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GREEN AGRICULTURAL INNOVATIONS TO FACE FOOD AND CLIMATE CRISES

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Summary

Abstract

Despite their crucial importance, the role of seed-borne microorganisms during seed germination processes is still largely unexplored, especially in the case of seeds of agricultural crops, which are in many cases treated with pest control active ingredients. In addition, with the increasing interest in using biodegradable and bio-based materials to replace synthetic polymers in seed treatment solutions, these aspects are expected to assume even more importance. Consequently, in view of these novel approaches, culturing methods and basic DNA approaches are likely to be inadequate for assessing microbial contamination of seeds or the effects of seed treatments on seed-borne microbes during germination processes.

The main objective of this study was to develop a novel seed quality approach and tool for elucidating the complex interactions between seed-borne microorganisms and treated seed, during germination processes. Additionally in this study, more emphasis was given to developing and designing a cost-effective and easy-to-use tool for achieving the main objective.

The study was organized as follows. The first chapter summarizes the development process of a novel germination apparatus specifically designed for both examining seed germination and the growth of seed-borne microorganisms. The apparatus was projected and realized using the 3D-printing technology and was adaptable to common single-use 50-mL centrifuge tubes and capable of preventing microbial contaminations from external sources. This germination tube is currently patent pending.

The second chapter examines seed-borne microorganisms, with more emphasis on endophytes. The use of the germination tube was combined with traditional microbiological techniques and advanced Next Generation Sequencing (NGS) metabarcoding analysis. This approach allowed the identification of the entire fungal community recovered from corn germinating seeds. The results highlight the importance of carefully monitoring the use of fungicides in seed treatments, as they could promote the selection of microorganisms with resistance, such as *Aspergillus flavus*, a fungus of agricultural and medical relevance.

The last chapter summarizes a study concerning the use of biochar in the treatment of corn seeds to improve germination rate. Being a bio-based solution, the study also investigated the potential growth of a key fungus of corn seeds, *Aspergillus flavus*.

In conclusion, the developed germination test allowed a more depth investigation of interactions between seed-borne microorganisms and seeds, during germination processes. Results from this series of studies opened the possibility to use an effective tool for evaluating seed quality with a modern DNA-based technique, the Next Generation Sequencing. This aspect is assuming importance with the increasing interest in using bio-based solutions in seed treatment and beneficial microorganisms to improve seed germination rate and seed protection.

Aim of the project

The idea to develop a novel germination apparatus for a rapid seed quality test came to me by the simple observation of two main needs in current seed testing methods:

1) Current seed testing methods are not suitable for analyzing microorganisms (both pathogenic and beneficial) that are associated with seeds. Today, microorganisms play an increasingly important role in seed technology and quality. For this reason, a new approach to seed testing is needed, one that ensures a controlled, sterile environment to minimize contamination.

2) Currently, there is no specific test designed to evaluate the effects of chemical treatments or coatings on seed quality. In most cases, treated or coated seeds are tested using the same methods as untreated seeds, often requiring the prior removal of the chemical treatment. Developing a new testing system is expected to reduce environmental interferences and provide more accurate assessments of how chemical treatments can impact seed germination and their overall quality.

To the best of my knowledge, there is no existing seed quality test that addresses these two aspects simultaneously.
During the development of the germination apparatus, my attention was specifically focused on the following aspects:

- sterilizable in an easy way;
- hermetic to prevent external fungal spores from contaminating the seeds;
- allows rapid observation of the germination process;
- occupies a minimal space and cost to fit into laboratory routines;
- being adaptable to the analysis of multiple species;
- being compatible with commercial DNA isolation kits.

Although the final result may seem simple, it was the product of extensive study, based on the fact that the seed is a living organism perfectly adapted to its soil environment.
Other important aspects that have been considered were**:**

- use of easy sterilizable and cheap materials compatible with 3D printing;

- an adequate substrate for the germination and first development of seedlings;
- calculate adequate light and water to supply for good germination.

