Therefore the seed is ready for emergence once it receives a second (and final) water stimulation when sowing occurs (Nerson, 2007).

- Seed treatment/Seed coating: This technique consists of the application of different products to the external part of the seed in different manners, attaching them to it and allowing it to be sown in a controlled, more environmentally safe and optimized way (Jambhulkar et al., 2016; Ma, 2019).

Seed Treatment

The aim of a seed treatment is to improve handling, protection, germination, plant establishment and finally, yield (Singh et al., 2016; Pedrini et al., 2017).

The seed treatment market is estimated to be valued at 6.4 billion USD (2020) and is projected to reach 11.3 billion by 2025, based on a CAGR of 12%.

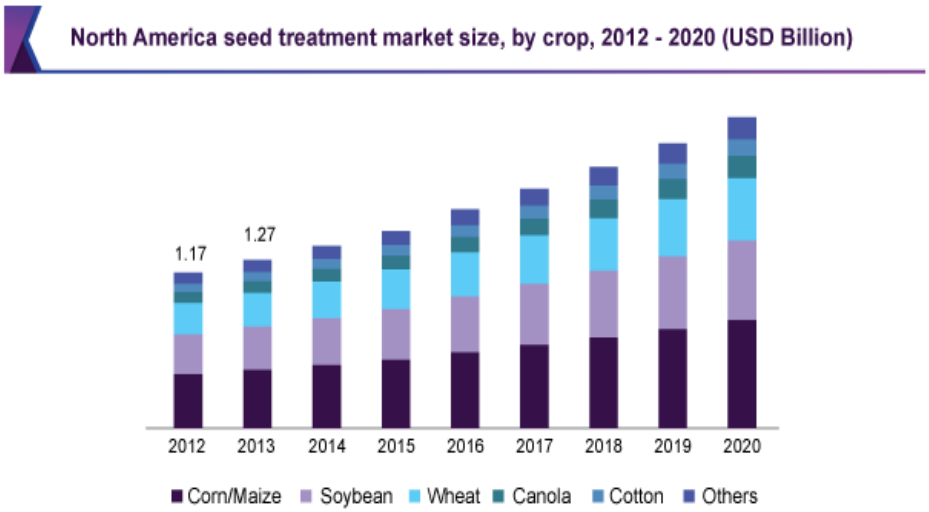

Figure 1. North America seed treatment market size by crop in the 2012-2020 period in USD billion (Seed Treatment Global Market Report, Grand View Research, 2017)

In the past, seed treatment was used to disinfect the seed to control certain pathogens that affected seed performance. In recent times, protection has been the objective, using different compounds and active ingredients. but now the objective is shifting towards stress prevention, helping the seed grow and ultimately enhancing crop yield (Seed Treatment Market, MarketsandMarkets.com, 2020).

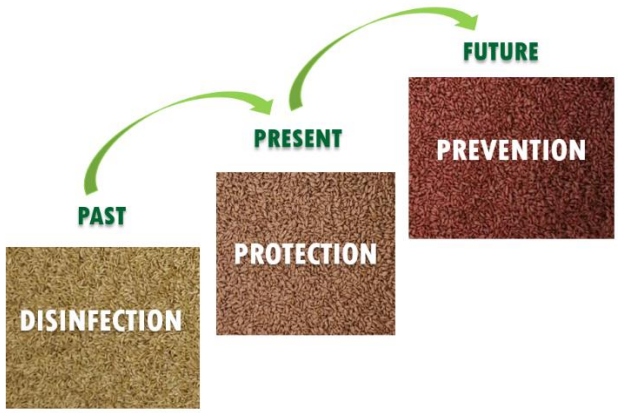

Figure 2. Seed Treatment - past, present and future expectations.

According to the type of compound applied there are several classes of products used in seed treatment:

- *Fillers*: powdery material of inert nature that is used mainly to increase seed weight from a physical point of view (e.g. clay or calcium carbonate-based powders).
- *Binders or stickers:* usually any compound used to provide support and adhere the rest of the compounds in the slurry to the seed.
- *Colorants and tracers*: encompasses those compounds that aim to increase the appeal of the seed visually and/or increase the traceability of seed batches, avoiding counterfeiting and mislabelling, respectively. Colorants also act as a way of highlighting the presence of harmful compounds to the end user and facilitate identification during sowing operations (Pedrini et al., 2017).
- *Active ingredients*:
    - Synthetic origin:
        - Pesticides: include fungicides, insecticides, nematicides, predator deterrents and herbicides. These compounds are usually of synthetic

origin and their use enhances the rate of germination and reduces predation and infection by soil pathogens (Pedrini et al., 2017).

- Organic origin:
  - Biostimulants: encompasses macro- and micro-nutrients and soil adjuvants (e.g. humic and fulvic acids (Berbara & García, 2014), protein hydrolysates and amino acids (Calvo et al., 2014)).
  - Microbial inoculants: the most common is rhizobia inoculation of legumes. Nowadays and with the improvement of coating techniques and formulations, other microbial inoculants have been used in seed treatments, predominantly bacterial and fungal inoculants. Typically microbial inoculants are classified as biocontrol agents (also called biopesticides) or biofertilizers (Calvo et al., 2014).

Currently seed treatment are mostly formulated with chemical compounds the goal of which is to protect the seed against seed- and soil-borne diseases (Krzyzinska et al., 2005; Zeun et al., 2013; Moya-Elizondo & Jacobsen, 2016).

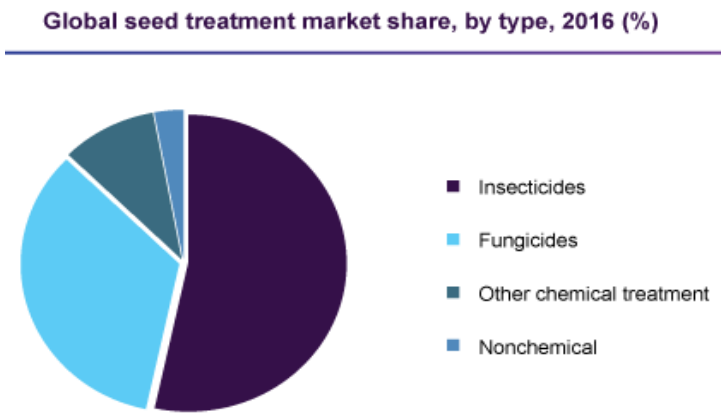

Figure 3. Global seed treatment market share by type of compound. Insecticide and Fungicide predominated the market in 2016 (Seed Treatment Global Market Report, Grand View Reseach, 2017)

Seed treatments can be classified depending on the resulting modification of the seed (size, shape, weight) and properties of the treatment (Pedrini et al., 2017).

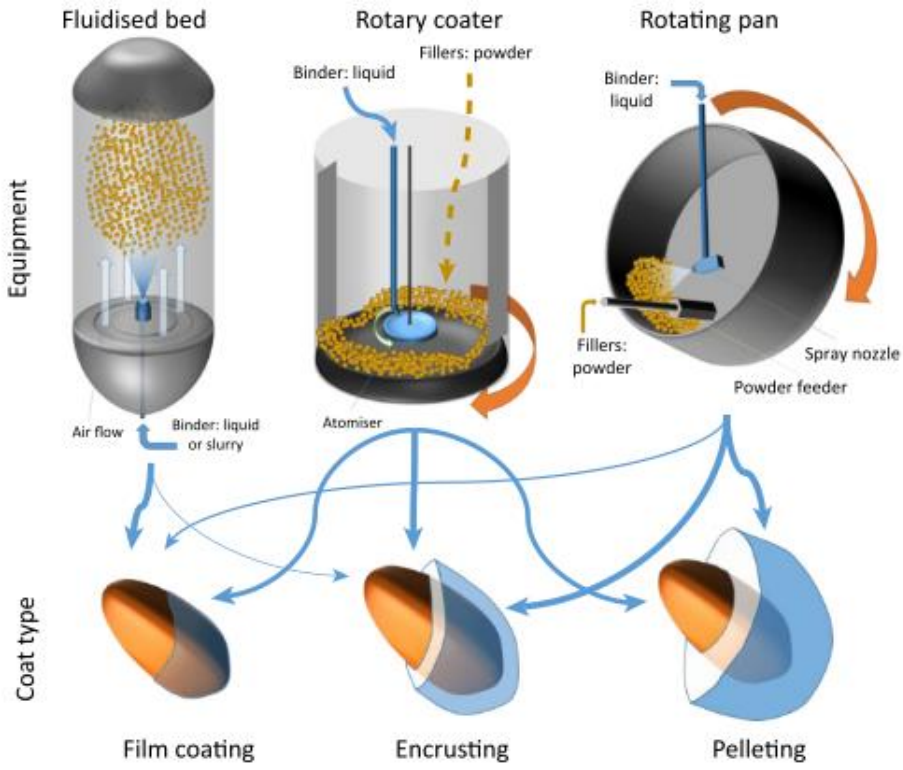

Figure 4. Seed Coating Mechanisms extracted Fig. 1b from Pedrini et al., (2017)

Firstly, *film coating* involves the application of a thin layer of slurry usually consisting of polymers, colorants and other synthetic compounds using a rotatory machine. This technique does not alter the size and/or shape of seeds much but it improves handling (Ma, 2019). It is usually applied to large seeds that are produced in large amounts, such as maize (*Zea mays*).

Figure 5: Examples of seeds with *film coating* seed treatment (*Zea mays)*.

Secondly, *encrusting* consists of the application of inert materials such as clay or calcium carbonate-based powders (among others) in order to increase the weight and modify the shape of the seed to improve precise mechanical sowing, protect the seed and create a more uniform batch. This is used mostly in seeds planted with a mechanical planter and that do not require thinning after emergence (Ma, 2019; Pedrini et al., 2018).

Figure 6: Examples seeds with *encrusting* seed treatment of Bermuda grass (*Cynodon dactylon*, left) and sunflower (*Helianthus annuus*, right).

Thirdly, *pelleting* consists (like *encrusting*) of the application of inert materials that modify the weight, size and shape of the seeds, where in this case, the seeds end up with a perfectly round shape, hence making this process more expensive than the previous one as more material is applied. This treatment facilitates the sowing of irregular or very small seeds that often have high value (Ma, 2019; Pedrini et al., 2018, 2017).

Figure 7: Examples of seeds with *pelleting* seed treatment of tomato (*Lycopersicum esculentum)*.

Microbes applied to seed treatment

Microbial inoculants have several advantages in comparison to chemical compounds: greater handling safety for humans, less environmental damage, targeted activity, effectiveness in small amounts, and self-multiplication controlled by the plant and the indigenous microbial populations (Singh et al., 2011).

Seed treatments have slowly but steadily been evolving to include the application of active biological substances, including *Azospirillium, Pseudomonas, Azotobacter, Bacillus*, *Aspergillus*, *Gliocladium* and *Trichoderma* spp., together with various adhesive agents (Taylor et al., 1991; Gholami et al., 2009; Hu et al., 2011; Cumagun, 2014; Singh et al., 2016; Moya-Elizondo & Jacobsen, 2016).

Bacterial and fungal inoculants

Many bacteria and fungi are used as commercial inoculants (Owen et al., 2015). Among bacteria, there are several genera capable of enhancing plant growth, such as the widely used *Rhizobium* sp. that reside inside the plant, forming specialized structures (nodules), and include nitrogen-fixing bacteria. On the other hand there are inter/extracellular bacteria that produce a plethora of compounds such as enzymes, hormones and antibiotics among others (Owen et al., 2015).

There are many kinds of mycorrhiza but microbial inoculants are predominantly formed by vesicular-arbuscular mycorrhizae (VAM) and ectomycorrhizae (EcM), which are the most widespread and ecologically important mycorrhizae (Owen et al., 2015). VAM fungi increase P uptake in diverse crops (Antunes et al., 2009).

In the case of *root-associated fungi* (RAF), many reside in the rhizosphere, in the rhizoplane and sometimes within root tissues as endophytes. In this group, the most predominant genus is without a doubt *Trichoderma*.

Trichoderma spp

The most successful genus of bio-fungicides is *Trichoderma*, being present in more than 60% of registered products around the globe (Verma et al., 2007). *Trichoderma* species are well known for multiple modes of action that can include mycoparasitism, antibiosis, competition, induction of plant resistance and endophytic activity (Verma et al., 2007; Waghund et al., 2016). Some species of *Trichoderma* can interact with the surrounding microorganisms in the soil and protect the plants against abiotic and biotic conditions and also enhance plant growth and yield (Harman et al., 2004; Hermosa et al., 2012; Fernández et al., 2014).

Table 2. Scientific studies including *Trichoderma* strains and effects observed in different crops.

Trichoderma strain	Crop	Observations	Reference
Trichoderma harzianum Rifai strain T-22	Tomato	Alleviate *Pythium ultimum* Abiotic stress in osmotic, saline, chilling and heat stress	Mastouri et al., 2010
Multiple *Trichoderma* spp strains with compost	Cucumber	Reduced damping-off by *Fusarium* spp Promoted fruit yield and quality	Elwakil et al., 2015
T. asperellum (previously *T. harzianum*) T-203	Cucumber	Enhanced root growth, root length, root surface area and number of root tips in greenhosue and hydroponic system	Yedidia et al., 2001
		Induction of systemic acquired resistance against *Pseudomonas syringae* pv. *lacrymans*	Yedidia et al., 2003
Commercial *Trichoderma sp* (TrichoFlow ®)	Cucumber	Enhanced total yield in three different cultivars in unheated glasshouse	Altintas & Bal, 2005
Trichoderma virens (two strains)	Cotton	Reduction of *Verticillium* Wilt symptoms	Hanson, 2000
Trichoderma spp (four species)	Flowering Chinese cabbage	Enhanced germination rate, height, fresh weight and yield	Ji et al., 2020

Trichoderma asperellum strain T34

Trichoderma asperellum strain T34 (T34) CECT Nº 20417 has been patented by the University of Barcelona (UB) and licenced to Biocontrol Technologies, S.L., a *spin-off* enterprise of the UB. The patent was first registered in Spain (ES 2188385 B1) and later expanded to several other EU countries (EP 1400586 B1) and finally the US (US 7553657 B2). This strain was discovered by M. I. Trillas and L. Cotxarrera.

T34 is authorized as a biological plant protection product in the EU (Spanish authorization ES-00283) and was included in Annex I by the Commission Implementing Regulation No. 1238/2012 on 19 December 2012 in accordance with article 80(1)(a) of Regulation (EC) No 1107/2009. It is currently available commercially in various EU states and other countries such as Canada, USA, Egypt and Peru. Several studies have reported on the performance of T34 under biotic stress (Cotxarrera et al., 2002; Trillas et al., 2006; Segarra et al., 2010; Borrero et al., 2012a; Segarra et al., 2013; Fernández et al., 2014) and under non-stressed conditions (de Santiago et al., 2011; García-López et al., 2015).

T. asperellum strain T34 has been observed to enhance Fe accumulation in cucumber depending on the soil and glucose addition (de Santiago et al., 2013), increase total P content in roots (García-López et al., 2015), activate plant defenses against fungi, bacteria and nematodes (Segarra et al., 2007; 2010; Martínez-Medina et al., 2017; Pocurull et al., 2020) and suppress *Rhizoctonia solani* in cucumber seedlings (Trillas et al., 2006). In pepper, T34 reduced the incidence of *Phytophtora capsici* (Segarra et al., 2013) and in tomato, T34 controls Fusarium wilt (Segarra et al. 2010) and *Botrytis cinerea* (Fernández et al., 2014). Moreover, maize seed treatment with T34 improved the relative water content in leaves, photosynthesis, the maximum quantum efficiency of photosystem II, and nutrient and kernel

parameters under well-watered and/or drought conditions (Estévez-Geffriaud et al., 2020).

2. Aims of this work

Aims of this work

The general aim of this thesis was to develop a seed treatment (seed coating) on maize (*Zea mays*) and cucumber (as representatives of field and horticultural crops, respectively) and to test it under several conditions in the greenhouse and field:

- Abiotic (drought) stress.
- Biotic (fungal pathogen) stress.
- Non-stressed conditions.

The specific aims were:

1. To develop a viable **seed treatment** with the biological plant protection product ***Trichoderma asperellum* strain T34** at high concentration.
2. To assay the seed treatment in a **greenhouse-grown corn line under well-watered conditions and drought-induced conditions** to determine its effects on plant physiology, nutrient status, and yield.
3. To assay the seed treatment in **field-grown maize lines with different levels of tolerance to a natural infestation of *Harpophora maydis*** and to evaluate the seed treatment regarding percentage disease incidence and final yield in different tolerant lines.
4. To assay the seed treatment in **greenhouse-grown cucumber parental and hybrid lines** to evaluate the effects of the seed treatment on fruit yield, seed yield and the seed bacteriome of the progeny.

3. Case Studies

Case Studies

General Remarks

Study I describes the (extended) data presented as a **short talk and poster** at the **International Symposium *MiCROPe*** (Microbe-assisted crop-production: opportunities, challenges & needs) in Vienna (Austria) in December 7th, 2017, titled: "Shelf-life and storage of *Trichoderma asperellum* strain T34 applied as a seed treatment on experimental corn seeds".

Study II was **published in PLANTA (Springer Editorial)**, which has an impact factor of **3.390 (2019).** This study evaluated the effect of a seed treatment with *Trichoderma asperellum* strain T34 on greenhouse-grown corn under drought and well-watered conditions (Estévez-Geffriaud, V., Vicente, R., Vergara-Díaz, O., Narváez, J.J., Trillas, M.I. Application of *Trichoderma asperellum* T34 on maize (*Zea mays*) seeds protects against drought stress. *Planta* **252,** 8 (2020). https://doi.org/10.1007/s00425-020-03404-3)

Study III comprises the **final report** of the field assays performed during the industrial PhD program. This study evaluated the effect of the abovementioned treatment on four corn lines grown in field conditions in a soil naturally infected with the fungal pathogen *Harpophora maydis* (formerly *Cephalosporium maydis*).

Study IV is **currently under review** at **Scientia Horticulturae (Elsevier Editorial)**, which has an impact factor of **2.769 (2019).** This study evaluated the effect of the abovementioned treatment on two parental lines of greenhouse-grown cucumber and the resulting hybrid.

Study I: Shelf-life and storage of Trichoderma asperellum strain T34 applied as a seed treatment on experimental corn seeds.

Shelf-life and storage of *Trichoderma asperellum* strain T34 applied as a seed treatment on experimental corn seeds.

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Abstract

Application of beneficial microorganisms to seeds is an efficient mechanism to allow microbial inoculation of roots and protect them against soil-borne diseases. However, survival of the microbial inoculant is a critical point to the success of biological seed treatments. Here, we use *Trichoderma asperellum* strain T34 in different formulations, doses, corn varieties and storage conditions through time to determine survival in the seed up to two years. Results show a viable treatment with a commercial corn fungicide at least up to a year, and up to two year with T34 alone. Finally, seed conservation conditions showed better survival than room temperature conditions.

Introduction

Maize is a very important crop mainly for human consumption. Application of beneficial microorganisms to seeds is an efficient mechanism to allow the microbial inoculant to colonize roots and protect them against soil-borne diseases. However, there are several issues to solve before making available seed treatment with microbial inoculants, particularly maintaining viability of the microorganisms (O'Callaghan, 2016).

The aim of this work is to evaluate the viability of biocontrol agent *Trichoderma asperellum* strain T34 (Biocontrol Technologies S.L.) as a seed treatment and along two-year period with different formulations, corn varieties and storage conditions.