Benefits initially not considered, but which have been observed:

- no need to regulate relative humidity;
- the ability to test the effects of pesticides or other compounds;
- compatibility with microbial metabarcoding sequencing applications.

These aspects highlight how the developed tool can address current limitations in seed testing methods, offering a practical and reliable tool for assessing seed quality and supporting agricultural research.

Figure a At the top, carrot (*Daucus carota*) seedlings were grown during a traditional paper roll germination test; at the bottom, carrot seedlings were grown using the developed germination tool.

Chapter 1: New approaches to seed testing

Highlights

- *Evaluating seed quality is an important prerequisite for farmers, producers, commercial stakeholders, and ensuring safety in international trade.*
- *Traditional seed testing methods are often inadequate for assessing seed microbial contamination or effects of seed treatments on seed-borne microbes.*
- *The development of a novel seed germination apparatus addresses the demands of modern seed technologies.*
- *3D printing can be an effective tool to develop novel seed testing technologies.*

1.1. Seed quality and testing

The quality of a seed lot is related to its market value, which is strongly linked to its suitability for planting. For this reason, both farmers and companies are interested in testing their seeds to determine their value and field performance (Elias et al. 2012). The main factors that influence seed quality are production, conditioning, transportation, treatment, and storage practices (Black et al. 2006).

Since the late 1800s, seed testing protocols have been developed to determine the quality of a seed lot (Elias et al. 2012). ISTA and AOSA, both founded at the beginning of the 1900s, are the major independent organizations that supervise and develop seed testing protocols (Elias et al. 2012).

Even though seed quality is usually related to its viability, there are various properties that determine seed quality and are tested. These include, first and foremost, germination ability, followed by vigor, varietal purity, analytical purity, seed health, moisture content, and uniformity of mixing and blending. In fact, alongside viability, it is important to test a seed lot's storage potential, overall quality, compliance with labeling laws, and to standardize seed marketing practices(Elias et al. 2012). In general, farmers and producers are particularly interested in the germination capability of a seed lot (Copeland and McDonald 2001, Elias et al. 2012). For this reason, the classical method for evaluating the germination capability of a seed lot is the germination test, which assesses germination under favorable conditions (Copeland and McDonald 2001; Elias et al. 2012). However, germination tests are conducted under ideal conditions, which may not accurately reflect real-world field conditions. To

address this limitation, certain tests, such as vigor tests, aim to minimize this discrepancy by providing more realistic estimates of seed performance under suboptimal conditions (Elias et al. 2012).

As stated before, as far as I'm aware, at the moment there are no specifically designed tests for treated seeds or for investigating seedborne microorganisms. Therefore, the decision was made to design the 3D-printed germination apparatus.

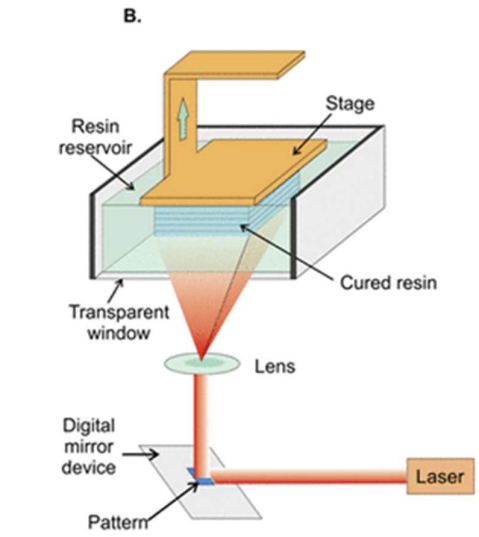
1.2. 3D printing applied to bio-research

1.2.1 3D printing history and applications

3D printing is a technique to build manufactures, it is based on overlapping multiple layers of different polymers, ceramics, metals, and a wide range of different materials (Pearce 2015; Pu et al. 2024).

Since its technical improvement in the early '90, 3D printing has found applications in various technical fields, such as prototyping in the automotive and aerospace industries, as well as, medicine, architecture, and fashion (Gross et al. 2014).

Two primary 3D printing techniques are Stereolithography (SLA) and Fused Deposition Modeling (FDM) (Gross et al. 2014; Hartings et al. 2019). The former, which uses the principle of the first 3D printer theorized by Hideo Kodama in 1980, was developed and patented by Charles Hull in 1986 (Gross et al. 2014; Hartings et al. 2019). SLA (Stereolithography) 3D printing operates by selectively solidifying layers of liquid resin using a process called photopolymerization. As illustrated in Figure 1.2.1 (top), a platform is positioned within a tank filled with liquid resin. During printing, this platform moves incrementally, lowering into the resin to allow each new layer to form and then raising slightly for the next layer. A laser or a light source is directed at specific areas of the resin, curing it layer by layer into solid plastic. (Melchels et al. 2010; Gross et al. 2014). However, FDM printing works by extruding thermoplastic material from a heated nozzle head. As shown in Figure 1.2.1 (bottom), this material is gradually building the 3D object from the bottom up (Gross et al. 2014; Carneiro et al. 2015).

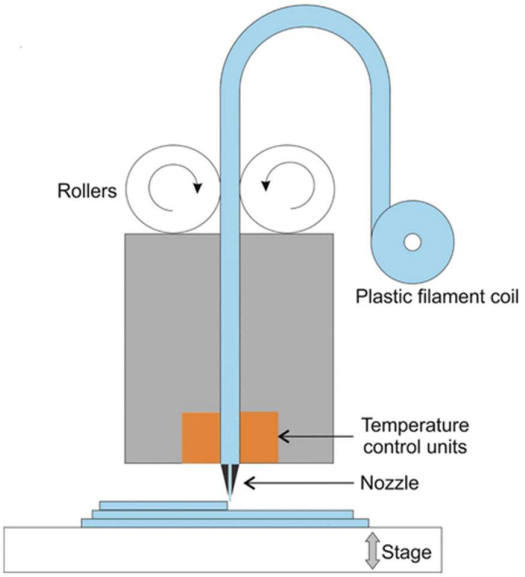


General schema of SLA printer

(Gross et al. 2014)

SLA 3D printer Form 3L

(Formlabs, Somerville, MA, USA)



FDM printer

(Gross et al. 2014)

Delta WASP 2040 INDUSTRIAL X

(WASP s.r.l, Massa Lombarda, RA, ITALY)

Figure 1.2.1 On the left, a schematic representation of general SLA and FDM printers. On the right, photographs of the 3D printer models used in this study.

Due to its high versatility, 3D printing has been widely applied in bioscience. An important example is bioprinting, which refers to the 3D printing of living materials by combining a polymer matrix with live cells (bioinks) (Pu et al. 2024). This technique has been applied to bacteria, fungi, and microalgae for various purposes, such as the degradation of organic pollutants, environmental monitoring, and bioelectricity production (Pu et al. 2024).

Design and printing a manufacturer need different steps of projecting and adapting the project to the 3D printer, using different software and file formats. After 3D printing, the object undergoes several refinement stages, including draining, washing, and removal of supports (Melchels et al. 2010).

1.2.2 Project Design

First, it is essential to consider both user needs and the feasibility of the object. In 3D printing, understanding how the technology operates is key to effective design. Material choice and print resolution can impact the project's requirements, particularly with details like small holes, thin walls, and the need for structural supports. After the expiration of key patents, 3D printing has gained popularity among hobbyists, resulting in many online resources, free designs, and adaptable guides. However, achieving optimal results may require multiple trials and adjustments.