Materials and Methods

Seed Treatment

We developed a seed treatment formulation protocol with an inert adhesive. We mixed a known quantity of seed (100 g) with the formulation compounds and mixed them with the formulated conidia of Trichoderma asperellum strain T34 (Biocontrol Technologies S.L). The dose of T34 was 10^9 or 10^{10} CFU/g formulated product. In some cases, we additionally mixed a commercial fungicide (CELEST XL: *Fludioxonyl* 25% and *Metalaxyl-M* 1%; Syngenta Crop Protection, Switzerland) alone or with an insecticide (SONIDO: *Thiacloroprid* (*Neonicotinoid*), Bayer CropScience, Germany) at the recommended dosages. After mixing the seed with all the compounds (Specific details are property of FITO SEEDS) the seed was dried at room temperature until it reaches again the same relative humidity (%) as before the treatment.

Scanning electron microscopy images

To check for the effective adhesion of T34 on the seed surface as well as the development of the fungus in radicle emergence, seed samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.4) at 4°C, then washed in phosphate buffer 5 times at 4°C and further processed with a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in phosphate buffer for 2 hours at 4°C and later washed with distilled water. Afterwards, the samples were dehydrated with ethanol and dried using the critical point procedure (Polaron Critical Point Drier). The samples were mounted in a standard Scanning Electron Microscope (SEM) support and coated with Au (Fisons Instruments SEM coating

system). The secondary electron images were obtained using a SEM JEOL 7001 FEG at the Electron Microscopy Unit of the Hospital Clinic, Barcelona.

Evaluation of the T34 seed load through time.

After the seed treatment, half of the seeds were stored at room temperature (RT) and other half at seed conservation conditions (SCC) in the headquarters of Fitó Seeds in Barcelona with low humidity and low temperature, optimal for seed conservation conditions. The treatment was performed in four different corn experimental lines, namely A, E, F and G, with a FAO cycle of 600, 700, 650 and 450, respectively.

The evaluation through time was performed on the seed in the different conditions, doses and treatments and measured through ten-fold serial dilution method and malt extract solid media for colony counting. Dilutions were initiated with 1 g of seeds in a tube containing 9 mL of autoclaved saline solution (NaCl 9 g·L-1) and agitated for 30 minutes in a vortex prior to ten-fold dilution series. This was performed twice for each condition. Finally, some seed treatments were performed in PYREX bottles while the last ones were performed in a laboratory-scale professional seed coater (HR160, Hoopman Equipment and Engineering, The Netherlands).

Results

SEM images (Fig. 1 top) showed the conidia of fungus attached to the seed in a stable and uniform manner. The adhesive applied surrounded the conidia and adhered it to the seed while developing hyphae and conidia can be seen in the radicle of seeds once germinated (Fig. 1 bottom). T34 seed load (CFU/seed) just after treatment at different concentrations and dosages, can be seen in Fig. 2. Results show that 'Treatment A' was the most effective to attach the highest amount of T34 conidia of the formulated product in the seed. Stability through time of T34 seed treatment in variety G up to two years can be found in Fig. 3. We observed a more

pronounced loss of T34 viable load in the seed at room temperature in both concentrations, being less pronounced in seed conservation conditions (SCC). Either way, a loss of viable T34 occurred in all treatments and conditions. T34 seed load (CFU/seed) just after treatment in different varieties (A, E and F) with T34 alone or with a mixture of fungicide - insecticide is shown in Fig. 4. Results show smaller variations in T34 seed load by varieties and treatments, but all values are between 10^6 and 10^7 CFU/seed. Stability through time of T34 seed treatment in varieties A, E and F with or without fungicide up to 310 days after treatment at two conservation conditions (RT and SCC) are displayed in Fig. 5. In variety A (Fig. 5a), E (Fig. 5b) and F (Fig. 5c), results show a diminished T34 load at 310 days more pronounced at RT while in SCC, the reduction of T34 is minimal. Variations among varieties and treatments can be observed, but in any case, in all conditions, varieties, and times, viable T34 load was above 10^4 CFU/seed. Although seed treatment with fungicide is still possible, lower populations of T34 are found in almost every storage condition and variety at every given time. Finally, results of T34 seed load at initial time between Pyrex bottle treatment (Fig. 2 and 3) and professional seed coater (Fig. 4 and 5) showed that the latter equipment higher values (above 10^6 CFU/seed) in T34 alone at the same dosages and concentrations.

Discussion

There are several issues regarding technical feasibility of a seed inoculant regarding microbial seed treatments. The most prominent one is maintaining viable the microorganism throughout commercial seed treatment processes and storage (O'Callaghan, 2016). The seed treatment developed in this work was successful as it allows to adhere between $5{\cdot}10^4$ and 10^7 CFU/seed (T34 formulated conidia) depending on the dosage and concentration of T34 at time 0. In agreement with our results, other authors reported high initial seed load of *Serratia plymuthica* in oilseed rape of 10^7 CFU/seed (Müller & Berg, 2008).

Observing SEM images, we can see the attachment of T34 conidia in the seed surface (Fig. 1 top), but also observe how the conidia can progress and develop hyphae once germination of the seed is initiated (Fig 2 – bottom), ensuring that the seed treatment does not prevent growth and development of the beneficial fungus. In the mentioned figure, we can observe conidia and hyphae developing along the radicle and surrounding it, which is a good indicator of what would happen in the inert substrate and/or soil once planted.

Regarding dosages and concentrations (Fig. 2), 'Treatment A' obtained, the highest attachment of T34 conidia of the formulated product, which was the treatment used for the Case Studies presented here (Study II, Study III) and with slight modifications in the last study (Study IV). In the T34 seed load, small variations can occur by corn variety (Fig. 4 and 5), probably due to differentiated kernel size (small, medium or large) and shape (round or flat) determined by the position of the kernels in the ear. In seed processing, lots are sorted by caliber (size and shape) so this characteristic change from one lot to another (internal information by Fito Seeds - seed processing and quality process team). Another source of variation can be found in the equipment used: In Fig. 4 and 5 seed treatment was not performed in a pyrex bottle as the previous Fig. 2 and 3 but with a seed coater (See Materials and Methods). This difference affected initial T34 seed load at time 0 (T34 alone), as we obtained around $7 \cdot 10^5$ and $4 \cdot 10^6$ CFU/seed with the pyrex bottle and the seed coater, respectively. In any case, the seed coater managed to provide a load above 10^6 CFU/seed and therefore, it seems to be a more efficient way of adhering T34 to the seed than the pyrex bottle, as expected.

Regarding storage conditions, there are little information about seed treatment shelf-life probably due to negative results. Moreover, it is well-known that lower temperature extends bacterial survival by reducing metabolic activity (O'Callaghan, 2016). Regarding our work, in T34 viability through time, the beneficial fungus remained viable in the seed up to two years (Fig. 3) in both storage conditions (RT

and SCC) although higher load was obtained in SCC, as expected, giving the low humidity and temperature present.

Other authors showed that *Pseudomonas putida* 40RNF in sugar beet treated with bacteria-sprayed film coating in pelleted seed or adding the bacteria into the filling material prior to pelleting achieved a population of $5 \cdot 10^7$ CFU/pelleted seed (Shah-Smith & Burns, 1997) which is a similar result regarding our work. Moreover, in the same work, storage during 52 weeks (13 months roughly) at 4°C, obtained a decline of one to two orders of magnitude while at 18-20°C storage, values were below detectable limits (<100/pellet) which is very different from our results, were storage at SCC for nearly a year (310 days) obtained a decline of one order of magnitude at most, while at room temperature the decline was between one and two orders of magnitude, being much more successful. Regarding *Trichoderma atroviride* spores, Swaminathan et al., (2016) highlighted that there are complex interactions between environmental conditions, formulation methods and excipients in wheat seeds. The best survival (up to six months) was obtained at low temperature and humidity with xanthan-gum-based formulation, which agrees with our results, were best storage was observed in SCC. Qualitatively, the treatment is successful regardless of the application of T34 and T34 with fungicides and insecticides in different varieties (Fig. 4 and 5) as T34 remains at high load in the seed along 310 days (at least). This demonstrates that the formulation of T34 and the protocol used for coating is effective, even with the presence of fungicides.

Finally, further work is needed to primarily address: a) how the seed treatment affects viability of the seed through time, b) if the differences are significant and c) for how long the T34 remains viable in the seed.

Conclusions

We have successfully attached between $5 \cdot 10^5$ – $1 \cdot 10^7$ CFU/seed of T34 at time 0 in different varieties and also ensured a load of T34 through time (stability) up to two years, even in detrimental conditions (room temperature); and up to a year (at least)

with or without seed treatment fungicide (CELEST XL) at both room temperature and seed conservation conditions. Minimal differences between experimental corn varieties have been found probably due to the seed lot caliber. Results by storage conditions showed greater loss of viable T34 in room temperature conditions than in seed conservation conditions. Finally, the film coating formulation allows the T34 to develop in the seed just after radicle emergence as seen in the SEM images. Further work is needed to address if a) the seed quality remains the same, b) these differences are significant and c) for how long the T34 remains viable in the seed in the case of the fungicide. However, these results show a promising industrial use of *T. asperellum* strain T34 as microbial seed treatment.

Figures

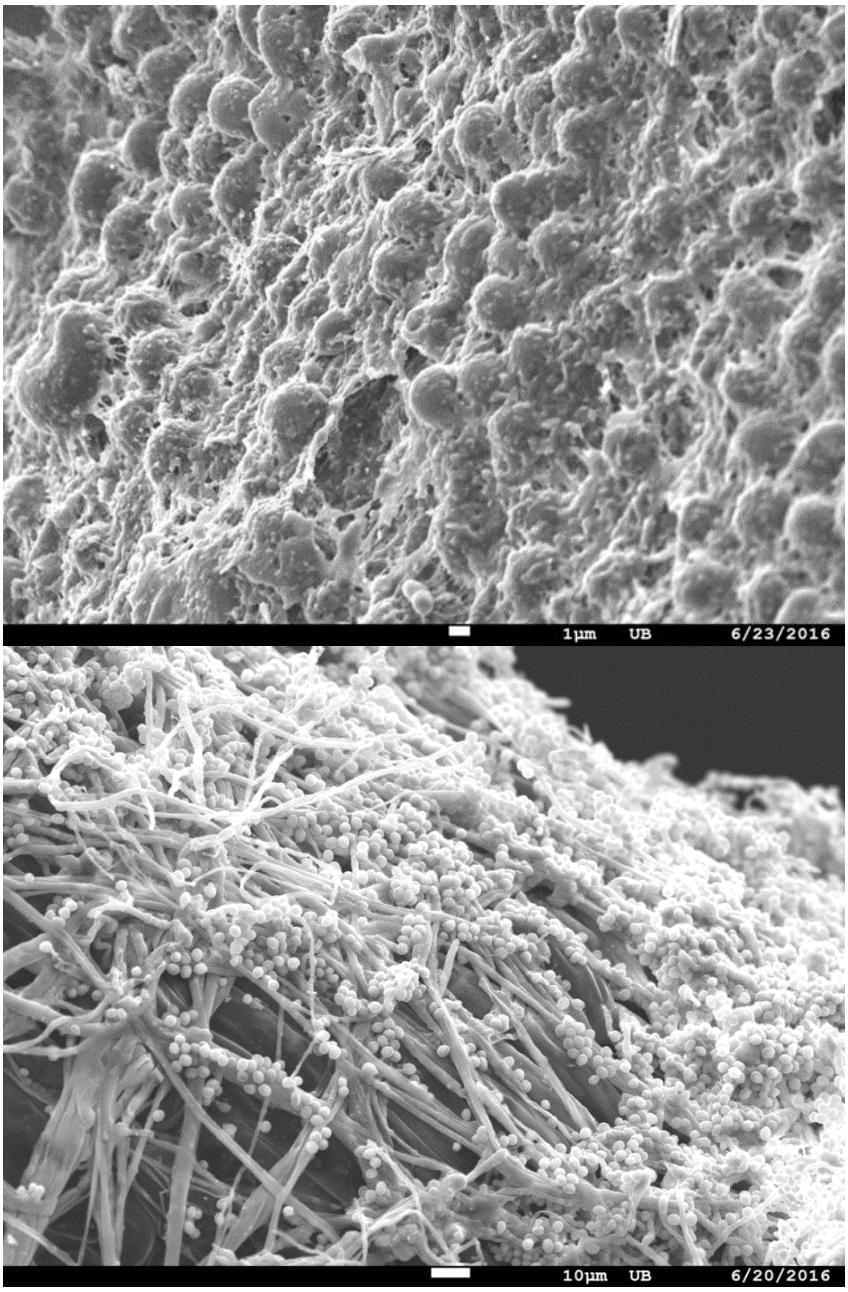

Figure 1. *Scanning Electron microscopy* (S.E.M) images of seeds with the seed treatment of T34 at time 0 (top) and from the radicle once emerged (bottom).

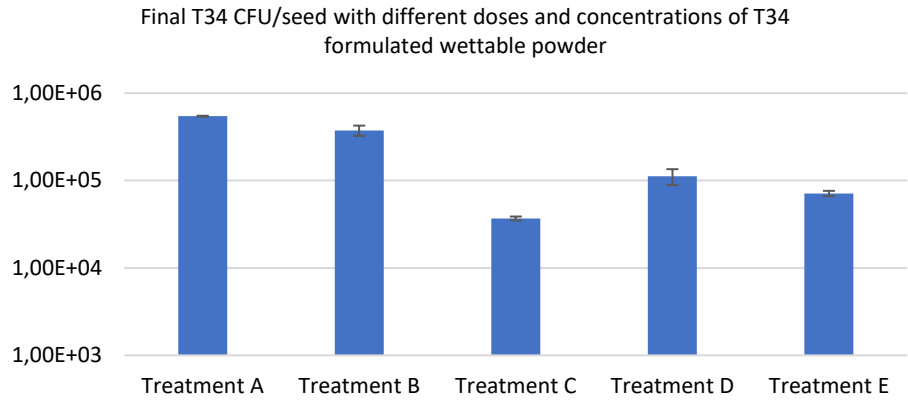

Figure 2. Final T34 CFU/seed at different applications doses and concentrations of T34 formulated wettable powder. Specifics details are property of FITO SEEDS. Variety G was used for the assessment at time 0 (just after treatment). Treatment was performed manually in a PYREX bottle. Mean ± SE.

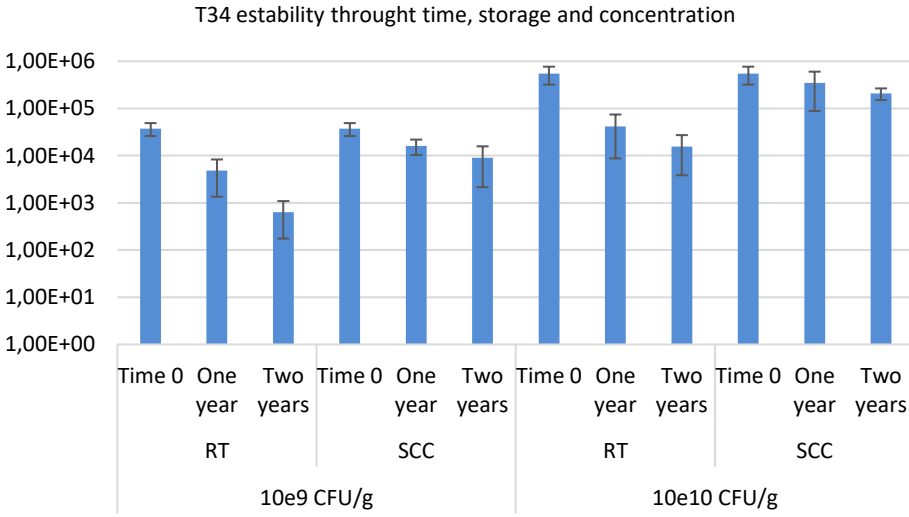

Figure 3. Results of T34 CFU/seed at different time (0, one year and two years after seed treatment), by T34 concentration (10^9 or 10^{10} CFU/g) and storage conditions (room temperature or seed conservation conditions). Treatment was performed manually on a PYREX bottle. Mean ± SE.

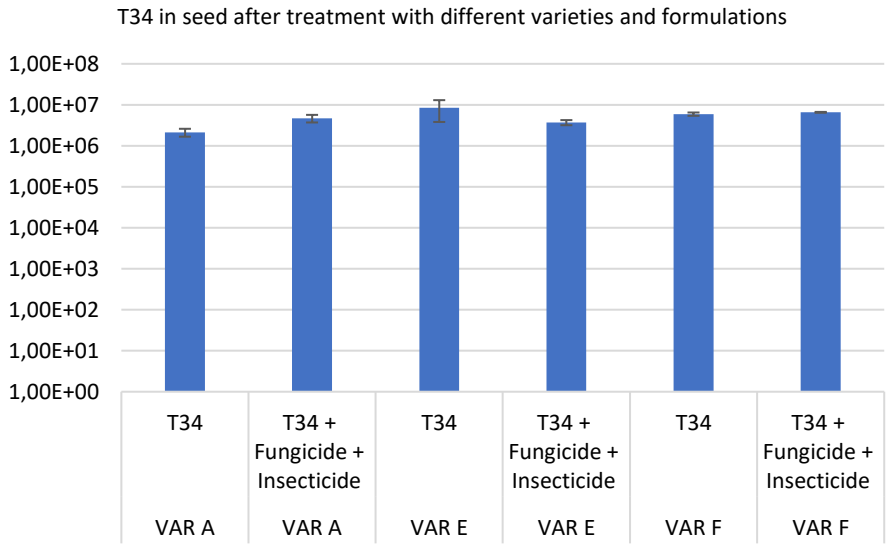

Figure 4. Results of T34 CFU/seed at different formulation (T34 and polymer alone or with fungicide and insecticide) by corn variety (VAR A, E or F) at time 0. Active ingredients for the Fungicide and Insecticide were *Fludioxonyl* plus *Metalaxyl* and *Thiacloroprid* (*Neonicotinoid*), respectively. Mean ± SE.

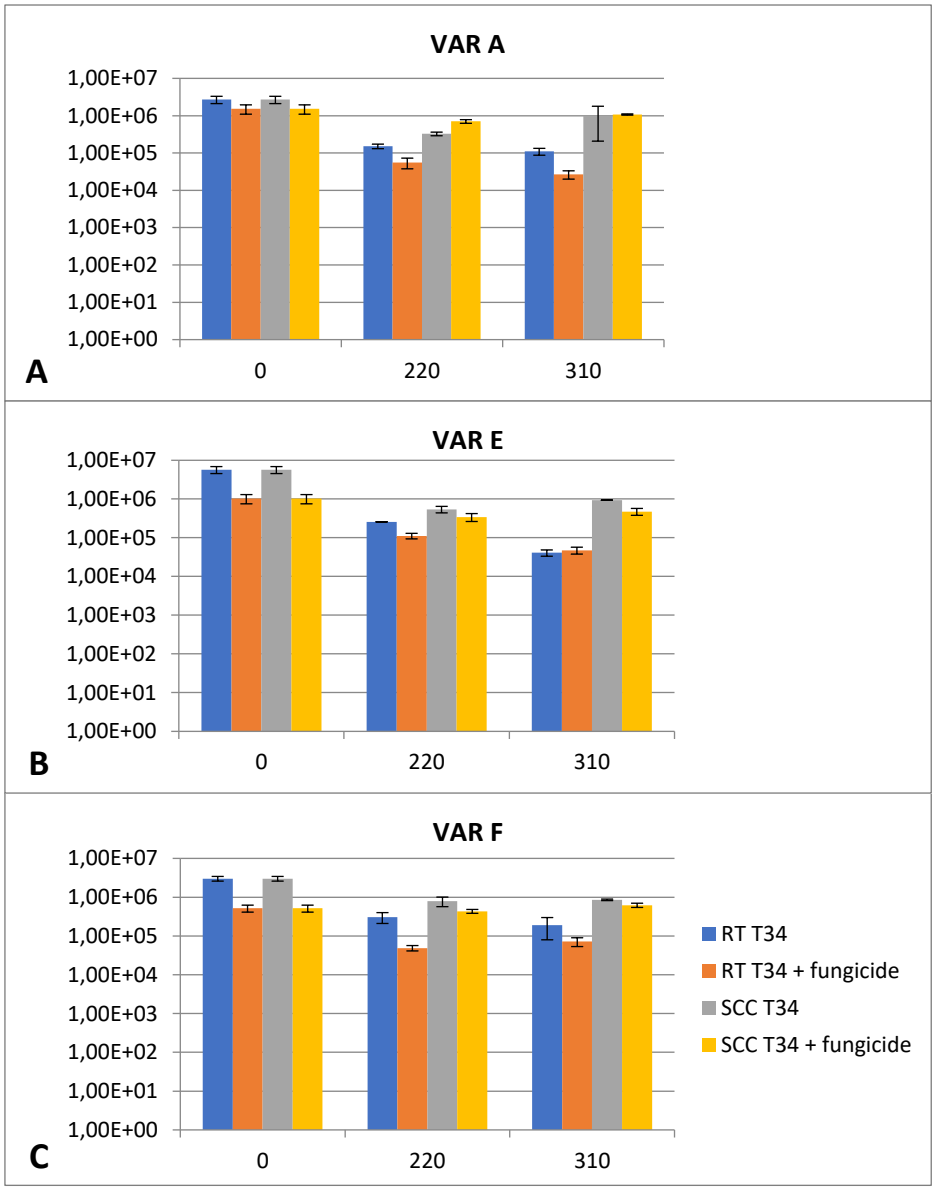

Figure 5. Results of T34 CFU/seed at different formulation (T34 and polymer only or T34 and polymer plus fungicide) by corn variety (VAR A, E or F) at different times (0, 220 and 310 days after treatment). **A**) VAR A; **B**) VAR E; **C**) VAR F. Active ingredients for the Fungicide were *Fludioxonyl* plus *Metalaxyl*. Mean ± SE.