1.2.3 Drawing and preparing for print

The prototype is created using computer-aided design (CAD) software, such as AutoDesk™ (Autodesk Inc., San Francisco, CA, USA), AutoCAD (Autodesk Inc., San Francisco, CA, USA), or more specialized programs like Fusion 360 (Autodesk Inc., San Francisco, CA, USA) and SolidWorks (Dassault Systèmes, Vélizy-Villacoublay, FR) (Gross et al., 2014). Additionally, there are several open-source program options available for CAD design, such as Blender (Blender Online Community) and FreeCAD (FreeCAD Team). The result is a file of various formats, such as .step or .f3d and others. This file is then converted into a file .STL format (Standard Tessellation Language or Stereolithography) (Gross et al. 2014). This type of file stores information about the surface geometry of the design, dividing it into triangles and saving the coordinates of each triangle within the file (Gross et al. 2014). The last step before printing is the conversion of the CAD file into a code format that communicates with the printer, usually, this process involves "slicing" (Baumann et al. 2016). In "slicing" software calculates the

printer tool paths and identifies the different “slices” (layers) as thick as the layer height of the printer (Baumann et al. 2016).

1.2.4 Printing and refining

The last step is printing, it could last minutes or hours depending on the vertical dimension of the object and the printer's velocity. In general, more higher the object respects the base, and longer the process. After printing, the object undergoes different processes depending on the material and type of printing (Gross et al. 2014). For example, FDM printing only requires the removal of any support structures mechanically or chemically, if present. SLA printing may require a post-curing process with the use of UV light to harder the resin (Gross et al. 2014; Pearce 2015). Figure 1.2.4a summarizes all the steps of the project, from designing to refining.

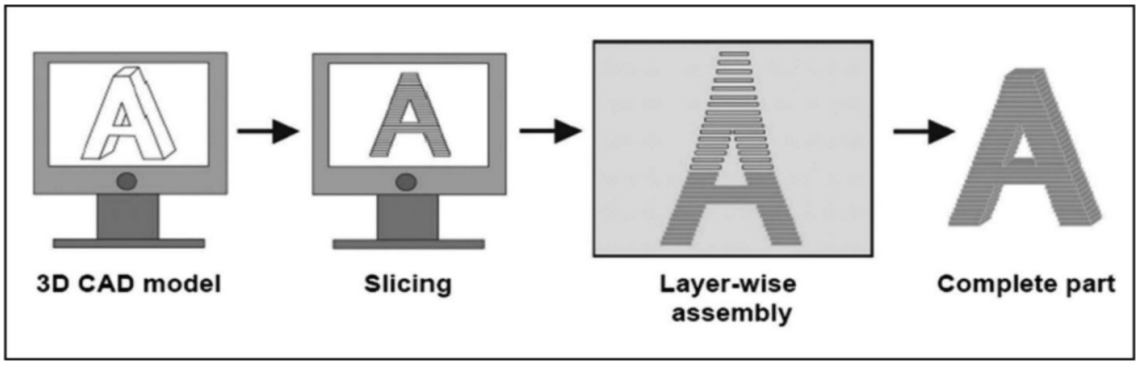


Figure 1.2.4a Summary of the 3D Printing process (Jadhav and Jadhav 2022).

Due to its versatility, 3D printing has found numerous applications across scientific fields, with some of the most sophisticated uses in biomedical engineering. In particular, advanced 3D printers can create tissue scaffolds, such as for bone, teeth, and organs, which are used in various patient treatments (Gross et al. 2014). Some chemical applications involve printing devices for studying microfluidics and electrochemical flow cells. In electronics, projects have been developed for building lithium batteries and microchips. (Gross et al. 2014).

In the research field, 3D printing can be applied to manufacturing custom supplies, equipment, and consumer goods. These can be more cost-effective than commercial alternatives, although the main advantage is obtaining the object more quickly than through ordering and shipping (Pearce 2015; Hartings et al. 2019).). Another significant advantage of this technology is its low cost, as many projects involving scientific equipment are open-source (Pearce 2015; Hartings et al. 2019). This approach allows for faster, more affordable custom

labware production, and in some cases, the specific items may not be commercially available due to their specialized use (Gross et al. 2014). Figure 1.2.4b displays some examples of 3D-printed labware.