Study II: Application of Trichoderma asperellum strain T34 on maize (Zea mays) seeds protects against drought stress.

Application of *Trichoderma asperellum* T34 on Maize (*Zea mays*) Seeds Protects Against Drought Stress

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Main conclusion

Coating maize seeds with the microbial plant protection product *Trichoderma asperellum* strain T34 is an effective form of inoculation that enhances plant performance when faced with drought stress, and it improves nutrient and kernel parameters differently in drought and non-stressed conditions.

Abstract

Drought is currently one of the biggest threats to maize production. *Trichoderma* spp. is mainly used in agriculture as plant protection product with secondary beneficial effects on plants: improved growth, nutrient uptake and plant immunity.

Here, we studied the physiological performance of maize plants under two different water regimes (fully irrigated and drought conditions) and three different seed treatments: application of *Trichoderma asperellum* strain T34, application of a chemical fungicide (CELEST XL) or the combination of both. Regardless of water regime, T34 treatment improved kernel P and C, kernel number and dry weight. Higher populations of T34 on the rhizosphere (T34 treatment) alleviated water stress better than lower T34 populations (T34+Q treatment). Under drought, T34 treatment improved leaf relative water content, water use efficiency, *PSII* maximum efficiency and photosynthesis. T34-treated maize seeds maintained sufficient T34 populations to alleviate drought throughout crop development suggesting an optimal dose of 10^4 and 10^5 colony forming units·g^{-1} dry weight of rhizosphere under the studied conditions. This work helps to demonstrate the beneficial interaction between *T. asperellum* strain T34 and maize plants under drought.

KEYWORDS: drought stress, elemental nutrient concentration, photosynthesis, gas exchange, kernel parameters, and leaf relative water content.

Introduction

Seed treatments are a suitable way to protect seeds against environmental adversities and ensure plant stand and seedling health. They mainly consist in the external application of a given compound (or more than one), which stays attached to the seeds (Zeun et al., 2013). Currently, most seed treatments are chemical formulations (Krzyzinska et al., 2005; Zeun et al., 2013; Moya-Elizondo & Jacobsen, 2016), the goal of which is to protect the seed against seed- and soil-borne diseases, and they are extensively used in all agricultural areas and with almost all crops (Zeun et al., 2013).

However, agricultural practices are changing, and more natural and sustainable seed treatments are required. Slowly but steadily, seed treatments have been evolving to include the application of active biological substances, including *Azospirillium*, *Pseudomonas*, *Azotobacter*, *Bacillus spp.* and *Trichoderma spp.*, together with

various adhesive agents (Taylor et al., 1991; Gholami et al., 2009; Hu et al., 2011; Moya-Elizondo & Jacobsen, 2016). Microbial inoculants have several advantages over chemical compounds: greater safety, potentially reduced environmental and human damage, targeted activity, effectiveness in small amounts, and self-multiplication controlled by the plant and the indigenous microbial populations (Singh et al., 2011).

Maize (*Zea mays*) is grown on over 170 million ha worldwide, of which 130 million ha are in less-developed countries (FAO, 2014). Low yields of these crops in sub-Saharan Africa are largely associated with drought stress and low soil fertility. Water stress decreases plant growth and development leading to a reduction in crop yield (Farooq et al., 2009). Plant tolerance to this abiotic stress is the result of extremely complex mechanisms involving molecular, biochemical and physiological processes (Medina et al., 2016). The degree of the reduction in crop yield due to this stress is dependent not only on the genotype but also on the severity and duration of the drought, the phase of plant growth and development, and environmental interactions with other factors (Medina et al., 2016).

Many studies of abiotic stress alleviation have used mycorrhizae, nitrogen-fixing bacteria (Ortiz et al., 2015), and increasingly, *Trichoderma* spp. in different crops (Pandey et al., 2016; Alwhibi et al., 2017; Fu et al., 2017). Some *Trichoderma* spp. are known to protect plants against pathogens through competing for nutrients, parasitism, antibiosis, enzyme activity and stimulating the innate immunity of plants; but they also promote growth (Howell, 2003; Segarra et al., 2007; Shoresh et al., 2010; Lorito et al., 2010). Products based on *Trichoderma* spp. are commercially available as plant protection products (PPPs) for a wide range of crops (horticultural, ornamental and agricultural crops, as well as fruit and vegetables during post-harvest storage (Harman et al., 2004; Verma et al., 2007). In particular, *Trichoderma asperellum* strain T34 (T34), has been widely studied under non-stressed conditions (de Santiago et al., 2011, 2013; García-López et al., 2015) and

under biotic stress (Cotxarrera et al., 2002; Trillas et al., 2006; Segarra et al., 2010; Borrero et al., 2012; Segarra et al., 2013; Fernández et al., 2014).

Here we aim to determine the effect of T34 on maize growth, kernel development, and both physiological and nutritional status under two irrigation conditions (well-watered and water-stressed conditions) via the application of three types of seed treatments: biological, chemical, and their combination. Our work focuses on a rapidly growing hybrid that has some tolerance to drought and which is widely used commercially in the Mediterranean region.

Materials and methods

Experimental setup

The experiment was performed in a glasshouse at the facilities of *Camps Experiementals, Universitat de Barcelona* (41º23'6.259''N; 2º7'12.434''E, 60 m above sea level) with maize (*Zea mays*) seeds treated or not with T34, over four months from June to October. The maize genotype was the hybrid SF9C031 from *Semillas Fitó* (Barcelona, Spain). T34 was coated on the seeds at a concentration of 10^{10} cfu·g^{-1} and applied at 4 kg·Tn^{-1} of seed; the chemical fungicide was applied at 10 L·Tn^{-1} of seed. Temperature and relative humidity were measured throughout the experiment with a datalogger (PCE-HT 71N, PCE Ibérica, Albacete, Spain) located in the center of the trial at canopy level, protected from direct light. During the 113 days after sowing (DAS), the average temperature was 25.3ºC (maximum 38.0ºC and minimum 14.5ºC) and the relative humidity was 63%; (maximum 92.5% and minimum 27.0%). The plants were covered with a shading screen extended in the hottest hours of the day, 12:00-16:00 h (UTC+2: CEST), and when necessary a cooling system was activated to maintain a suitable temperature.

Three seed pools were used: seeds treated with the commercial biological fungicide (T34), active ingredient *T. asperellum* strain T34 (Biocontrol Technologies, S.L., Barcelona, Spain); seeds treated with a commercial chemical fungicide (Q), CELEST

XL active ingredient Fludioxonil 25% and Metalaxyl-M 1% (Syngenta Crop Protection, Basel, Switzerland); and a combination of both products (T34+Q). Previous to seed treatment, all seeds were surface disinfected before any application with 2% sodium hypochlorite (commercial bleach) for 10 min, and then rinsed three times with sterile distilled water before air drying in a laminar flow hood at room temperature for 2 h. Maize seeds (one per pot) were sowed 10 cm from the top of the substrate, using 10 L pots with coconut fiber:perlite:vermiculite (2:1:1, v/v) as an inert substrate with a pH of 6.5 (25ºC) and an electrical conductivity of 1 $mS \cdot cm^{-1}$. The plants were watered with Hoagland's solution diluted 1:2. There were two irrigation conditions: fully irrigated and drought condition, with 20 pots per treatment, giving a total of 120 pots. The pots were placed in two blocks in order to fit two independent lines of drip irrigation. The pots within each block were placed randomly. The chemical fungicide was applied in such a way as to mimic commercial practices with a well-used broad-spectrum fungicide.

The irrigation was the same in all pots for 35 DAS (from VE to V8 maize scale (Hanway & Ritchie, 1986)) using two drips per pot, which provide approximately 160±8.3 ml of water per application three times a day. The fertirrigation was applied automatically in the early morning, at the middle of the day, and in the late afternoon. Then, half watering was applied by removing one of the drips in half of the pots (mild water-stress) for a 28-day period (35-63 DAS, from V8 to R1 (silking) maize stage (Hanway & Ritchie, 1986). In this period, fertirrigation was applied three times per day, at a dose of 146±5.2 $mL \cdot pot^{-1} \cdot application^{-1}$, except for the stressed plants, where half of this watering was applied. After that, the drought stress was intensified by removing the remaining drip from the drought treatment pots (intensified water-stress) from 63 to 113 DAS (from R1 to R5 maize scale (Hanway & Ritchie 1986)). The well-watered plants (WW) continued receiving the fertirrigation three times per day with two drips per pot at a dose of 355±7.6 $mL \cdot pot^{-1} \cdot application^{-1}$. Nevertheless, both WW and water-stressed (WS) plants received additional water supply (one $application \cdot day^{-1}$, during five days distributed uniformly in this last 50-

day period) to avoid complete plant desiccation in WS. The drought stage lasted 78 days and the study ended at 113 DAS. A soil moisture meter (Jellas Soil pH Meter, Jellas Technology Limited, Hong Kong) was regularly used to monitor the soil moisture in the pots. Normal soil moisture levels (4 to 7 on a scale of 0-10) were always maintained for WW, while in WS values reached "dry" (<3) during the measurements and sampling of material.

T34 population

To assess the establishment and maintenance of the fungus, its population was determined by the serial dilution method and plate counting in *Trichoderma* spp. selective medium (Chung & Hoitink, 1990). Populations were evaluated in seeds at sowing time (3 to 5 seeds per treatment, T34, Q and T34+Q) as well as at the end of the experiment in the plant rhizosphere for five pots per treatment and condition and repeated twice. Dilutions were initiated with 1 g of seeds in a tube containing 9 mL of autoclaved saline solution (NaCl 9 $g \cdot L^{-1}$), or with 10 g of substrate from the soil rhizosphere (without removing the roots) in 90 mL of autoclaved saline solution and agitated for 30 minutes in a rotatory shaker.

Scanning electron microscopy images

To check for the effective adhesion of T34 on the seed surface, seed samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.4) at 4°C, then washed in phosphate buffer 5 times at 4°C and further processed with a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in phosphate buffer for 2 hours at 4°C and later washed with distilled water. Afterwards, the samples were dehydrated with ethanol and dried using the critical point procedure (Polaron Critical Point Drier). The samples were mounted in a standard Scanning Electron Microscope (SEM) support and coated with Au (Fisons Instruments SEM coating system). The secondary electron images were obtained using a SEM JEOL 7001 FEG at the Electron Microscopy Unit.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange parameters were determined to provide key information about plant physiology and photosynthetic machinery. Maize plants were measured with a Li-Cor 6400 system (Li-Cor, USA) using the youngest fully developed leaves. The measurements were recorded from an intermediate leaf position on one side of the central nerve. Four to five plants were used per treatment and water condition at three different stages: 63 DAS, at the end of the mild water stress (R1, silking), and at two points during intensified water stress, at 85 DAS (R3, milk stage) and, 104 DAS (R4, dough stage). The measurements were photosynthesis rate (An), transpiration (E), stomatal conductance (g_s), ratio of intercellular/extracellular CO_2 concentration (C_i/C_a), maximum quantum efficiency of photosystem II (Fv'/Fm'), and water use efficiency (WUE), calculated as the ratio An/E. These measurements were taken at saturating 1,500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance and 25ºC using a 2 cm^2 leaf chamber, and performed on clear days between 3 and 9 h after dawn, when photosynthesis was most likely to peak.

Water status, yield parameters and mineral nutrients

A simple and classic method for evaluation of plant hydric status is the leaf relative water content (RWC). It was measured on the same leaves as for the gas exchange measurements. RWC was calculated using the formula: RWC (%) = ((FW-DW) / (TW-DW)) x 100; where FW is the leaf fresh weight, TW is the leaf turgid weight after 24 h imbibition in water at 4ºC, and DW is the leaf dry weight after drying the sample to constant weight in an oven at 60ºC (Barrs & Weatherley, 1962).

Since drought and T34 application can affect nutrient absorption, mineral nutrients were determined in leaves at two different growth stages (85 and 104 DAS; R3 and R4, respectively) and in kernels at harvest (113 DAS) in the *Centres Científics i Tecnològics de la Universitat de Barcelona* (CCITUB, Spain). Moreover, at the end of the experiment (113 DAS) the dry weight/number of kernels per plant, plant height and shoot fresh/dry weight were determined for each treatment and condition

combination. Leaf samples were taken from the dried leaves used for the gas exchange, chlorophyll fluorescence and RWC measurements. All the samples were finely powdered using a Mixer Mill MM400 (Retsch GmbH, Germany). Then 100 mg per sample was digested with 2 mL concentrated HNO_3 and 2 mL H_2O_2 in a Teflon container at 90°C for 3 days. Analysis of Ca, K, Mg, Fe, P, Na and S was performed by inductively coupled plasma optical emission spectrometry (ICP-OES) using an Optima-3200RL (Perkin Elmer). Analysis of B, Cu, Zn and Mn was performed by ICP mass spectrometry (ICP-MS) using an Elan 6000 (Perkin Elmer).

Carbon and nitrogen content and isotope composition

The carbon and nitrogen concentration and the stable isotope composition (‰) of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) were determined in the leaves and kernels using an elemental analyzer (EA1108, Series 1, Carlo Erba Instruments) coupled to an isotope ratio mass spectrometer (IRMS, Delta C, Finnigan MAT) as described in Medina et al. (2016). The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were expressed in δ notation for carbon and nitrogen as follows: δ (‰) = ((Sample Isotope Ratio / Standard Isotope Ratio) –1) x 1000; where the standard refers to the international secondary standards of known stable carbon and nitrogen ratios (Medina et al., 2016).

Statistical analysis

Treatment main effects and interactions were determined by one- or two-way analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) test ($P < 0.05$). Results of T34 populations, nutrient elements and isotope parameters are mean values of three replicates per treatment and condition. Nutrient and isotope determination in kernel was performed on three ears of different plants per treatment and condition, being each of them a pooled sample of all grains in each ear. Results of gas exchange and water parameters are mean values of three to five replicates per treatment and condition. Results of kernel dry weight and number are mean values of four to seven replicates per treatment and condition. Results of plant height and plant shoot weight are mean values of seven

and three replicates per treatment and condition, respectively. Normality and homoscedasticity of the data was assumed in order to perform the ANOVA. Moreover, each plant represented an independent sample. The analysis was performed with GenStat 7th Edition software (VSN International Ltd., United Kingdom), while the graphics were produced with Sigma-Plot 11 software (Systat Software Inc., USA).

Results

Seed treatment and T34 population

SEM images (Fig. 1) showed the conidia of fungus attached to the seed in a stable and uniform manner. The adhesive applied surrounded the conidia and adhered it to the seed (Fig. 1a, b) while developing hyphae and conidia can be seen in the seeds once germinated (Fig. 1c, d).

Fungal population per seed (Table 1) just before sowing showed equal populations in both inoculated treatments (T34 and T34+Q) as expected. The Q treatment, on the other hand, presented negligible populations of T34, probably due to accidental contributions, but insignificantly in comparison to the treated seeds. In the rhizosphere, T34 populations were higher in T34 seed treatment, followed by seeds with T34+Q, and then Q. Interestingly, the application of the chemical reduced the proliferation of T34 by a factor of 10. Apart from that, significant results regarding water regime and treatment unexpectedly differentiated T34 populations, but to a lesser extent than for treatment only.

Physiological plant measurements

Gas exchange and chlorophyll fluorescence measurements were firstly recorded at a very early stage (35 DAS) before the application of the different water regimes (Suppl. Table S1). At this stage, these parameters were not significantly modified by the treatment factor. At 63 DAS, An and E increased significantly in the T34 treatments in comparison to Q treatment, especially under water stress conditions

in T34 (Fig. 2). C_i/C_a ratio and leaf RWC were slightly reduced by WS compared to WW and C_i/C_a ratio and g_s increased in T34 compared to T34+Q and Q (Fig. 3, Suppl. Table S1). At a more advanced reproductive stage (85 DAS), An, E, g_s and Fv'/Fm' decreased under WS compared to WW (Fig. 2, Suppl. Table S1). The highest E value was reached in the treatment T34+Q under WS conditions. C_i/C_a ratio decreased under WS, with the highest value obtained in T34 under WW, although it was significantly decreased by WS. Leaf RWC was similar between treatments under WW conditions, but interestingly it increased in T34 treatments (T34 and T34+Q) while it decreased in Q under WS (Fig. 3). These changes were similarly achieved for WUE, although they were not statistically significant. In the last growth stage, at 104 DAS, when the cob was ripening and the plant was starting to dry out, we found that An and g_s (Fig. 2) decreased compared to previous growth stages. We were surprised to find that An in T34 and T34+Q under WS was higher than in the Q treatment. However, g_s was similarly decreased by WS in the different treatments but not significantly (Suppl. Table S1). Associated with these changes under WS, we observed higher Fv'/Fm' and WUE in the T34 and T34+Q treatments compared to Q, with these values being even higher when only T34 was applied. Leaf RWC was strongly decreased by WS compared to WW, while it was higher in T34 treatments, particularly in T34+Q (Suppl. Table S1).

Elemental nutrient composition and isotopic measurements

Leaf mineral nutrient content and C and N isotope compositions at 85 and 104 DAS (Table 2) showed that the water regime significantly affected nutritional components including N, C/N, K, P, S, Mn, B, and Zn, as well as $\delta^{15}N$. Particularly, WS decreased N, P, S, Mn, B and Zn in all the treatments at 85 DAS which continued decreased at 104 DAS but now also K was increased. The $\delta^{15}N$ increased under WS at both times, while the significant decrease in N led to an increase in the C/N ratio in leaves (Suppl. Table S2). Meanwhile, treatment and interaction effects were barely significant: S and Zn were significantly modified by treatment, increasing their

content in leaves in T34+Q at 85 DAS, but this effect disappeared at 104 DAS (Suppl. Table S2). B was higher in T34+Q compared to the other treatments under WW at 104 DAS, while it was higher in T34 under WS (Suppl. Table S2). Results of kernel mineral nutrient content and C and N isotope compositions showed significant differences by treatment and water regime (Table 3, Suppl. Table S3 and S4). C and P contents were higher in T34 and T34+Q treatments than in Q treatment, being particularly higher in T34. WS conditions altered kernel nutrient and isotope composition (Table 3): N, P, Mg, Zn and $\delta^{13}C$ decreased by WS while Fe, C/N and $\delta^{15}N$ increased. The treatment x water regime interaction did not modify these parameters in the kernel, except for the $\delta^{13}C$ (Suppl. Table S3 and 4). It was strongly affected by WS in Q treatment and in a lesser extent in T34+Q, being not affected in T34.

Yield parameters

WS did not affect plant height but decreased shoot fresh/dry weight and dry weight of kernels per plant (Fig. 4, Suppl. Table S5). Shoot fresh weight was higher in T34 and T34+Q compared to Q, while a similar tendency was observed for shoot dry weight although it did not reach statistical significance. T34 treatment showed the highest number of kernels per plant compared to Q and T34+Q (Fig. 4), while the effects of water regime or the interaction were not significant (Suppl. Table S5). Dry weight of kernels per plant was significantly higher in T34 and T34+Q compared to Q under WW, while a similar non-significant tendency was observed under WS.

Discussion

The seed coating applied in this assay with *T. asperellum*, strain T34 was effective and the presence of the chemical fungicide did not affect the initial populations of maize plants (Fig. 1; Table 1). In general, many previous studies (Lifshitz, 1986; Harman, 1991; Prasad et al., 2002; Mastouri et al., 2010) showed that seed coatings excipients used in scientific publications (talc, pelgel, gum arabic, corn starch, methyl cellulose, polyethylene glycol) differ from those used industrially in seed

companies (synthetic microplastic-based polymers like the used here) and therefore are not comparable with the seed treatment used in our work. Chemical treatment alone presented low T34 populations due to initial cross-contamination, but it was insignificant compared to the treated seeds. Nevertheless, those populations in the rhizosphere remained in a similar range to naturally occurring populations of *Trichoderma* spp. in soil: 10^2-10^3 CFU·g^{-1} dry weight of soil (Longa et al., 2009) and were not altered by water regime (Table 1). At the end of the assay, the rhizosphere populations were significantly much higher in the seed treatments with T34 than in T34+Q, demonstrating the capacity of T34 to survive and establish itself in an inert soil from only an impregnated seed. As expected, the presence of the chemical fungicide lowered its populations. Interestingly, the interaction showed that populations increased under WS in T34+Q, but decreased in T34 in comparison to their respective WW treatment. This could mean that the plant may modulate T34 populations as needed, suggesting also that 10^4 cfu·g^{-1} may be inadequate for WS and 10^5 cfu·g^{-1} may be preferable. Other experiments with field trials showed that T34 can be found at 10^4 CFU·g^{-1} rhizosphere dry weight at the beginning of the maize reproductive stage (Fitó seeds, unpublished data) with the same maize hybrid, seed coating (T34+Q) and in optimal conditions. In Pandey et al., (2016), dose-dependent *T. harzianum* strain Th-56 showed a differentiated water-stress response in different rice genotypes, suggesting the need for a minimal dose of *T. harzianum* to influence crop physiology under drought, which correlates with the effects observed in our experiment.

In our experiment, water and treatment effects were more evident in late stages (grain filling) when drought was more evident and can strongly decrease kernel yield, in agreement with previous reports (Anami et al., 2009; Bista et al., 2018). However, in the case of photosynthetic capacity, An was significantly modified in early (63DAS) and late (104 DAS) stages, but not at 85 DAS, which could be associated with the specific genotype used in this experiment. In this sense, the fact that Fv'/Fm' and g_s were mainly affected in late growth stages when water supply

was very limited demonstrated that the photosynthetic machinery of our maize hybrid showed a certain drought tolerance. WS only decreased the C_i/C_a ratio and leaf RWC compared to WW at 63 DAS, changes that did not modify photosynthetic capacity. At 85 DAS, An decreased slightly under WS together with E, mainly due to stomatal closure (lower g_s) and a lower maximum quantum efficiency of photosystem II (Fv'/Fm'; Fig. 1, Suppl. Table S1). When water deprivation was intensified, coinciding with grain filling stage (104 DAS), we observed the same changes on photosynthetic capacity as before but more severe, also explained by a decrease in leaf water status (WUE, RWC) and CO_2 available for its fixation by Rubisco (C_i/C_a ratio). It is known that An decreases when water is scarce, due mainly to stomatal closure or inhibition of CO_2 metabolism (Asada et al., 2000; Javed et al., 2011; Vitale et al., 2011; Aslam et al., 2013), which is in agreement with our results. $\delta^{13}C$ values are known to decrease with increasing water stress in maize and other C_4 plants (Dercon et al., 2006). In our study, kernel $\delta^{13}C$ decreased under WS (Table 3 and Suppl. Table S3), highlighting that the plants suffered water stress during the experiment regardless of the treatment.

Regarding the treatment effects, at early stages (63 DAS) T34 treatments (T34 and T34+Q) promoted photosynthesis and transpiration compared to Q, particularly under WS (Fig. 1, Suppl. Table S1). Anyhow, T34 treatments showed some features suggesting a better water status, as an increase in E in T34+Q under WW or leaf RWC in T34 and T34+Q compared to Q. During the key growth stage of grain filling (104 DAS), and especially when water was severely limited in WS conditions, mimicking real conditions for field-grown maize in the Mediterranean basin, greater differences were observed. Although T34 seemed to not have an effect on photosynthetic capacity when water supply was optimal, it really influenced it under WS conditions. Indeed, application of T34 (T34 and T34+Q treatments) increased An and E by an improvement of light harvesting (Fv'/Fm') and leaf water status (WUE and RWC). These changes can also suggest the absence or reduction of damage to the photosynthetic machinery caused by drought stress. Similar improvements in

quantum efficiency were observed with *T. harzianum* T22 in *Arabidopsis* (Shoresh et al., 2010). Moreover, kernel $\delta^{13}C$ results showed higher values in the T34 and T34+Q treatments than in Q under WS (Suppl. Table S3), which may indicate that T34 improved water status throughout the experiment, since $\delta^{13}C$ provides information on the photosynthetic carbon assimilation and is a time-integrated indicator of the A_n/g_s ratio and WUE (Serret et al., 2018). The increase in shoot fresh weight in T34 and T34+Q compared to Q, but not significantly in shoot dry weight, clearly indicated that water content at whole plant level was promoted by T34 application. In carnation plants, enhancement of water uptake related to T34 is known, even in the presence of biotic pressure (Sant et al., 2010).

Leaf mineral nutrient content was modified by WS, indicating that the uptake of several nutrients (i.e., N, P, S, Mn, B and Zn) decreased due to this stress, except in the case of K at 104 DAS (Table 2 and Suppl. Table S2). $\delta^{15}N$ was modified by water conditions in leaves at two growth stages and in kernels at harvest. The nitrogen isotope composition ($\delta^{15}N$) is used to trace shifts in nitrogen metabolism, although it could be related to several processes and the underlying biochemical mechanisms is still not very well understood (Medina et al., 2016; Serret et al., 2018). In our experiment, its changes highlighted that it is a good indicator of changes in N metabolism, since the N content was affected significantly in leaves and kernels (Tables 2 and 3). Drought decreases nutrient uptake in most plants for several reasons, including a reduction of nutrient supply through mineralization and reduced nutrient diffusion in the soil (Bista et al., 2018). Related with this, E and g_s decreased in our experiment, which could directly influence nutrient uptake. These changes affected final kernel quality, decreasing the content of nutrients such as N, P and Zn, although Fe increased (Table 3). In many cases, drought stress lead to Fe deficiencies in plant (Ahanger et al., 2016). However, in our case its higher kernel content could reflect a greater Fe uptake and/or allocation during this experiment and can be related with the lower content of P and Zn, which are known to have opposite effects on Fe uptake (Ahanger et al., 2016).

The effects of T34 application on mineral nutrient content in leaves was scare. Although some nutrients (S and Zn) increased in T34+Q leaves compared to the other treatments at 85 DAS, these changes disappeared at 104 DAS. In the case of B, it increased in leaves of T34+Q under WW and T34 under WS. Its uptake is mostly a passive process, so it is greatly determined by the rate of water uptake by roots (Ahanger et al., 2016). Its higher content in leaves, at least in T34 under WS, could be related with the improvement of photosynthesis by the stabilization of membrane integrity and reduction of ROS (Ahanger et al., 2016), although further studies are needed to corroborate this hypothesis. C and P contents were higher in kernels in T34 and T34+Q compared to Q (Table 3), suggesting higher C assimilation, P uptake and/or translocation to kernels. Similarly, García-López et al., (2015) showed that plants treated with T34 improved P absorption in the presence of non-available P forms in cucumber. In Fernández et al., (2014), improvements of nutrient assimilation and C allocation, as well as other growth parameters were reported in T34-treated tomato plants. In spite of this better C content in kernels and photosynthetic machinery and leaf water status under WS, T34 did not prevent a decrease in kernel weight, suggesting that the drought stress performed in this study was severe. The maize responses to T34 under WS reported here demonstrate that the T34 population levels we measured are adequate to prevent water loss and promote photosynthetic capacity and stress avoidance in maize leaves but not sufficient to improve kernel dry weight and, then, grain yield under WS conditions. Nevertheless, T34 did promote kernel yield when the water regime was more favorable (i.e. WW conditions), highlighting the potential of T34 to improve maize yield under future climate change scenario and/or to reduce environmental contamination. Speculating about the mechanism by which T34 could alleviate drought stress in our experiment, authors such as Alwhibi et al., (2017) observed that *T. harzianum* increased phenol and flavonoid content in tomato plants affected by drought. Meanwhile, *T. asperelloides* T203 ameliorated plant growth under biotic stress by lowering deleterious ethylene (that accumulates under stress) thanks to

increased antioxidant activity (Shoresh & Harman, 2008; Brotman et al., 2013) which is again related to higher phenol synthesis (Ahmad et al., 2017). Increased antioxidant levels would degrade higher amounts of ROS (reactive oxygen species) and therefore protect photosynthesis. Harman et al., (2004) suggested that *Trichoderma* spp. metabolites or root colonization could modulate cellular and molecular changes in plants through modification of plant gene expression, thus causing a shift in plant responses.

In conclusion, in this work we observe the potential of T34 seed treatment under optimal conditions and abiotic stress in a key crop such as maize, which is susceptible to water stress around the world. T34 enhanced hydration and photosynthetic capacity under WS conditions. However, it was not sufficient to avert kernel dry weight decrease, probably due to the severity of the stress. In the context of climate change in Mediterranean regions, where periods of drought are increasingly present, the use of biological plant protection products against soil diseases (such as T34) can have secondary benefits for crops either under optimal conditions or under drought stress. Further studies are necessary to evaluate the positive effects of T34 application on leaf water status and photosynthetic capacity under different water regimes from mild to severe water stress, to understand its effects on plant metabolism and yield improvements, particularly in field-grown conditions and using a wider spectrum of maize genotypes with differentiated drought sensibility.

Acknowledgements

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(*Centre Científic i Tecnològic de la UB*) for performing electron microscopy observations and mineral nutrient and isotope determinations.

Author contributions

VE performed the assays, collected kernels, sampled, and analyzed tissues, analyzed the data and wrote the manuscript. RV and OV performed the gas exchange measurements and contributed to the writing and critical revision of the manuscript. JJN helped design and perform the seed treatments and critically reviewed the manuscript. MIT provided technical advice concerning T34 application, experimental design and analysis of the results, and reviewed the manuscript.

Abbreviations

WW: well-watered conditions/plants

WS: water-stressed conditions/plants

RWC: relative water content

An: rate of photosynthesis

E: transpiration

Fv'/Fm': maximum quantum efficiency of photosystem II (PSII)

$\delta^{13}C$: stable carbon isotopic composition

$\delta^{15}N$: stable nitrogen isotopic composition

Figures

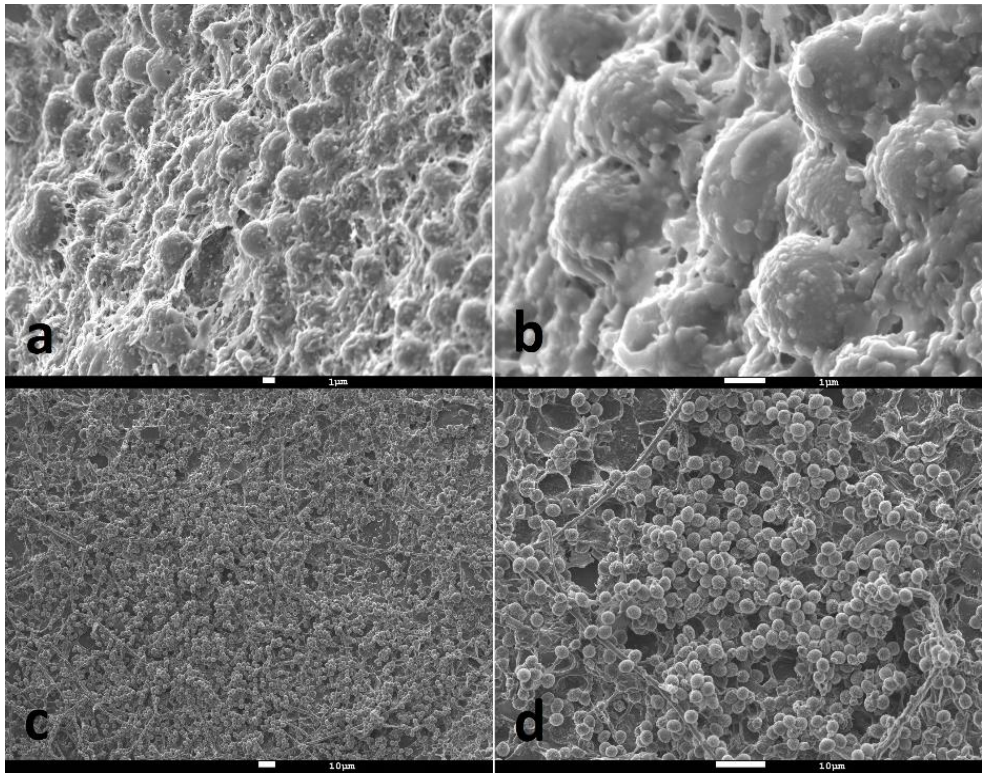

Figure 1. Scanning electron microscopy (SEM) images of corn seed treated with *T. asperellum* strain T34. (a) and (b): Conidia adhered to the surface of a seed corn just after T34 treatment. (c) and (d): Germinated hyphae and/or conidia of T34 on germinated corn seed.

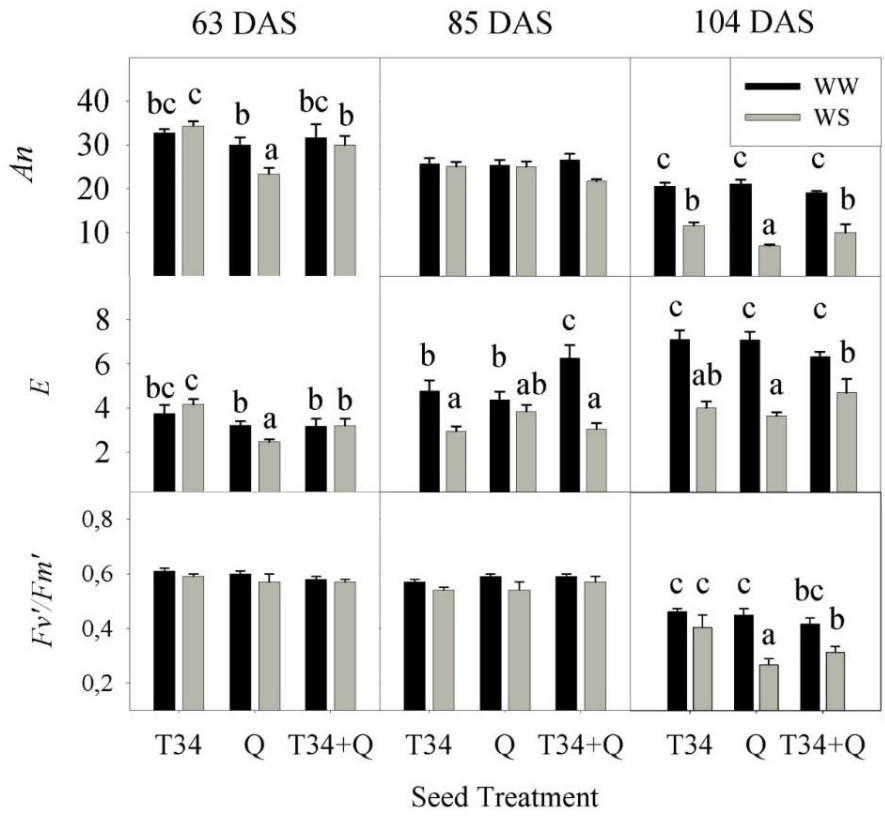

Figure 2. Gas exchange and fluorescence measurements. Rate of photosynthesis (*An, µmol $CO_2 \cdot m^{-2} \cdot s^{-1}$*), transpiration (*E, nmol $H_20 \cdot m^{-2} \cdot s^{-1}$*) and maximum efficiency of photosystem II (*Fv'/Fm'*) were recorded in well-watered (WW) and water-stressed (WS) plants at three time points (63, 85 and 104 days after sowing: DAS) and for three different seed treatments (*T. asperellum* strain T34 (T34); chemical fungicide (Q); and their combination (T34+Q)). Plants were subjected to mild stress for 28 days (until 63 DAS), and intensified stress for the rest of the assay (until 85 and 104 DAS). Each value is the mean of 3-5 replicates per treatment and condition. Significant effects were determined by two-way ANOVA ($P < 0.05$). Different letters denote statistical significance for the interaction. Figures with no letters showed no significant differences in the interaction. Mean values in Suppl. Table S1.

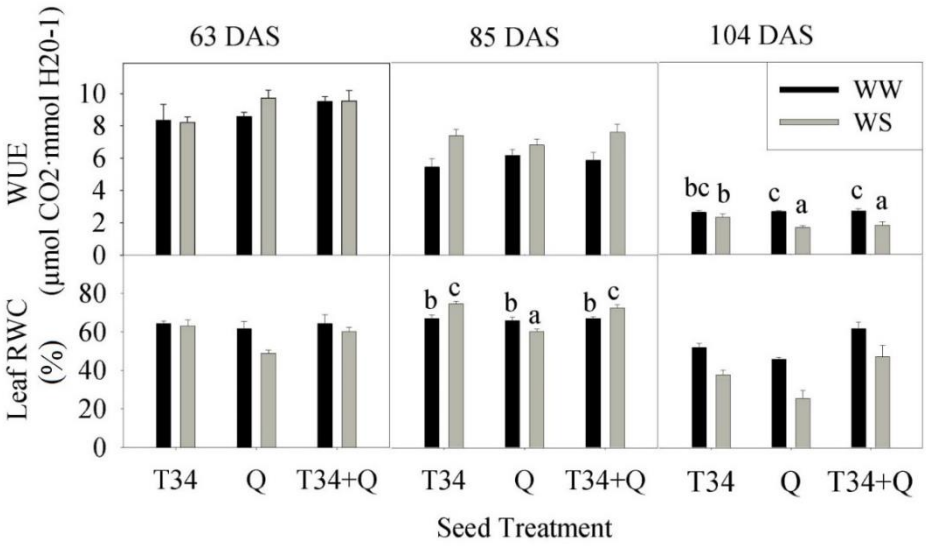

Figure 3. Water use efficiency (*WUE*) and leaf relative water content (*RWC*) were recorded in well-watered (WW) and water-stressed (WS) plants at three time points (63, 85 and 104 days after sowing: DAS) with three different seed treatments (*T. asperellum* strain T34 (T34); chemical fungicide (Q) and their combination (T34+Q)). Plants were subjected to mild stress for 28 days (until 63 DAS), and intensified stress for the rest of the assay (until 85 and 104 DAS). Each value is the mean of 3-5 replicates (n = 3-5). Significant effects were determined by two-way ANOVA ($P < 0.05$). Different letters denote statistical significance for the interaction. Figures with no letters showed no significant differences in the interaction. Mean values in Suppl. Table S1.