Figure 1.2.4b Some 3D printed laboratory objects. A. one of the first objects 3D-printed by Kodama in the early 1980s. B. Laboratory supplies C. 3D-printed fluidic device D. 3D-printed 'reactionware' for performing a multistep, organic synthesis and purification (Hartings et al. 2019).

In agriculture, low costs and the diffusion of open-source designs are also driving factors in the development of 3D printing projects. Due to its affordability, 3D printing, especially PLA, has been utilized for manufacturing low-cost equipment and consumer goods in developing countries (Pearce 2015). Additionally, 3D printing technologies play a role in modern agriculture by enabling integration into smart farming. Examples include custom irrigation systems, crop-specific planting devices, and precision farming tools such as GPS equipment (Padhiary et al. 2024).

1.2.5 Protocol for quality and molecular analysis using the new germination apparatus

The final design of the 3D-printed germination apparatus resulted from prototyping and testing various shapes and materials. At the end of the prototyping phase, the most effective tool for seed germination consisted of two cylindrical PLA[1] components: an inner and an outer cylinder. The outer cylinder ends with fillets at both extremities, making it compatible with standard 50 mL tubes. This design allows the seedling to develop in isolation from the

[1] Polylactic acid

environment. The central cylinder includes two sided slots for cotton swabs, which can be soaked with sterile water using an automatic pipette. Additionally, two sterile paper sheets (0.5 cm × 1.5 cm) can be placed in contact with the seed to enhance microbial isolation during DNA isolation. Each cylinder is designed for the germination of a single seed, that grows between the cotton swabs. The apparatus can be used for seeds of different sizes and species ensuring that the direction of root growth points downward as illustrated in Figure 1.2.5a.

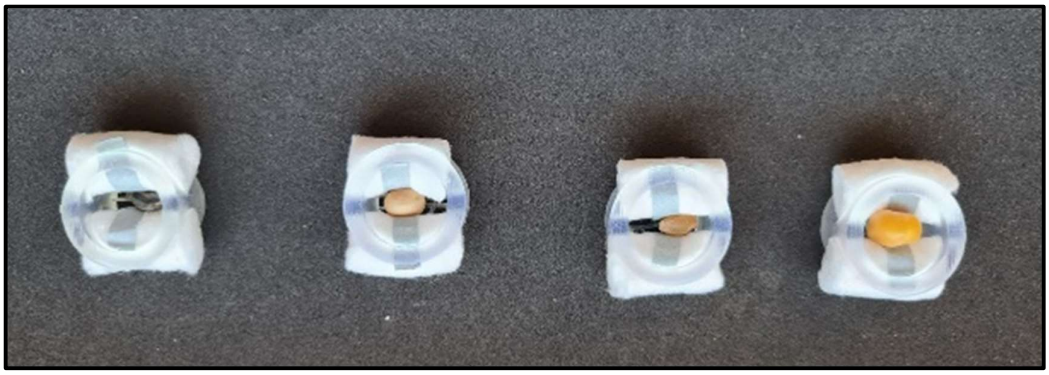


Figure 1.2.5a In the photo, the inner cylinder is prepared with the seeds of four different species: sunflower, protein pea, wheat, and corn.

In the next step, the inner cylinder is placed in the space between the fillets of the outer cylinder, and the swabs are hydrated with a sufficient amount of water. For corn, it has been empirically determined that each cotton swab requires between 1.5 and 2 mL of water, resulting in a total of up to 4 mL (Figure 1.2.5b).

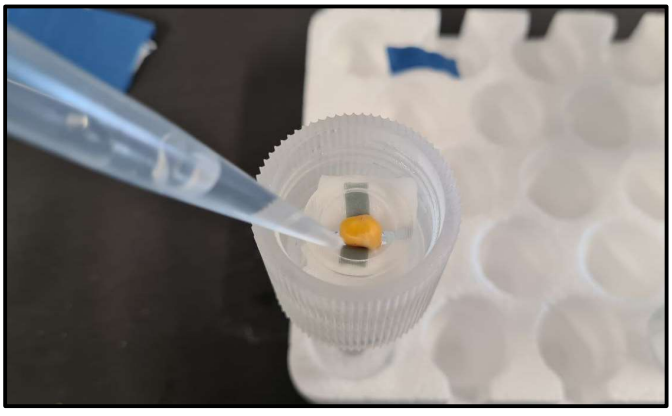


Figure 1.2.5b The cotton swabs can be soaked with a precise amount of water, dispensed using an automatic pipette.

The two sides of the outer cylinder can be sealed with standard 50 mL tubes, as the fillets are compatible with various commercially available brands (Figure 1.2.5c, left). On the right, Figure 1.2.5c illustrates how the apparatus's dimensions and shape allow multiple replicates to be arranged in a small space. The transparency of the closure tubes allows light to pass through. It is worth noting that only a temperature-controlled chamber is needed for growth, with no need to regulate relative humidity.

Figure 1.2.5c On the left, a corn seed is shown before starting germination in the apparatus. On the right, multiple replicates are packed in a small space.

Sampling can begin a few days after the emergence of the radicle. In Figure 1.2.5d (left), it is shown how the normality of the obtained seedling can also be evaluated. The swabs and/or paper sheets can be sampled (Figure 1.2.5d, right).

The same seedling can be preserved for further analyses, and it is remarkable that this method is non-destructive. DNA can be isolated using a standard kit or a quick isolation protocol developed for microbial isolation. In the second case, the two cellulose papers can be vortexed for 30 minutes in 1 mL of a 0.02% Tween 20 solution (Sigma-Aldrich, St. Louis, MO, US). The liquid phase is centrifuged for 10 minutes at 12,000 rpm, the supernatant is discarded, and the pellets are resuspended in 50 µL of Triton X-100 buffer, composed as described by Goldenberg et al., 1995: 10 mM Tris-HCl (pH 8.00), 1 mM EDTA, and 1% Triton X-100.