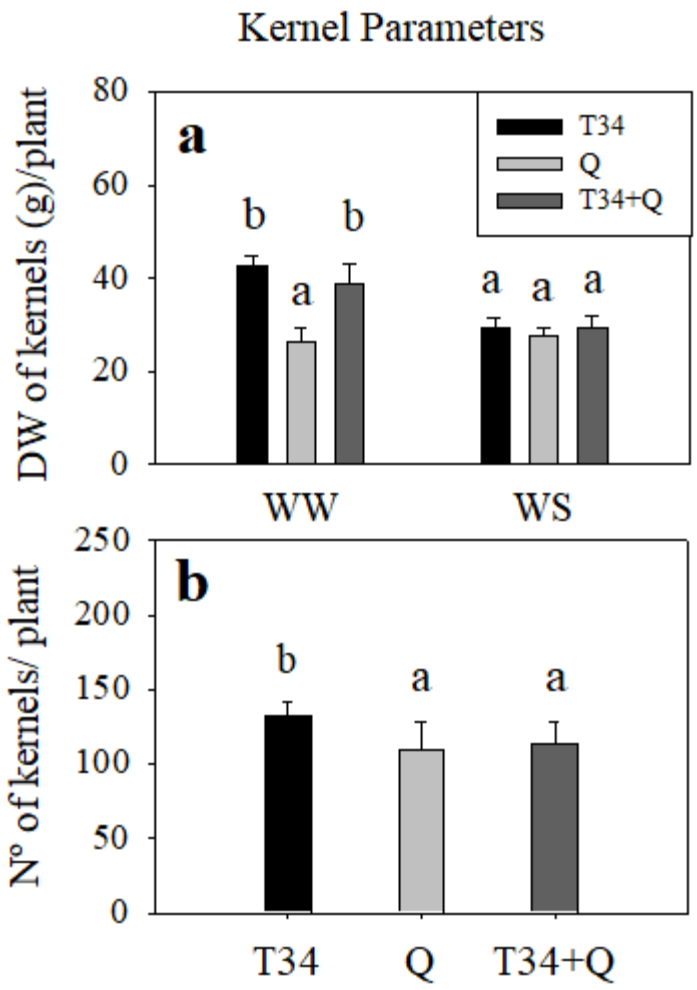

Figure 4. Kernel parameters: dry weight of kernels (a) and number of kernels (b) at the end of the greenhouse study (113 days after sowing: DAS) of an experimental maize (*Zea mays*) variety grown under different seed treatments (*T. asperellum* strain T34 (T34); chemical fungicide (Q); and their combination (T34+Q)). The plants were subjected to mild stress for 28 days (until 63 DAS), and intensified stress for the rest of the assay (until 85 and 104 DAS). Each value is the mean of 4-7 replicates. Significant effects were determined by two-way ANOVA ($P < 0.05$). Different letters denote statistical significance for the interaction. Mean values in Suppl. Table S5.

Table 1 T34 population in the seeds at the beginning of the experiment and in the rhizosphere at 104 days after sowing. T34: *T. asperellum* T34 seed treatment; Q: chemical fungicide seed treatment; T34+Q: combination of *T. asperellum* T34 and chemical fungicide; WW: well-watered, WS: water-stressed (WS). CFU: colony forming units of T34 per gram of dry weight of substrate.

Beginning of assay (treated seeds)[a]				
Log (CFU of T34·seed^{-1})				
T34	6.08 ± 0.07 b		P-value	LSD value
Q	1.09 ± 0.11 a	Log (CFU of T34·kernel^{-1})		
T34+Q	6.31 ± 0.03 b	T	<0.001	0.2497
End of assay (rhizosphere)[b]				
Log (CFU of T34·(g of dry weight of substrate)$^{-1}$)				
T34	5.22 ± 0.03 c		P-value	LSD value
Q	2.14 ± 0.03 a	Log (CFU of T34·(g of dry weight of substrate)$^{-1}$)		
T34+Q	4.30 ± 0.05 b	T	<0.001	0.0981
WW	3.87 ± 0.22 a			
WS	3.90 ± 0.22 a	C	0.461	0.0801
T34 WW	5.33 ± 0.03 e			
T34 WS	5.10 ± 0.04 d			
Q WW	2.17 ± 0.03 a			
Q WS	2.10 ± 0.06 a			
T34+Q WW	4.11 ± 0.06 b			
T34+Q WS	4.50 ± 0.03 c	T*C	<0.001	0.1387

[a,b] For the treated seed and rhizosphere populations, values were log-transformed mean values ± SE of 3-5 seeds or five pooled pots, respectively, for each treatment and condition, repeated twice. Significant effects were determined by two-way ANOVA ($P < 0.05$) plus Fisher's LSD. Different letters in each section indicate significant differences ($P < 0.05$).

Table 2 P-value of leaf concentration, stable isotopic composition (‰), macro- and micronutrients at 85 and 104 days after sowing (DAS). T: seed treatments (*T. asperellum* strain T34, chemical fungicide and their combination), C: water condition (well-watered and water-stressed), and TxC: interaction.

	85 DAS			104 DAS		
	T	C	TxC	T	C	TxC
C	0.562	0.739	0.308	0.424	0.086	0.221
$\delta^{13}C$	0.861	0.943	0.648	0.284	0.474	0.702
N	0.694	**0.027**	0.313	0.356	**<0.001**	0.543
$\delta^{15}N$	0.289	**<0.001**	0.446	0.445	**<0.001**	0.646
C/N	0.896	**0.007**	0.421	0.633	**<0.001**	0.637
K	0.657	0.45	0.424	0.563	**0.029**	0.286
Ca	0.105	0.107	0.826	0.218	0.113	0.643
Mg	0.873	0.086	0.598	0.371	0.131	0.09
P	0.176	**<0.001**	0.668	0.681	**<0.001**	0.144
S	**0.031**	**<0.001**	0.257	0.817	**<0.001**	0.286
Fe	0.719	0.369	0.178	0.765	0.095	0.102
Mn	0.556	**<0.001**	0.163	0.416	**<0.001**	0.658
Cu	0.724	0.056	0.798	0.288	0.303	0.141
B	0.602	**<0.001**	0.533	0.5	**<0.001**	**0.01**
Zn	**<0.001**	**<0.001**	**0.013**	0.546	**0.018**	0.974

Each value is the mean of 3 replicates. Significant effects were determined by two-way ANOVA. Different letters denote a statistical significance in Fisher's LSD *post hoc* ($P < 0.05$). C, N, K, Ca, Mg, P, S, Na and Fe: mg of nutrient· (g leaf dry weight)$^{-1}$. Mn, Cu, B and Zn: µg of nutrient·(g leaf dry weight)$^{-1}$. Mean values can be found in Suppl. Table S2.

Table 3 C and N content and stable isotopic composition (‰), C/N ratio, and content of other macro- and micronutrients in kernels at the end of the assay (113 days). Measurements were taken in well-watered (WW) and water-stressed (WS) plants, with the following seed treatments: *T. asperellum* strain T34 (T34), chemical fungicide (Q) and their combination (T34+Q).

	C		$\delta^{13}C$		N		$\delta^{15}N$		C/N	
T34	**425.14 ± 2.58**	**b**	-11.799 ± 0.04	a	15.83 ± 0.39	a	2.06 ± 0.45	a	26.98 ± 0.60	a
Q	**416.04 ± 2.24**	**a**	-11.883 ± 0.09	a	15.70 ± 0.44	a	1.79 ± 0.50	a	26.67 ± 0.69	a
T34+Q	**422.03 ± 1.93**	**ab**	-11.757 ± 0.06	a	16.39 ± 0.50	a	2.14 ± 0.44	a	25.95 ± 0.73	a
WW	420.86 ± 1.82	a	**-11.662 ± 0.03**	**b**	**16.48 ± 0.39**	**b**	**0.69 ± 0.08**	**a**	**25.72 ± 0.57**	**a**
WS	421.28 ± 2.30	a	**-11.964 ± 0.04**	**a**	**15.46 ± 0.27**	**a**	**3.30 ± 0.17**	**b**	**27.35 ± 0.44**	**b**
	K		Ca		Mg		P		S		Fe		Zn	
T34	0.96 ± 0.08	a	5.84 ± 0.44	a	0.31 ± 0.02	a	**0.85 ± 0.05**	**b**	0.24 ± 0.01	a	5.24 ± 0.53	a	5.05 ± 0.33	a
Q	0.84 ± 0.04	a	6.25 ± 0.26	a	0.33 ± 0.01	a	**0.72 ± 0.03**	**a**	0.28 ± 0.01	a	5.88 ± 0.59	a	6.01 ± 0.60	a
T34+Q	0.89 ± 0.07	a	5.61 ± 0.43	a	0.30 ± 0.02	a	**0.80 ± 0.02**	**ab**	0.28 ± 0.02	a	6.12 ± 0.85	a	5.85 ± 0.48	a
WW	0.92 ± 0.06	a	6.31 ± 0.29	a	**0.34 ± 0.01**	**b**	**0.85 ± 0.03**	**b**	0.28 ± 0.01	a	**4.48 ± 0.34**	**a**	**6.25 ± 0.39**	**b**
WS	0.87 ± 0.05	a	5.49 ± 0.28	a	**0.29 ± 0.01**	**a**	**0.73 ± 0.03**	**a**	0.25 ± 0.01	a	**7.01 ± 0.29**	**b**	**5.03 ± 0.30**	**a**

Each value is the mean of 3 replicates. Significant effects were determined by two-way ANOVA. Different letters denote a statistical significance in Fisher's LSD post hoc ($P < 0.05$). C, N, K, Mg, P and S (mg of nutrient) and Ca, Fe and Zn (µg of nutrient· (g kernel dry weight)-1). Mean values of the interaction and p-values in Suppl. Tables S3 and S4.

Study III: Field Evaluation of a biological seed treatment with Trichoderma asperellum strain T34 on maize (Zea mays) against Late Wilt (Harpophora maydis)

Field Evaluation of a biological seed treatment with *Trichoderma asperellum* strain T34 on maize (*Zea mays*) against Late Wilt (*Harpophora maydis*)

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Abstract

Late Wilt of Maize is a fungal disease caused by *Harpophora maydis* (formerly *Cephalosporium maydis*) that produces rapid wilting of plants at the late stages of corn development before ear maturity, causing significant losses in yield. Here we evaluated the beneficial fungus *Trichoderma asperellum* strain T34 applied as a seed treatment in natural infected field with four experimental corn varieties with different disease tolerance. Results show diminished disease incidence with T34 in all corn lines in all years. Regarding yield (g/plant), T34 improved final production per plant in variety B and D in both years (medium and tolerant lines, respectively) while no improvement was found in variety A and C (sensible and medium lines), possible explained by genotype specificity.

Key words: Field assay, *Cephalosporium maydis*, final yield, percentage of disease incidence.

1. Introduction

Late Wilt of Maize, also called black bundle disease, is a fungal disease of corn caused by the vascular soil-borne *Harpophora maydis* (formerly known as

Cephalosporium maydis) (Samra et al., 1962). The only known hosts of the pathogen are *Lupinus* (lupine plant) and *Zea mays* (corn) (Sabet et al., 1966). The disease is characterized by rapid wilting of maize plants, typically late in the crop cycle, just before tasselling and until shortly before maturity where normal dehydration of the plant occurs (Sabet et al., 1970). The drying of the plant starts at the bottom or lower parts to the upper part of the plant (Sabet et al., 1966).

2. Materials and Methods

2.1 Plant Material and seed treatment.

Four maize experimental lines were used in field assays provided by Fito Seeds (SEMILLAS FITÓ). Specific agronomic details of each assay can be found in **Table 1**. Seeds were treated with CELEST XL (Syngenta) and CELEST XL plus T34 in a film coating at 0.4 g/100g maize seeds of T34 at 10^{10} CFU/g. Specific details of the seed treatment are owned by SEMILLAS FITÓ.

2.2. Field assays and plot design

Field assays were carried out in the same location in Seville, along three years (two of them consecutive). The field has been presenting presence of Late Wilt of maize (*Harpophora maydis)* for at least five consecutive years. The field does not present other pathologies other than sporadic observations of *Ustilago maydis* (personal communication – Fitó Seeds employees in Don Benito, Extremadura). The type of soil was Calcaric Fluvisol (Jc) following WMS Soil map visualization tool (Junta de Andalucía 2005) (**Fig. 1**). Each plot size was 4 rows of 2 m x 7 m long, 220 seeds were planted per plot giving a density of 15.7 plants/m^2. The field design was an unbalanced complete design as plot number changed through the three-year assays: 3 and 12; 2 and 3 and 3 and 18 (treated and untreated, respectively) replicates were done per treatment and variety. Other treatments were tested but are not presented here (data not shown). Assays were surrounded by 4 to 6 rows of another corn line sowed transversally to the plots to reduce variability in field for the plot assay. In all years, Variety A and D (sensible and tolerant lines) were present.

Only in the last two consecutive years, two more corn lines were included (B and C, medium tolerance lines). Specific information for the assays were detailed in **Table 2**. Climatic conditions for air temperature, relative humidity, precipitation and solar radiation for the two consecutive field assays with four corn lines were extracted from the nearest SiAR (*Sistema de información Agroclimática para el Regadío*) station SE22 "*Villanueva del Río y Minas*" (Position: UTM X: 262609; Y: 4164000; Time Zone: 30; Altitude: 31; Installation date: April 2011, Meteorological station property of the Ministry of Agriculture, distance from field assay: 6.5 km aprox) and are presented in **Fig. 2**.

2.3 Disease incidence and final yield determination

Incidence determination was done at 98, 111 and 137 days after sowing corresponding to the counting all fully wilted plants and total number of plants from the two central rows of each plot (**Fig. 3**). Disease incidence was expressed as follows: Disease Incidence (%) = (fully wilted plant number/total plant number) x 100 for each plot. Irrigation was done by flooding as needed. Yield determination was done harvesting all ears from each plot and calculating percentage of relative humidity of the grain (% Moisture), weighting all cobs per plot and adjusting for 14% moisture. Yield per plot at 14% Moisture = ((100 - % Moisture of plot)/ (100 – 14))*Weight (g) per plot. Yield by plant was expressed as follows: Yield (g/plant) = total weight by plot at 14% /total number of plants in each plot. Test Weight (Kg/hl) was determined by measuring the weight and volume of a representative sample of grains from each plot. Results of disease incidence and yield were discussed qualitatively as small and unequal sample size was obtained.

Results

In the comparison of variety A and D (sensible and tolerant lines) three year assay results (**Fig. 4 - left**) showed that variety A had increased levels of disease incidence (%) in comparison to variety D which was the tolerant line. Percentage of fully wilted plants ranges from 18 - 22% and 7 – 8% (Control and T34, respectively) in Variety A

and 0 – 6% and 0 – 3 % (Control and T34, respectively) in variety D. T34 treatment presented diminished percentage of disease incidence in every year and variety.

In the comparison of the two year field assay with four varieties (**Fig. 5**): Assessment in the field showed higher incidence in Variety A (sensible), followed by maize Variety B (medium tolerance), followed by Variety C (medium tolerance) and finally Variety D (tolerant) in both years. Percentage of fully wilted plants ranges from 0 to 22% in both years at the measured time. T34 treatment presented diminished percentage of disease incidence in comparison to control treatment in every corn line in the trial, in both years.

Regarding mean yield results per plant (g/plant) (**Fig. 6**) lower global values were observed in the second year. In the first year of the trial, higher values were obtained in T34 treatment in all maize lines except for the sensible line. In the second year, highest values were obtained in T34 treatment in the Variety D (Tolerant) and Variety B (medium tolerance), followed by Variety A (sensible line). Lower values in yield were obtained in Variety C (medium tolerance). Minimum, maximum, and mean relative humidity (%) of the cob was: 12, 23, 19% and 10, 13, 12% for the first year and second year, respectively. Minimum, maximum, and mean test weight (Kg/hl) (weight/volume) was 64, 82 and 74 for the first year. Yield measurements and Test weight measurements for the first and last year (respectively) of could not be properly collected.

Discussion

Regarding the results on percentage of disease incidence (**Fig. 4 and 5**), we observed that maize line B has a greater sensibility to Late Wilt disease than Variety C that obtained more similar data with the tolerant line (Variety D). Our results agree with the previous information we had regarding the varieties (**Table 1**).

In agreement with García-Carneros et al., (2012) initial incidence of late wilt symptoms in maize plants depends on the isolate of *C. maydis* and the maize variety,

in accordance with our results, with the same infested field similar values of disease incidence were found in different years.

Previous work with *Trichoderma* strains in corn affected by *Harpophora maydis (*non-natural affectation*)* by Elshahawy & El-sayed, (2018) observed that four *Trichoderma* strains (*T. harzianum, T. viride, T. virens* and *T. koningii*) applied separately by soaking 1mL/seed of 10×10^4 propagates/mL of dH_20, reduced disease incidence percentage at 80 and 110 days after sowing (in greenhouse and field assay, respectively) with a decrease of 28 – 43 % and 16 – 23 % depending on the *Trichoderma* strain. In our work in a natural infected field, the reduction in T34-treated seeds was to up to 13%. Although these authors reported greater disease incidence reduction, artificial inoculation and genotype used influenced disease incidence of control plants (73 and 48 % in greenhouse and field, respectively) while in our work we obtained up 18-22% in the sensible (Variety A) corn line.

Regarding yield data (**Fig. 6**), heterogeneous number of plants and yield was observed (data not shown) between years where environmental and soil conditions (apart from disease incidence) affected final harvest. As exposed by Elshahawy & El-sayed, (2018), four different *Trichoderma* strains (previously described above) improved average grain by plant in comparison to control plants in artificially-infected field assay where an increase between 7 and 21 % was obtained (depending on the *Trichoderma* strain) which is partially in agreement with our results, where we obtained an improvement in both years in two corn lines (B and D) of 8 – 43% of final yield per plant where in the remaining corn varieties (A and C) obtained mixed results as final yield per plant varied between -29 and 13 %, possible by a T34-genotype specificity.

In this report we observed how a microbial seed treatment based in T34 can improve maize behaviour in a natural infected field by a soil pathogen like Late Wilt of maize (*Harpophora maydis*) either by reducing disease incidence, and therefore symptoms of the disease and/or improving its yield in two consecutive years in a

field that is naturally infected for at least five years. Further research is needed in order to study if these changes are significantly different and how this enhanced tolerance correlates with T34 seed treatment. Also, if T34 improvement of yield (g/plant) is genotype-specific by corn line.

Conclusion

In our work, T34 seed treatment diminished percentage of disease incidence in four experimental corn lines (with differentiated disease tolerance) in a naturally infected field in all years. These results show that this biological seed treatment is a functional method for fighting soil borne fungal disease *Harpophora maydis*. Results by yield in g/plant, showed improvement in variety B and D (medium and tolerant lines, respectively) while in variety A and C (sensible and medium lines) we obtained mixed results. These results suggest T34-genotype specificity.

Finally, further work is needed to study a) whether differences are significant, b) the mechanisms of this increased stay green by T34 and c) how correlates yield with plant host genotype.