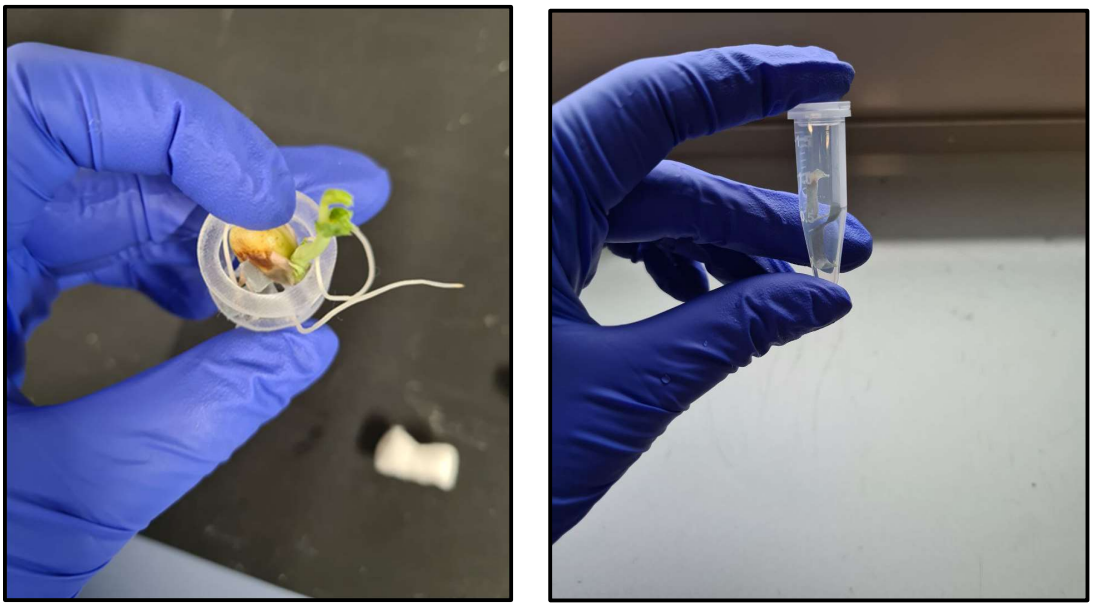


Figure 1.2.5d On the left, a proteic pea seedling was obtained in the germination apparatus after four days. On the right, paper sheets in contact with the seeds during germination were sampled for further molecular analysis.

Samples are incubated at 95°C for 30 minutes with agitation and then placed on ice for 30 minutes (Goldenberg et al., 1995; Li et al., 2017). The obtained DNA template was amplified using Open qPCR (Chai Bio, Santa Clara, CA, US) or with a standard endpoint PCR. For the use of DNA isolated with the quick method in NGS applications, further analyses are required; however, preliminary results are promising.

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1.3. Patent n. 102023000000675, *SEME RIVESTITO, METODO E MACCHINA PER RIVESTIRE UN SEME*.

12

[…]

Secondo un aspetto della descrizione, la descrizione riguarda un
10 dispositivo per valutare la germinabilità dei semi.
Nel seguito si farà riferimento indistintamente al dispositivo per valutare la germinabilità dei semi anche con l'espressione Seed Roll[2].
Il dispositivo può essere impiegato per valutare la germinabilità, ad esempio, di semi rivestiti con una pluralità di filamenti realizzati come
15 sopradescritto, non escludendo l'impego per semi conciati con metodi convenzionali (semi conciati con film-coating mediante polimeri di sintesi e non e semi confettati).
Secondo un aspetto, il dispositivo comprende una prima provetta, una seconda porzione a provetta, un connettore interposto tra la prima e la
20 seconda provetta ed un alloggiamento discoidale per il seme e due tamponi o substrati spugnosi.
Ad esempio, la prima e seconda provetta possono essere tubi da centrifuga, preferibilmente da 50 mL.
Ad esempio, la prima e la seconda provetta possono essere realizzate in
25 materiale plastico.
La prima e la seconda provetta sono accoppiate al connettore da parti opposte, preferibilmente avvitate ad esempio per mezzo di una filettatura. Secondo un aspetto, il dispositivo comprende un componente discoidale, disposto all'interno connettore per accogliere il seme rivestito.
30 Secondo un aspetto, il componente discoidale presenta una prima ed una seconda apertura.

13

Il dispositivo comprende un primo ed un secondo tampone spugnoso alloggiati rispettivamente nella prima e nella seconda apertura.

[2] In the Italian patent pending, the designed germination apparatus is referred to as the 'Seed Roll'

I due tamponi o substrati di forma cilindrica (diametro di circa 1 cm e lunghezza di circa 2 cm) sono disposti in modo da contattare il seme.

I due substrati possono essere in acetato di cellulosa, cotone, poliuretano espanso.

Saturando i substrati porosi con acqua viene permessa l'imbibizione del seme.

Secondo un aspetto, il dispositivo comprende una griglia disposta in

corrispondenza di una base del componente discoidale. Vantaggiosamente, il seme può germogliare e le radici sviluppano attraverso la grigia in direzione della provetta.

Vantaggiosamente la griglia permette di mantenere in posizione il seme durante la germinazione senza ostacolarne la crescita.

Secondo un aspetto della descrizione, la descrizione riguarda un metodo per valutare la germinabilità di un seme.

Secondo un aspetto, il metodo comprende:

- una fase di predisporre un dispositivo per valutare la germinabilità dei semi secondo gli aspetti sopra descritti;
- una fase di inumidire il primo ed il secondo substrato del dispositivo;
- una fase di posizionare il seme tra il primo ed il secondo substrato;
- una fase diposizionare il dispositivo in un fitotrone o una camera di crescita a temperatura, luce e umidità controllata.