Acknowledgements

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Figures

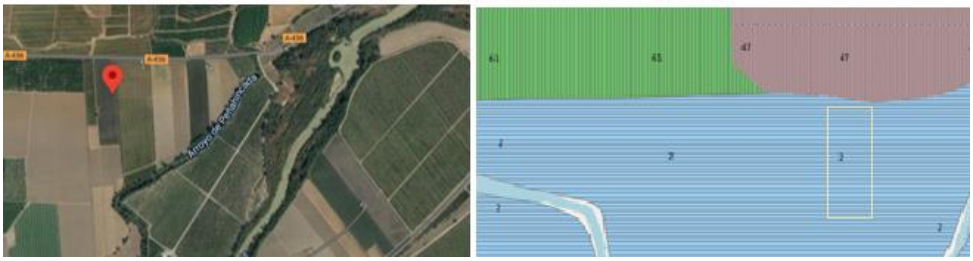

Figure 1. Upper image of the location of the field assays. Right image is the soil type visualization map of the location of the field assays. Field assays performed in the highlighted brown quadrant.

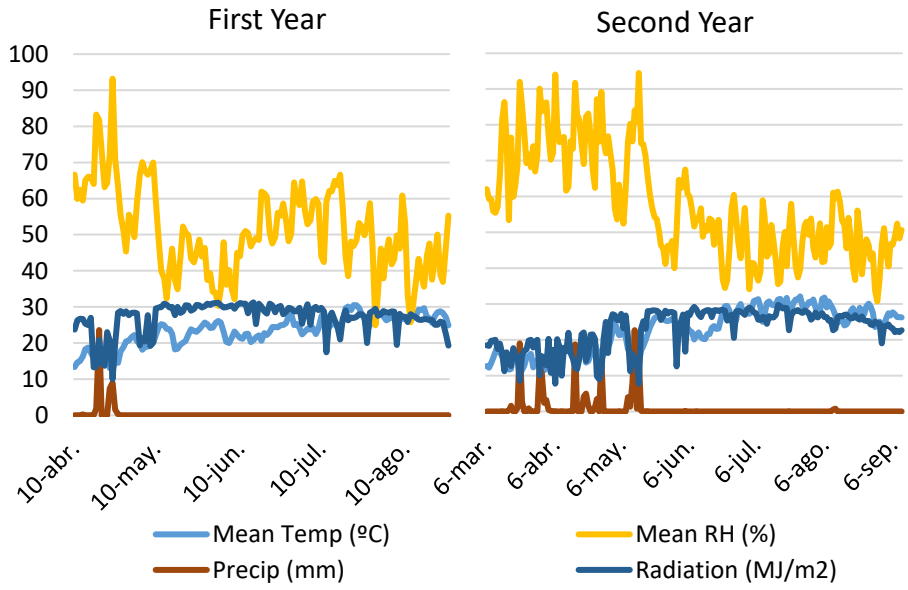

Figure 2. Climatic data from the two field assays from sowing and until harvest approx. Mean temperature (ºC), Mean relative humidity (%), Precipitation (mm) and solar radiation (MJ/m2). All variables follow left axis except for solar radiation that follows right axis. All values are daily measurements.

Figure 3. Second year first assessment images. Left image shows a lane of separation, fully wilted plants can be seen on the left. Right images show the upper aerial part and lower part (stalk) of wilted maize plants (right part) and healthy plants (left part) taken at 137 days after sowing.

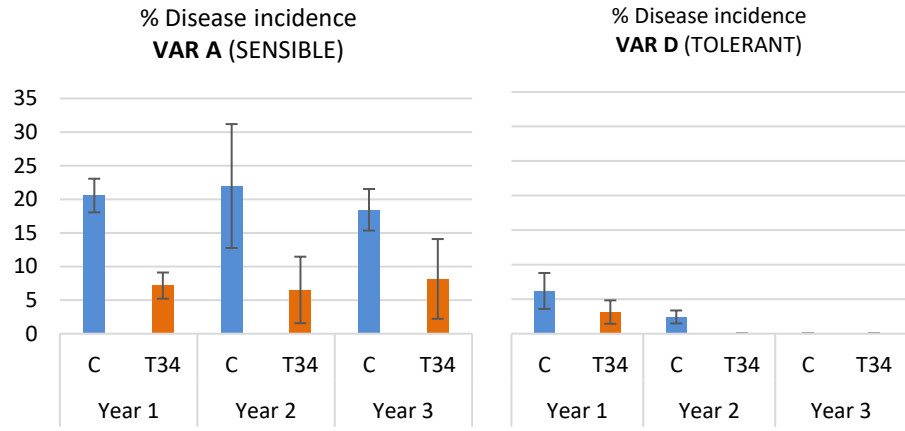

Figure 4. Percentage of Incidence of *H. maydis* disease by counting fully wilted plants at 98, 111 and 137 DAS (days after sowing) corresponding to the three-year assays respectively in Variety A (left) and Variety D (right).

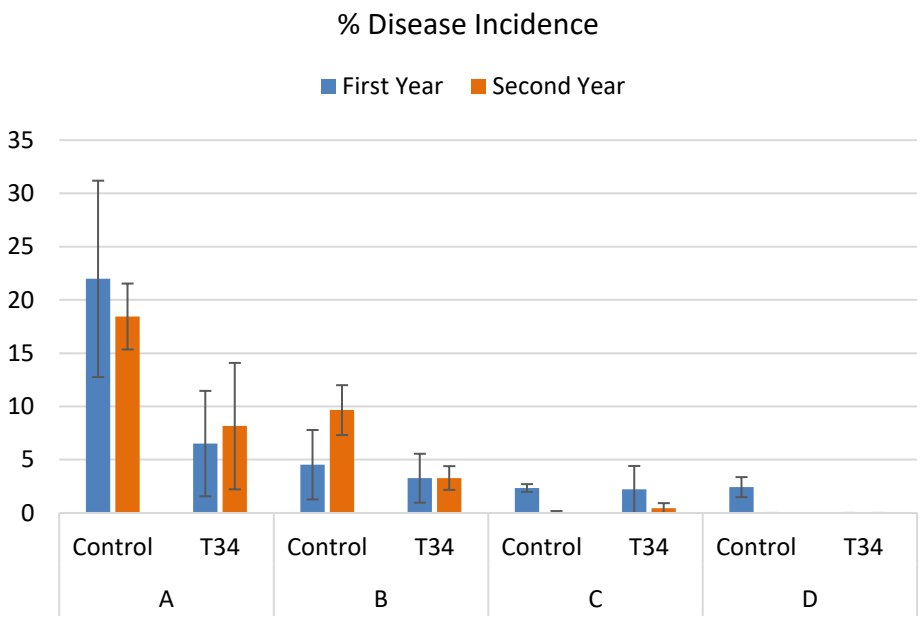

Figure 5. Percentage of Incidence of *H. maydis* disease by counting fully wilted plants at 111 and 137 DAS (days after sowing) corresponding to the first and second year of field assays.

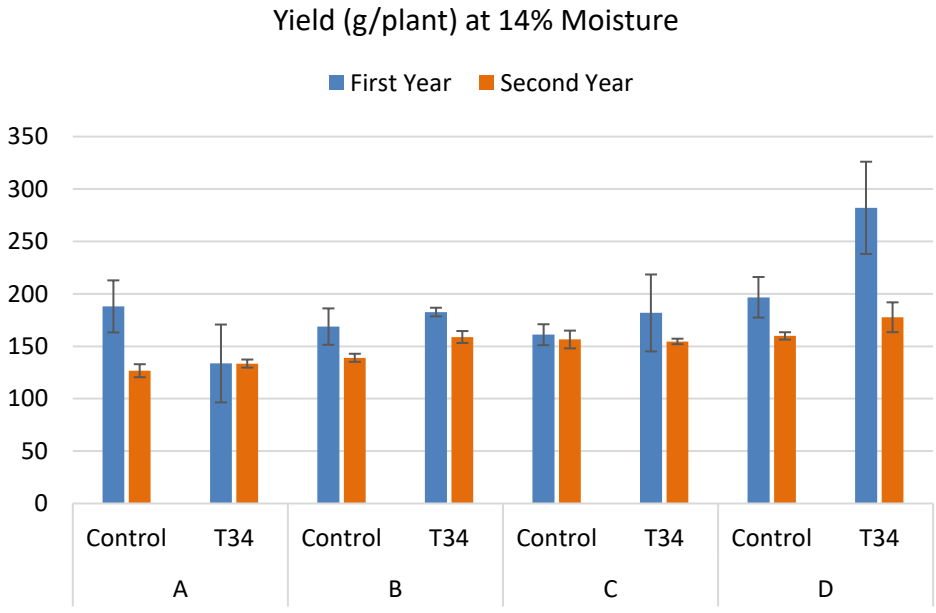

Figure 6. Mean Yield at 14% Moisture (g/plant) at 138 and 174 DAS (days after sowing) corresponding to the first and second year of field assays.

Tables

Table 1. Agronomical characteristics of the four varieties used in field assays. Tol: corn genotype tolerance for Late Wilt (Sen: sensible; Med: medium; Tol: Tolerant). H: Plant Height. SG: stay green of the plant. Use: G: grain; S: silage. W1000: weight of a thousand grains/kernels. TW: test weight (density of grains). NREAR: number of rows per ear.

Var	Tol	FAO	Vigor	H (m)	Stay green	Use	W 1000 (g)	TW (Kg/hl)	Kernel shape	NREAR
A	Sen	600	+++	2,70	Good	G	382	75	Toothed	18
B	Med	700	++++	2,70	Excellent	G	420	74	Toothed	16-18
C	Med	600	++++	2,80	Good	G	490	78	Vitreous	16
D	Tol	700	+++	3,00	Medium	G, S	380	78	Toothed	16-18

Table 2. Assay location and planting details. Field assays were carried out in *Alcolea del Río, Sevilla* (Spain).

Assay	GPS location	Sowing date	Disease Evaluation	Harvest date	Variety
1	37.617478, -5.620986	May, 30	September, 5	-	A, D
2	37.617478, -5.620986	April, 10	July, 30	August, 26	A, B, C, D
3	37.614470, -5.621984	March, 6	July, 21	August, 27	A, B, C, D

Study IV: Microbial seed treatment in parental and hybrid lines of cucumber (Cucumis sativus) influence fruit and seed production and final seed microbiome.

Microbial seed treatment in parental and hybrid lines of cucumber (*Cucumis sativus*) influences fruit and seed production and final seed microbiome

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Abstract

Cucumber is one of the most important crops from the *Cucurbitaceae* family, with Europe having the second largest share in cucumbers. The *Trichoderma* genus is the most successful biofungicide used worldwide. Certain species can protect several crops against multiple types of biotic and abiotic stress as well as promote plant growth and yield. In this work, we studied the effects of *Trichoderma asperellum* strain T34 as a seed treatment in parental lines of cucumber and their resulting hybrid. T34 influenced flower number, significantly increasing early fruit and seed yields in the parental lines and total fruit yield and diameter in the hybrid grown under springtime greenhouse conditions. Analysis of the progeny seeds showed no differences in germination and vigour, although we observed a modified seed bacteriome compared to the seeds obtained from untreated plants. There was an increased presence of the Bacteroidetes, Firmicutes and Actinobacteria phyla at the detriment of Proteobacteria (particularly *Enterobacteriaceae*), indicating a far-reaching impact of T34 on plant physiology and seed microbiome.

Key words: Mediterranean greenhouse*, Trichoderma asperellum* strain T34, seed microbiome, seed yield, fruit yield.

1. Introduction

Plant material

Cucumber (*Cucumis sativus*) is one of the most economically important members of the *Cucurbitaceae* family. In 2017, 84 million tonnes of cucumbers and gherkins were produced globally. Europe has the second highest share of cucumbers (7.4%), with Asia being the largest producer (87.9%) (FAO, 2019). The *Cucurbitaceae* family is usually distributed in the warmer regions of the world, given their sensitivity to frost. Gynoecious cucumber varieties (which produce only female flowers) are commonly used in seed production and are grown under protected conditions. Moreover, staminate flower (male flower) induction is necessary in the production of F1 hybrids (Verma et al., 2018). Modern cucumber cultivars also tend to have varying levels of parthenocarpy (cucurbit fruit set that can occur without pollination or fertilisation) (Nerson, 2007). Cucumber production requires a high level of fertilisation due to the high plant density and long production times (Emad et al., 2017). Breeding and cultivation techniques have been extensively studied in cucumber fruit and seed production (Ruiz & Romero, 1998; Nerson, 2007; Kaddi et al., 2014; Emad et al., 2017; Verma et al., 2018). There is currently an increasing amount of research on targeted and/or sustainable applications.

Seed treatment

Seed treatment (or coating) is the most prominent example of targeted applications. Seed coating consists of applying one or more natural and/or synthetic products to the seed to impregnate it before sowing. The aim is to improve handling, protection, germination, plant establishment and yield (Malusá et al., 2012; Cumagun, 2014; Singh et al., 2016; Pedrini et al., 2017). Transfer of plant pathogens through seeds is a major mode of transmission and it is therefore important to predict disease emergence and spread that can potentially affect seed quality (Shade et al., 2017).

Applications of *Trichoderma* spp.

This genus is the most successful biofungicide used globally, occurring in more than 60% of registered products worldwide (Verma et al., 2007). *Trichoderma* fungi are antagonists against several soil-borne plant pathogens, being involved in mycoparasitism, antibiosis, competition and also the induction of plant resistance (Verma et al., 2007; Waghund et al., 2016). Certain *Trichoderma* species can interact with other microbes in the rhizosphere, protecting plants against biotic and abiotic stress as well as promoting plant growth and yield (Harman et al., 2004; Hermosa et al., 2012a; Fernández et al., 2014). For example, *Trichoderma harzianum* Rifai strain T-22 has been reported to alleviate the disease caused by *Pythium ultimum* and protect against abiotic stress such as osmotic, saline, cold and heat stress among germinating seeds and seedlings in tomato (Mastouri et al., 2010). Furthermore, the combination of different antioxidants with compost and different strains of *Trichoderma* has been observed to reduce the incidence of the damping-off disease caused by several *Fusarium* species in cucumber, even promoting fruit yield and quality (Elwakil et al., 2015). Other studies have observed that *T. asperellum* (previously *T. harzianum*) T-203 increases cucumber root growth, root length, root surface area and the number of root tips in both greenhouse and hydroponic experimental systems in the absence of any stress (Yedidia et al., 2001). Moreover, it can induce systemic acquired resistance against the bacterial plant pathogen *Pseudomonas syringae pv. lachrymans* (Yedidia et al., 2003). Altintas & Bal, (2005) noted that the total yield of cucumbers grown in an unheated glass greenhouse was enhanced by the soil application of commercial *T. harzianum* (TrichoFlow WP, Agrimm Technologies, New Zealand) in three different cultivars. Likewise, *T. asperellum* strain T34 has been observed to increase Fe accumulation in cucumber depending on the soil and glucose addition (Santiago et al., 2013), increase total P content in roots (García-López et al., 2015), activate plant defences against fungi, bacteria and nematodes (Segarra et al., 2009; Martínez-Medina et al., 2017; Pocurull et al., 2020), and suppress *Rhizoctonia solani* in cucumber seedlings (Trillas et al., 2006). T34 reduces the incidence of *Phytophtora capsici* in pepper (Segarra et al.,

2013) and protects against *Fusarium* wilt (Segarra et al., 2010) and *Botrytis cinerea* (Fernández et al., 2014) in tomato. Moreover, maize seed treatment with T34 improves the relative water content in leaves, photosynthesis, maximum quantum efficiency of photosystem II, as well as nutrient and kernel parameters under well-watered and/or drought conditions (Estévez-Geffriaud et al., 2020).

Microbiome modification

Studies aiming to gain a deep understanding of plant-microbe interactions have highlighted the importance of the plant rhizosphere microbiome in successful plant growth (Mitter et al., 2017). *T. harzianum* CCTCC-RW0024 has been observed to induce changes in the maize rhizosphere microbiome, increasing plant growth, promoting rhizobacterial and maize plant growth, as well as decreasing the incidence of pathogenic *Fusarium graminearum* (Saravanakumar et al., 2017). Recent studies have even described an effective modification of the seed microbiome by the transfer of beneficial endophytic bacteria (*Paraburkholderia phytofirmans* PsJN) from the parent plant to its progeny (seeds) in wheat, maize, pepper and soy, with the bacteria introduced by spraying the plants in their flowering period (Mitter et al., 2017).

To further explore the effects of the rhizosphere coloniser *Trichoderma asperellum* strain T34 on plant physiology and the production of parental and hybrid cucumbers, we studied the influence of T34 seed treatment on (i) early flower, fruit and seed production of the progeny seeds of two parental gynoecious cucumber lines, (ii) fruit yield of the hybrid (of the parental lines) grown in greenhouse conditions and (iii) the hybrid seed bacteriome compared to the hybrid seeds obtained from untreated parental cucumber lines.

2. Materials and methods

2.1. Plant material

Cucumber (*Cucumis sativus*) parental lines and its hybrid (HOL14038) were supplied by SEMILLAS FITO S.A. Hybrid seeds were obtained from the parental lines grown in the previous year. Cucumber plant material corresponded to a 'Dutch' cucumber type of fruit.

2.2. Seed microbial inoculation

Treated seeds were disinfected with 85% potassium bicarbonate (ARMICARB, CERTIS Europe, Spain) at a dose of 3 g/L for 20 minutes at 50ºC in a thermal bath. The seeds were then rinsed with distilled water and dried at room temperature for 24 h. After that, the seeds were coated with the beneficial fungus *Trichoderma asperellum* strain T34 (Biocontrol Technologies S.L.) at a ratio of 0.4 g/100 g of seed and a non-toxic, synthetic and biodegradable polymer sticker at a ratio of 6 mL/100 g of seed. A small glass beaker was used to coat the seeds, which were then kept at room temperature for 15 minutes to dry the coating. Control (untreated) and treated (T34-treated) seeds were sowed first in coconut fibre trays before transplanting them to the greenhouse.

2.3. Greenhouse assays and management

The experiments were conducted under plastic greenhouse conditions (latitude 41.557547, longitude 2.482687 in *Sant Andreu de Llavaneres,* Barcelona) between April and August 2019. Throughout these five months (transplant to harvest), the mean, maximum and minimum values of air temperature (ºC) and humidity (%) were recorded with a TESTO 175 H1 data logger (TESTO Instruments, Spain). These values were 24.5ºC, 38.0ºC and 11.7ºC for temperature and 67.9%, 97.3% and 18.4% for relative humidity, respectively.

For the greenhouse assays, non-sterilised coconut fibre bags (1-m long, coir-based growing medium, Dutch Plantin B.V., the Netherlands), previously disinfected with hydrogen peroxide for ten minutes and then rinsed thoroughly with tap water, were used. Three plants per bag were transplanted into the greenhouse with two

irrigation drips per bag located between the plants (**Fig. 1**). One empty bag separated each bag with the treated plants in the same line of irrigation to avoid contact between the treated plants. Plants were left with two stems per plant. A plastic net and plastic ropes were placed vertically along each plant stem to allow the vertical maintenance and growth of the crop. Bags were placed in a row and the temperature range of the substrate was 20.3-24.1ºC (air temperature was 24.4ºC), the pH range was 6.5-7 and the electrical conductivity range was 0.42-1.03 mS/cm. The ferti-irrigation pH and electrical conductivity (EC, mS/cm) were recorded daily before the hybridisation and until the end of the experiment. Fertilisation (ICL Specialty Fertilizers) was performed throughout the entire crop development, adjusting the NPK by plant stage and irrigated as needed. Pest management was performed with Swirski Ulti-Mite (Koppert Biological Systems) against thrips (*Thysanoptera* sp.) and white flies (*Trialeurodes vaporariorum* and *Bemisia tabaci*). Disease management was performed preventively with TAKUMI (CERTIS Europe) against powdery mildew. The dosage and time of the application followed the manufacturer's instructions.

2.4. Experimental design

2.4.1. Parental assay

Twenty plants were sowed for each treatment group (control and treated) in the parental female line, while seven to nine plants in the parental line were treated to produce male flowers per treatment group (with a seven-day delay between the male and female sowing). Transplanting was performed seventeen days after sowing. Manual hybridisation was stopped when at least four flowers in each stem were hybridised in every single stem of each plant.

2.4.2. Hybrid assay

Hybrid seeds were obtained in the same scheme as the parental assay described here, but grown in 2018. For the hybrid assay, twelve plants were planted per treatment group (female), while six more plants per treatment group were used to

produce male flowers (without sowing delay). Transplanting was performed eight days after sowing. All flowers were hybridised.

2.5. Male flower induction and hybridisation

Male flower induction in the hybrid and parental male (pure parental line that served as the male) lines was achieved by spraying the plants with silver thiosulphate solution (STS) containing 0.008% sodium thiosulphate and 0.01% silver nitrate at 10, 17, 20, 31 and 34 days after transplanting (DAT) approximately. Hybridisation was performed manually during nine and eleven consecutive days in all the flowers present in the parental female plants and in the hybrid plants, respectively. During the hybridisation period, the mean, maximum and minimum temperatures were 21.8ºC, 33.3ºC and 12.7ºC, respectively, and the mean, maximum and minimum relative humidity values were 65.8%, 91.7% and 18.4%, respectively, among the parental plants. In the hybrid plants, the mean, maximum and minimum temperatures were 26.5ºC, 37.9ºC and 19.7ºC, respectively, while the mean, maximum and minimum relative humidity values were 69.6%, 92.3% and 32.4%, respectively. Pollen viability was checked shortly after hybridisation using the tetrazolium pollen viability test (Norton, 1966), incubating the samples for 2 h at room temperature prior to the readings. Observations were made with an optical monocular microscope (MOTIC), which indicated 94-99% pollen viability for the STS-treated plants (parental and hybrid).

2.6. Flower and fruit production

Flower count on the male parental line was performed at 40 DAT. Measurements were made by height, using the plastic net as reference. Flower count was performed between the fourth and eighth squares (each square measuring 15 x 15 cm) of the net, starting 10 cm above the substrate. Due to overlapping leaves and stems, the flower count was performed globally by treatment and side, as the treated plants were divided into two segments parallel to one another. Fruit count per stem was performed preliminarily at 58 DAT and 84 DAT in the parental female

line. In the first count, the size of the fruit was assessed using a four-point scale, with 1 indicating a gynoecious or female flower that had just opened or been manually pollinated, 2 indicating a developing cucumber fruit that was not fully enlarged, 3 indicating a developed cucumber fruit that was fully enlarged but not completely thickened, and 4 corresponding to a developed cucumber fruit that was fully enlarged and thickened. Harvest was performed at 95 DAT for the female parental line (cucumber maturation lasted between 52 and 60 days) and 105 DAT for the hybrid (cucumber maturation lasted between 40 and 50 days). In the parental female line, fruit number, fruit weight, seed weight and the number of seeds per stem and treatment were assessed. In the hybrid, fruit number, weight and diameter were assessed in each treatment group globally.

2.7. Seed yield, quality and bacterial microbiome

Hybrid seeds obtained in the parental assay were tested for seed germination and vigour, following the conditions of the International Seed Testing Association (ISTA, 2014). The microbiomes of the (hybrid) seeds from the untreated / treated fruits from the female parental line were analysed using an ISO 17025-accredited external laboratory (Eurofins Genomics Europe). The analysis code was BJNM “non-targeted microbiota screening”. Samples corresponded to one pooled sample of 100 g of seeds from all the plants per treatment group (control and treated). DNA was extracted with the commercial next generation sequencing (NGS)-specific extraction kit (Eurofins). 16S targets were amplified by PCR from the samples using target-specific NGS primers and analysed by Amplicon sequencing on the Illumina MiSeq platform (specific primers and other details of the protocol are the property of Eurofins Genomics Europe). The sorting of each sequence set was performed using the NCBI database to obtain a taxonomical assignment. If an assignment could not be solved at the species level, a higher taxonomic rank was reported (e.g., genus). Species with a fraction of at least 0.1% of all the assigned reads are reported. The purified amplicons were then pooled into equimolar concentrations and the

final concentration of the library was determined using a quantitative PCR NGS library quantification kit. Unclassified sequences or sequences belonging to the *Eukaryota* or *Achaea* domains, chloroplasts, or mitochondria were discarded (filtered operational taxonomic units (OTUs)). Sequences were divided into groups according to their taxonomical rank and then assigned to OTUs at a 97% identity cut-off for the 16S rRNA gene. Only samples with more than 5,000 reads per sample are displayed. Two regions of the 16S rRNA gene were amplified: V1-V3 and V3-V4. The seed microbiota composition is displayed as the mean taxonomic abundance of the bacterial taxa by Krona radial space-filling displays. The percentage of abundance was calculated by dividing the number of reads in the OTUs of a specific BLAST hit with a 97-100% identity by the total number of reads in the OTUs of that amplified region (V1-V3 or V3-V4) and then multiplying that by 100. Species richness and the Shannon diversity index were calculated. The Shannon diversity index was calculated using log in base 2. As one replicate was used per region and treatment group, no statistical tests were performed.

2.8. T34 populations

Seed and rhizosphere T34 populations were determined with the serial dilution method and plate counting in *Trichoderma* spp. selective medium (Chung & Hoitink, 1990). One gram of T34-treated seeds was sampled after the coating process. Ten grams of rhizosphere samples were collected at the end of the experiment from three different bags in each treatment group (control and treated), discarding the first few centimetres of the top layer and collecting coconut fibre rhizosphere from the middle plant of each bag.

2.9. Statistical analysis

A non-parametric median test (*agricolae* R package) was performed on the flower count in the male-induced line due to the small sample size. The Kruskal-Wallis test was used for the data on fruit size per stem with Dunn's post-hoc test (Bonferroni correction). For the rest of the analyses, analysis of variance (ANOVA) and the post-

hoc Fisher's Least Significant Difference (LSD) test were used, assuming a normal distribution and homoscedastic data.

3. Results

3.1. Seed treatment effects on flower and fruit production in the parental lines

The mean flower count per side (of the line of the irrigation system) and height (**Fig. 2**) in the male parental line was significantly higher at 40 DAT for the T34-treated plants, especially in the fourth and eighth quadrants, 10-30 and 90-110 cm above the substrate, respectively. The mean flower count per plant regardless of height (mean ± SE) was 12.41 ± 1.41 for the control and 15.86 ± 3.52 for the T34-treated plants. The mean number of fruits per stem and fruit size at 58 DAT (**Fig. 3a**) in the parental female line were significantly different between the control and treated plants. Higher mean values were observed in the T34-treated plants for the medium fruit size and lower values were observed in the control plants for the very small fruit size. The fruit count per plant (**Fig. 3b**) in the parental female line at 58, 84 and 95 DAT showed significant differences between the T34-treated and control plants. Statistical analysis was performed independently on each day because the fruit counts at 58 DAT included flowers with an initial fruit forming through manual hybridisation (with pollen from the induced male flowers) as well as through non-manual hybridisation (non-controlled). Spontaneous hybridized fruits were discarded later, decreasing the fruit count over time. The parameters of the harvested fruits and seeds at 95 DAT (**Table 1**) showed significant differences in the number of fruits (nº fruits·plant^{-1}) and seeds (nº seeds·plant^{-1}), but not in the other parameters measured. Germination (%) and vigour (%) of the seeds were 100 ± 1.0 and 100 ± 1.0, respectively, for the control plants and 99 ± 0.5 and 99 ± 1.0, respectively, for the T34-treated plants, with no significant differences between them (data not shown).

3.2. Seed treatment effects on the microbiome of the progeny seeds of the parental lines

The bacterial microbiome of the seeds obtained from the parental female line showed an increased number of OTU reads (classified) in the seeds originating from T34-treated plants (**Table 4**). The richness was also increased in these seeds in the V1-V3 region, but remained the same in the V3-V4 region. The Shannon diversity index showed increased diversity in both regions in the seeds from the treated plants. The bacteriome (**Fig. 4**) of the seeds from the T34-treated plants presented increases in the percentage of Bacteroidetes (+11.4 - 14.4%), Firmicutes (+4.7 – 5.8%) and Actinobacteria (+0.1 - 0.7%) at the detriment of Proteobacteria (for both the V1-V3 and V3-V4 regions of 16S) (**Supplementary Table 1**). Regarding the Proteobacteria subgroups, Gammaproteobacteria dominated in both treatment groups and in the datasets of both regions (ranging from 92 to 94%). Betaproteobacteria were not found in the V1-V3 dataset for seeds from either control or treated plants. More Alphaproteobacteria (+0.9 – 1.4%) and Betaproteobacteria (+1.4%) were found based on both regions (V3-V4 and V1-V3) and the V3-V4 region, respectively, in the seeds from the treated plants, while fewer Epsilonproteobacteria were found based on both regions in the seeds from the treated plants.

3.3. Seed treatment effects on hybrid fruit production

Significant differences were observed between T34-treated plants and control plants in the mean hybrid fruit weight ($g \cdot fruit^{-1}$) and diameter ($mm \cdot fruit^{-1}$) (**Table 2**). The final fruit yield ($kg \cdot m^2$) also showed higher values for T34-treated plants. Technical difficulties in the harvest period prevented the collection of data regarding the exact number of fruits per plant.

3.4. T34 populations in the seed and rhizosphere

As shown in **Table 3**, there were $8.1 \cdot 10^7$ CFU of T34 per seed after the seed treatment and just before sowing. At the end of the study, there were 10^6 CFU/g of rhizosphere dry weight in the treated plants and 10^2 CFU/g of rhizosphere dry weight in the control plants that had not received a T34 application.

4. Discussion

4.1. Seed treatment and T34 populations

In this work, there were 10^6 CFU of T34 per gram of rhizosphere dry weight at the end of the cucumber harvest (**Table 3**), which is in accordance with the results of a previous study applying the same seed treatment with T34 in inert substrate in maize (Estévez-Geffriaud et al., 2020), although the populations reported in that study were lower (10^4 CFU/g of rhizosphere dry weight). This difference is probably due to plant physiology, as corn completes its nutrient acquisition when entering the flowering period, while the cucumber used here does not or at least not forcefully, as it has an indeterminate type of growth and continues nutrient assimilation and compound exudation (Mengel & Kirkby, 2001; Kowalczyk et al., 2020). Seed treatment is part of the methods being currently developed for successful target applications (Pedrini et al., 2017) in an industry worth 1.8 billion USD (2019) (Seed Coating Market, MarketsandMarkets.com).

4.2. Fruit and seed production

Previous studies have demonstrated that the root zone application of *T. harzianum* significantly increases the final fruit yield, also improving the early fruit yield, but not significantly (Altintas & Bal, 2005). This is consistent with our results, which showed significant increases in both the final fruit yield (in the hybrid) and early fruit yield (in the parental line) (**Tables 1** and **2**). The key factor influencing fruit yield is the number of fruits per plant (Mengel & Kirkby, 2001). In *Pelargonium peltatum* (ornamental plant), increases in bud and flower numbers were observed with the inoculation of arbuscular mycorrhizal fungi in peat substrate (Perner et al., 2007),

possibly through an increased nutritional status and hormone balance. In our work, we observed an increase in flower/fruit numbers in both parental lines in T34-treated plants (**Fig. 2** and **Fig. 3**), suggesting a root-mediated stimulation. In line with this, Lu et al., (2018) observed that plant rhizosphere microbiota can modulate flowering time through IAA production in *Arabidopsis thaliana*, indicating that such an influence is indeed possible. Poulton et al., (2002) observed changes in male and female reproductive characteristics in tomato plants inoculated with arbuscular mycorrhizal fungi. For example, mycorrhizal inoculation and high soil P conditions were shown to improve the female total fruit mass and seed production per plant by increasing total flower production per plant. This is in agreement with our results, which revealed increased female fruit and seed production per plant (**Table 1**) due to increased flower production per stem and plant (**Fig. 3**) at the observed times. Poulton et al., (2002) also observed an increased number of seeds per fruit, which we did not observe. However, our results were only related to early production as only four flowers were hybridised per stem. In the case of male tomato plants, Poulton et al., (2002) reported an improvement in the total flower production in the inoculated plants, which is consistent with our finding of an increased number of flowers at 40 DAT (**Fig. 2**). Other tomato cultivars inoculated with mycorrhiza have demonstrated increases in fruit production (through an increased flower number) and the number of seeds per fruit, as well as a reduction in the number of days of flowering (Bryla & Koide, 1990), which we also observed in both the male and female counts (**Fig. 2** and **Fig. 3a**).

4.3. Seed microbiome modification

The seed microbiome has an important role in the resistance against colonisation by pathogens and has a strong influence on the response to biological seed treatments as well as in production practices. Modification of the final seed microbiome can have a pronounced effect on germination and the future plant (Barret et al., 2015; Nelson, 2018; Arif et al., 2020). There are currently no studies

looking at the effect of seed treatment on the progeny seeds of parental lines. However, previous studies have shown that beneficial microorganisms can be passed on to the progeny seeds (Mitter et al., 2017b) and that seed microbiome modification happens in chemically-treated seeds (Chen et al., 2020). In our work, seed-associated bacteria included Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes, which is in agreement with current knowledge regarding the bacteria associated with seeds (Barret et al., 2015; Johnston-Monje et al., 2016). We observed equal or higher bacterial abundance and diversity in the seeds obtained from T34-treated plants, consistent with the findings of Rybakova et al., (2017), who found that the seed bacteriome of oilseed rape (*Brassica napus* L.) was dominated by Proteobacteria (58%). Curiously, Rybakova et al., (2017) also found that among the three cultivars, the one with the lowest bacterial abundance and diversity presented the highest incidence of potential pathogens among the seeds, which was not established in our findings. These results suggest that modifying the seed microbiome could lead to a further shift in plant responses into the next generation. Strong genotype-specific seed microbiomes have been observed in 14 genotypes of *Cucurbita pepo* (Adam et al., 2018), with Proteobacteria predominating (83%), followed by Firmicutes (11%) and Actinobacteria (2%). These observations are in accordance with our results, which showed that Proteobacteria predominated (67-89%). However, in our case, they were followed by Bacteroidetes (6-21%), Firmicutes (0.9-10.5%) and, finally, Actinobacteria (1.6-2.4%). Adam et al. (2018) hypothesised that fewer enterobacterial pathogens and/or promoting higher microbial diversity in seeds could benefit breeding strategies. We noted a lower enterobacterial abundance (4.2% and 16.2% less, based on the V3-V4 and V1-V3 regions, respectively, for *Enterobacteriaceae*) in the seeds from T34-treated plants than those from control plants (**Fig. 4**). Current research is focusing on the possibility of engineering plant microbiomes (Arif et al., 2020). Johnston-Monje et al., (2016) suggested that maize rhizosphere optimisation should focus on the seed microbiome rather than on the soil microbiome. Our results support the idea that

modifying the seed microbiome through changing the soil microbiome of the previous generation of plants alters the bacteriome of the progeny seeds, which could potentially lead to greater benefits. However, this remains to be established.

In summary, more work is needed to address how T34 interacts with the cucumber plant, especially with regards to the flower number, plant hormone balance, plant nutrient status and the effects of the seed microbiome.

5. Conclusion

Cucumber (*Cucumis sativus* L.) seeds treated with *Trichoderma asperellum* strain T34 and planted in the spring in coconut fibre bags in a greenhouse produced plants that showed an increased number of flowers, resulting in an increased early yield of fruits and seeds in the parental male and female lines as well as an improved final fruit yield in their hybrid. At sowing time, there were $8 \cdot 10^7$ CFU of T34 per seed. At the end of the study, there were 10^6 CFU of T34 per gram of rhizosphere dry weight, demonstrating successful inoculation. The microbiome of the progeny seeds from T34-treated plants showed a decreased percentage of Proteobacteria, specifically *Enterobacteriaceae*, compared to that of the control seeds, but a higher percentage of Bacteroidetes and Firmicutes. Further studies are needed to confirm how a root-associated fungus like T34 can influence flower number. This could be widely used in breeding/production programmes and, therefore, in fruit and seed production of commercially grown cucumbers and potentially other horticultural crops under protected conditions.

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The authors declare no conflicts of interest.

Figures

Figure 1. Experimental set-up of cucumber plants grown under greenhouse conditions. **(a)** Control treatment of the parental female line at 84 DAT. White bar represents the scale of the net used for counting the flowers by height (162 pixels = 20 cm). **(b)** T34 treatment of the parental female line at 84 DAT. **(c)** Control treatment of the female hybrid at 71 DAT. **(d)** T34 treatment of the female hybrid at 71 DAT.

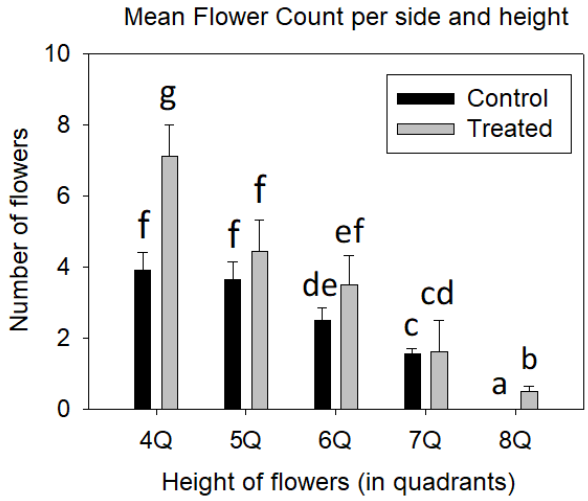

Figure 2. Flower count of the parental male line at 40 DAT. This was performed using the plastic net as reference, starting at the fourth square of the net (20 x 20 cm), 10 cm above the substrate, and ending at the eighth square. Values represent the global count per two sides (each side had 7 to 9 plants per treatment group).

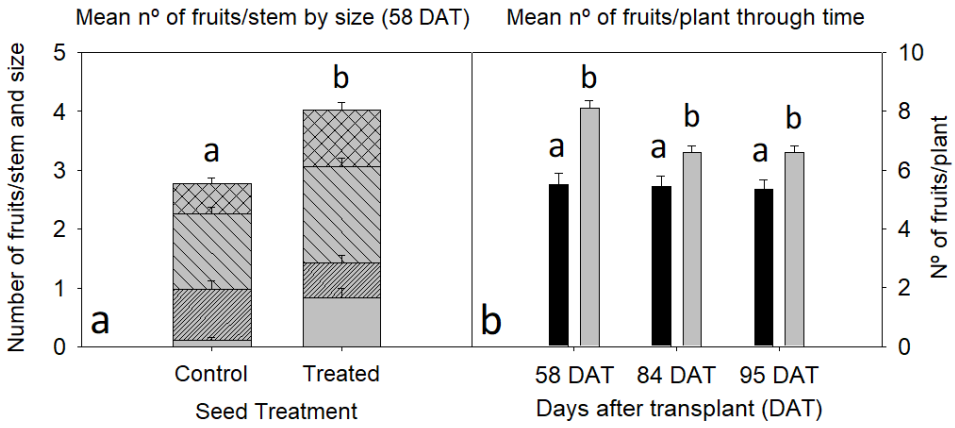

Figure 3. Fruit count in the parental female line. **(a)** Mean fruit numbers by stem and size range ± SE at 58 DAT in the parental male line. Fruit size was assessed on a grey pattern scale (accumulated), from bottom to top: 1, gynoecious or female flower; 2, developing cucumber that is not fully enlarged; 3, developed cucumber that is not completely thickened; and 4, developed cucumber that is fully enlarged and thickened. **(b)** Mean fruit numbers per plant ± SE at 58, 84 and 95 DAT in the parental female line. Significant effects were determined by two-way ANOVA. Different letters denote a statistical significance with Tukey's HSD test ($P < 0.05$).

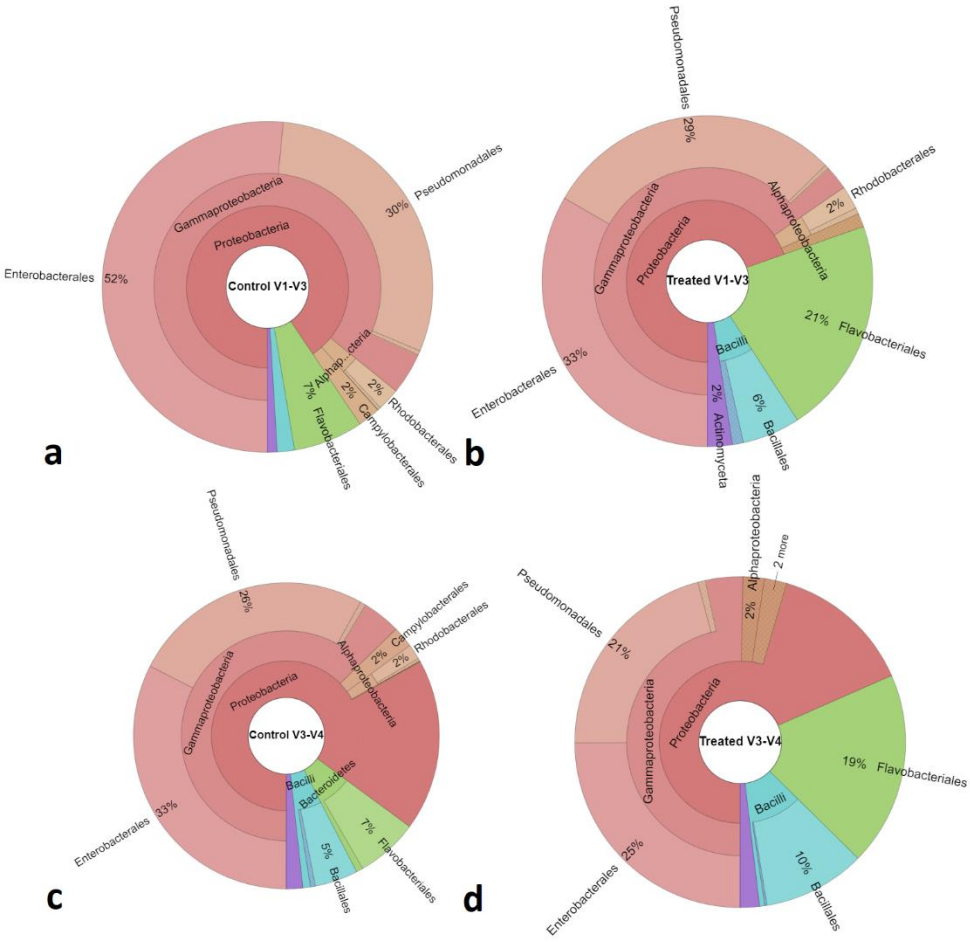

Figure 4. Krona radial space-filling displays of the relative abundance of the OTUs. The different displays represent different regions amplified from the 16S rRNA of the endophytic bacteria from the seeds obtained from control or treated plants. **(a)** Amplified V1-V3 region from the seeds of control plants (*Blue region: 1% *Bacillales*; Purple region: 2% *Actinomyceta*). **(b)** Amplified V1-V3 region from the seeds of treated plants. **(c)** Amplified V3-V4 region from the seeds of control plants (*Purple region: 2% *Actinomyceta)*. **(d)** Amplified V3-V4 region from the seeds of treated plants (*Purple region: 2% *Actinomyceta*). *Information omitted in the figure.

Tables

Table 1. Parameters of harvested fruits and seeds at 95 DAT.

Parental Female line							
Parameter	CONTROL			Sig.	T34			Sig.
number of fruits per plant	5,2	±	0,3	a	6,6	±	0,3	b
mean weight of fruit (g)	1109,9	±	40,7	a	1006,6	±	43,6	a
number of seeds per plant	876,8	±	57,3	a	1033,9	±	55,1	b
mean weight of seed (mg)	27,5	±	0,3	a	26,9	±	0,2	a
number of seeds/fruit	166,7	±	6,7	a	168,2	±	6,5	a

Significant effects were determined by two-way ANOVA. Different letters denote a statistical significance with Fisher's LSD test ($P < 0.05$). n= 17-18 (T34 and Control treatments, respectively).

Table 2. Hybrid Fruit Parameters at 105 DAT.

Treatment	Mean Weight of fruit (g)				Mean Diameter of fruit (mm)				Final Fruit Yield (Kg/m2)
	mean		SE	Sig.	mean		SE	Sig.	
CONTROL	791	±	17,4	a	56,8	±	0,47	a	16,8
TREATED	842,1	±	18,9	b	58,3	±	0,58	b	19,1

n = 15 (CONTROL) and n = 12 (T34).

Table 3. Seed and rhizosphere T34 populations at the end of the cucumber assay.

Time		T34 population (±SE)		Unit
Seed (before sowing)		$8.1 \cdot 10^7$ (±4.8)		CFU/seed
Rhizosphere (End of assay)	CONTROL	$9.3 \cdot 10^2$ (±2.1)	a	CFU/g DW of soil rhizosphere
	TREATED	$6.7 \cdot 10^6$ (±3.8)	b	

Table 4. Richness and Shannon index for relative abundances by Taxonomical Genera and total number of OUT reads (classified).

Region	Treatment	Richness	Shannon	OTU reads
V1 - V3	CONTROL	31	3,584	16038
	TRACTADA	36	4,010	16417
V3 - V4	CONTROL	37	3,499	28014
	TRACTADA	37	3,829	52293

4. General Discussion

General Discussion

Owen et al. (2015) suggested that commercial bio-inoculants should be seen as a component of integrated management strategies but indicated that there is a lack of consistency in application rates, testing and production methods for the different products, a lack of consistency in performance between (batches and trial) designs, a lack of information on the stability of products and, above all, intellectual property difficulties.

We therefore took a cross-sectional approach to major issues arising from seed treatment development, ranging from the application dose, stability in the seed and performance at different scales in representative crops (a cereal and a vegetable) under abiotic, biotic and seed/fruit production systems that would help tackle the potential use of our microbial seed treatment in today's agricultural environment.

Fungal shelf-life and establishment of T34 on maize (*Zea mays*) under different storage conditions and greenhouse conditions

Information on the global seed treatment market mentions that one of the restraints for a successful treatment is the limited life of the treated seed. This refers not only to the viability of the seed in terms of germination and vigour but also microbe viability, which is affected by the physical and chemical properties of its formulation (Seed Treatment Market, MarketsandMarkets.com, 2020). In **Study I**, we presented a seed treatment with a formulated wettable powder of *Trichoderma asperellum strain* T34 conidia. Our approach was to evaluate a well-known commercial *Trichoderma* sp. with a proven background as a plant growth promoter in many crops that is also an effective solution against many plant diseases and more importantly, registered in the European market for use in agriculture, which is a critical step in seed treatment development. Regarding viability, we carefully selected the components of the treatment to avoid damage to the microbial

inoculant, which unfortunately cannot be discussed in depth due to intellectual property issues.

The viability of T34 in the seed treatment under different inoculant concentrations, doses, varieties, storage conditions and times showed that a higher concentration (10^{10} CFU/g) and dose allows higher adherence to the seed (up to $1 \cdot 10^7$ CFU/seed), and that maize variety and equipment produced small differences probably due to differences in the size and shape of the seed and efficiency of the seed treatment process, respectively. SEM images showed hyphae developing around the emerging radicle, which is a good indicator of what would happen in inert substrate/soil conditions once planted. As 'Treatment A' (Fig. 2) led to the highest attachment of conidia of the formulated product, this treatment was used for the other case studies presented here (**Study II, Study III**) and with slight modifications in the final study (**Study IV**). We also found that viability was less affected when seed was stored at low temperature and humidity (seed conservation conditions, SCC) rather than at room temperature (RT), as expected based on information from other studies of *Trichoderma* sp. (Swaminathan et al., 2016). In terms of formulation, at time 0 the addition of fungicide (CELEST XL) alone or with insecticide (SONIDO) resulted in a similar final T34 population on the seed as with T34 alone. However, the presence of fungicide in the seed reduced the viability of T34 in the seed stored at RT and in SCC conditions at both 220 and 310 days after treatment, which demonstrates that although seed treatment is viable it is unlikely that we would obtain the same benefits in the interaction with the plant for at least the same amount of time as for T34 alone, simply because a decline in T34 means less inoculum in the rhizosphere. In this study the T34-treated seed was viable for up to two years (T34 alone) in both storage conditions, which was higher than reported by other authors; for example, Shah-Smith & Burns (1997) recorded $5 \cdot 10^7$ CFU/pelleted seed with *P. putida* but after roughly 13 months its viability had decreased one to two orders of magnitude when stored at 4°C and below detectable limits (<100 CFU/pellet) when stored at 18–20 °C, much lower than in our results.

Regarding cucumber (**Study IV**), the initial load of T34 in the seed with T34 (alone) was $8 \cdot 10^7$ CFU/seed, which was even higher than in corn, probably due to the difference in seed morphology, which facilitates microbial loading.

In **Study II**, treatment with CELEST XL led to significantly lower final T34 populations in the rhizosphere of maize than without it (10^4 and 10^5 CFU/g DW respectively), as previously mentioned. Interestingly, differences by interaction (treatment and water regime) were also observed, meaning that the plant may modulate populations and suggesting that 10^5 CFU/g DW rhizosphere may be adequate under WS conditions. This dose-dependent behaviour was also observed in *T. harzianum* Th-56 in rice under drought (Pandey et al., 2016). Although the final populations in the corn field (**Study III**) were not determined, assays performed with the same treatment under non-stressed conditions showed that T34 populations were present in the rhizosphere at corn flowering time and prior to harvest (data not shown).

In **Study IV**, T34 populations in the rhizosphere of cucumber at the end of harvest were even higher than in maize (10^6 CFU/g DW rhizosphere), which probably reflects the differences in crop physiology: corn stops acquiring water and nutrients when entering the reproductive stage whereas cucumber does not, or at least not forcefully, as it has an indeterminate type of growth (Mengel & Kirkby, 2001).

Further work is now needed to address: a) seed viability following treatment over time, b) compatibility assays with other formulation compounds, c) T34 evaluation in soil and/or field conditions to check survival in the rhizosphere and d) the minimum dose at which T34 benefits the plant.

Genotype drought tolerance and effect of T34 on maize (*Zea mays*) physiology under well-watered and water-stress conditions in the greenhouse

In **Study II**, water and seed treatment effects were more obvious in late stages (grain filling) when drought was more pronounced, with a resulting decrease in kernel

yield, consistent with previous reports (Anami et al., 2009; Bista et al., 2018). The fact that Fv'/Fm' and g_s were mainly affected in late growth stages demonstrates that the photosynthetic machinery of our maize hybrid showed a certain degree of drought tolerance. Kernel $\delta^{13}C$ decreased under WS, highlighting that the plants suffered water stress during the experiment regardless of the treatment.

Regarding treatment effects by WS, the application of T34 (T34 and T34+Q treatments) increased An and E by improving light harvesting (Fv'/Fm') and leaf water status (WUE and RWC). These changes also suggest the absence or reduction of damage to the photosynthetic machinery caused by drought stress. Similar improvements in quantum efficiency were observed with *T. harzianum* T22 in *Arabidopsis* (Shoresh et al., 2010). Moreover, kernel $\delta^{13}C$ was higher in the T34 and T34+Q treatments than in Q under WS, which may indicate that T34 improved water status throughout the experiment, since $\delta^{13}C$ provides information on photosynthetic carbon assimilation and is a time-integrated indicator of the A_n/g_s ratio and WUE (Serret et al., 2018). The significant increase in shoot fresh weight in T34 and T34+Q compared to Q, but not in shoot dry weight, clearly indicated that water content at the whole plant level was promoted by T34 application. Enhancement of water uptake related to T34 has been reported in carnation plants, even in the presence of biotic pressure (Sant et al., 2010).

Effect of T34 on maize (*Zea mays*) nutrient and isotope composition and final yield under well-watered and drought conditions in the greenhouse

Study II found that the effects of T34 application on the mineral nutrient content in leaves was minimal. Although some nutrients (S and Zn) were found at higher concentrations in T34+Q leaves compared to the other treatments at 85 DAS, these changes disappeared at 104 DAS. Nutrient uptake is mostly a passive process, so it is greatly determined by the rate of water uptake by roots (Ahanger et al., 2016). C and P contents were higher in kernels in T34 and T34+Q compared to Q, suggesting higher C assimilation, P uptake and/or translocation to the kernels. Similarly, García-

López et al. (2015) found that plants treated with T34 improved P absorption in the presence of non-available P forms in cucumber. In Fernández et al. (2014), improvements in nutrient assimilation and C allocation, as well as other growth parameters, were reported in T34-treated tomato plants. Despite this better C content in kernels and improved photosynthetic machinery and leaf water status under WS, T34 did not prevent a decrease in kernel weight, suggesting that the drought stress induced in this study was severe.

Further studies are necessary to evaluate the positive effects of T34 application on leaf water status and photosynthetic capacity under different water regimes from mild to severe water stress, and to understand its effects on plant metabolism and yield improvements, particularly in field-grown conditions and using a wider spectrum of maize genotypes with differentiated drought sensitivity.

Reduction of incidence of *Harpophora maydis* by T34 seed treatment in different tolerant lines of maize (*Zea mays*) in a naturally infected field

In **Study III**, we found that disease incidence was greatest in maize line A, followed by line B, C and finally D, which was consistent with previous information regarding genotype tolerance (sensitive, medium, medium and tolerant line, respectively). In that regard, maize line B is more sensitive than maize line C. Previous work with *Trichoderma* strains in corn affected by *Harpophora maydis* (artificially infected) by Elshahawy & El-sayed (2018) found that four *Trichoderma* strains (*T. harzianum, T. viride, T. virens* and *T. koningii*) applied separately reduced disease incidence by 28–43% and 16–23% depending on the *Trichoderma* strain and design (greenhouse and field, respectively). In our work in a naturally infected field, there was up to 13% reduction of disease in plants grown from T34-treated seeds, supporting current literature about *Trichoderma* spp.

Improvement of maize (*Zea mays*) yield in different lines of T34-treated plants in a field naturally infected with *Harpophora maydis*

Regarding yield data (**Study III**), variations in the number of plants and yield were observed (data not shown) between years as environmental and soil conditions (apart from disease incidence) affected the final harvest. Elshahawy & El-sayed (2018) found that four different *Trichoderma* strains (described above) improved average grain yield in comparison to control plants in an artificially infected field assay, reporting an increase between 7 and 21% (depending on the *Trichoderma* strain), which is partially in line with our results, as we found an improvement in both years in two maize lines (B and D) of 8–43% of final yield per plant, while in the remaining maize varieties (A and C) the final yield per plant varied between -29 and 13%, due to a possible T34-genotype specificity.

To sum up, further research is needed to explain a) the mechanisms underlying the ability of T34 to reduce disease incidence and b) the mechanisms by which T34 influences yield per plant, especially by maize genotype.

Effect of T34 on flower and fruit/seed yield of parental and hybrid lines of cucumber (*Cucumis sativus*) grown under protected conditions

In **Study IV**, increased flower/fruit numbers were obtained in both parental lines (and the hybrid) in T34-treated cucumber plants, which suggests root-mediated stimulation as T34 is a root-associated fungus. It is known that rhizosphere microbiota can modulate flowering time in *Arabidopsis thaliana* through IAA production (Lu et al., 2018), which indicates that this influence is indeed possible. Tomatoes inoculated with mycorrhizae showed increased total flower production per plant (Poulton et al., 2002), as also observed in our study (along with increased fruit production) in the parental male and female lines of cucumber and the hybrid.

Regarding yield, other authors have previously described an improvement in cucumber yield following the application of *Trichoderma harzianum* (Altintas & Bal, 2005), consistent with our results. On the other hand, in tomato inoculated with mycorrhiza, this improvement was due to increased seed number per fruit as well

as earlier flowering (Bryla & Koide, 1990), whereas in the current study, only the latter could be deduced from the flower count per size in the parental lines.

Effect of T34 on bacteriome modification in seeds of cucumber (*Cucumis sativus*) hybrid progeny

The seed microbiome plays an important role in the resistance to colonization by pathogens. Moreover, the seed microbiome has a strong influence on the response to biological seed treatments and production practices, which can also be responsible for the modification of the final seed microbiome. This modification can have a pronounced effect on germination and the future plant stand (Barret et al., 2015; Nelson, 2018; Arif et al., 2020). Previous studies have shown that the introduction of microorganisms into progeny seeds is possible (Mitter et al., 2017) and that seed microbiome modifications take place in chemically treated seed (Chen et al., 2020). In **Study IV**, seed-associated bacteria of cucumber hybrid seed resulting from the female parental line (hybridized with the pollen of the male line) included *Proteobacteria* (67–89%), *Bacteroidetes* (6–21%), *Firmicutes* (0.9–10.5%) and *Actinobacteria* (1.6–2.4%), in line with current knowledge about the predominant seed microbiome phyla (Barret et al., 2015). Regarding the bacteriome of seeds originating from T34-treated plants, an equal or higher abundance and diversity was found on these seeds in comparison to control plants. Curiously, Rybakova et al. (2017) found that *Proteobacteria* was the dominant phylum (58%) in the seed bacteriome of oilseed rape (*Brassica napus* L.), and that of three cultivars, the one with the lowest seed bacterial abundance and diversity had the highest number of pathogens in the seed bacteriome; meanwhile Adam et al. (2018) hypothesized that having fewer enterobacterial pathogens and/or expressing a higher microbial diversity in the seeds could benefit breeding strategies. In our work, a lower enterobacterial abundance was found in seeds from T34-treated plants (4.2% and 16.2% less in V3–V4 and V1–V3 region, respectively, in *Enterobacteriaceae*) along

with higher diversity in comparison to control seeds. On the other hand, *Bacteroidetes* and *Firmicutes* were seemingly enriched in seeds from treated plants.

More work is now needed to understand: a) how T34 can influence cucumber plant development, especially in terms of flower number, through plant hormone balance and/or plant nutrient status and b) the long-term benefits of modification of the T34-mediated seed bacteriome in the plant rhizosphere of the previous generation.

5. Conclusions

Conclusions

Regarding the specific aims of this thesis:

a) We developed a viable maize (*Zea mays*) seed treatment with a T34 load of up to $1 \cdot 10^7$ CFU/seed immediately after treatment.
b) T34 is viable in the maize (*Zea mays*) seed for at least two years (without a fungicide) or for up to 310 days (with fungicide CELEST XL), depending on storage conditions and dose.
c) T34 is able to colonize an inert substrate and maintain its population during crop development. Larger populations alleviated water-stress better than smaller populations.
d) Regardless of water regime, T34 maize treatment improved kernel P and C content, kernel dry weight and number.
e) Under drought, T34 maize treatment improved water status and photosynthesis parameters but was not able to avert a decline in yield in this particular corn genotype under the conditions of the current study.
f) T34 reduced disease incidence (by up to 13%) relative to untreated maize plants in a field naturally infected with *H. maydis*.
g) T34 improved yield per maize plant (g/plant) relative to untreated plants in a field naturally infected with *H. maydis*. Different responses were observed between different maize genotypes.
h) T34 enhanced early flower count and early fruit yield in both parental lines of cucumber (*Cucumis sativus*) in comparison to untreated plants.
i) T34 increased final fruit yield, mean fruit weight and diameter in comparison to untreated cucumber plants.
j) The cucumber seed bacteriome from T34-treated plants showed a lower relative abundance of Proteobacteria (specifically Enterobacteriaceae family), and a higher abundance of Bacteroidetes and Firmicutes in

comparison to control seeds, potentially benefiting the growth of the future plant.

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7. Appendix

Appendix

Supplemental data Study II

Supplemental information for this Study can be found at:

https://link.springer.com/article/10.1007/s00425-020-03404-3#Sec17

Supplemental data Study IV

Supplemental information is under review where it will be available after publication.

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2020