Essendo il dispositivo Seed Roll chiuso ermeticamente, con questo

dispositivo il controllo dell'umidità nel fitotrone o camera di crescita non è strettamente necessario.

Nei Grafici 1 e 2 sotto riportati, sono rappresentati i risultati delle prove di germinazione effettuate con il dispositivo sui semi trattati con lo slurry repellente con o senza fibre di cotone.

I dati presentati nel grafico sono stati ottenuti dalla media dei valori di germinazione e di tempo medio di germinazione di 100 semi per ciascun

trattamento.

Nel Grafico 1 è stata indicata in ordinata la percentuale di germinazione dei semi di mais trattati, mentre in ascissa con Ctr i semi non trattati, con SR i semi trattati con la soluzione repellente, con SR/FC i semi trattati con

5 soluzione repellente e fibre di cotone.

Dal Grafico 1 si nota che i trattamenti con la soluzione o slurry repellente (SR) e con o senza l'aggiunta di fibre di cotone (SR/FC) non hanno mostrato degli effetti negativi sulla percentuale di semi germinati rispetto al controllo (Crt).

10 Risultati simili si notano nel Grafico 2 in cui è rappresentato il Tempo Medio di Germinazione, anche in questo caso i risultati ottenuti con i semi trattati con la soluzione repellente (SR) non si discostano da quelli ottenuti con i semi di controllo (Crt). Questo effetto è da considerare positivo dal punto di vista agronomico in quanto riduce il tempo di germinazione dei

15 semi e quindi anche l'intervallo di massima rischiosità dei semi nei confronti di attacchi di patogeni, insetti e predazione da parte di uccelli. Durante la germinazione e fino al completo attecchimento dei germinelli, il seme presenta infatti massima vulnerabilità nei confronti degli agenti biotici ed abiotici.

20 Per quanto riguarda i semi trattati con la soluzione repellente con l'aggiunta di fibre di cotone (SR/FC) si nota una diminuzione del tempo medio di germinazione, probabilmente dovuto dal fatto che le fibre di cotone permettono una maggiore imbibizione dei semi.

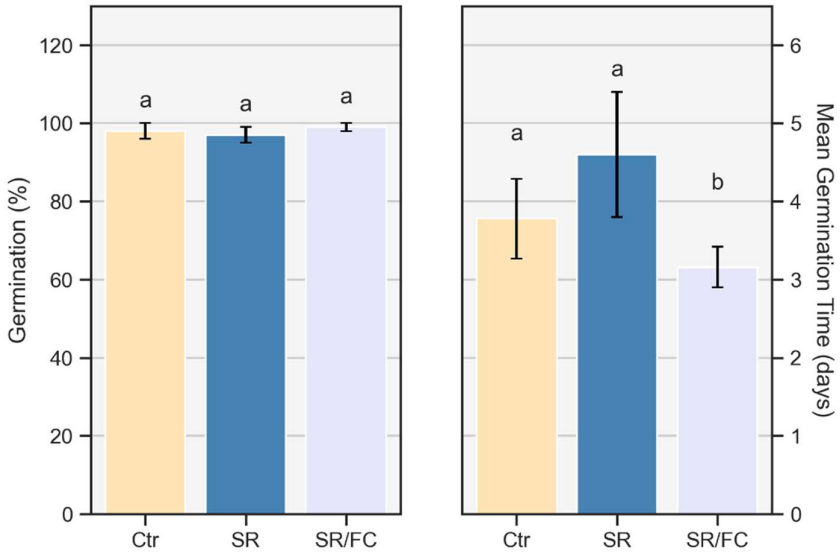

Grafico 1: Germinabilità

Grafico 2: Tempo Medio di Germinazione

Per validare il test con Seed Roll, e quindi confermarne i risultati sulla germinazione dei semi trattati con lo slurry repellente, con o senza

l'aggiunta di fibre, è stata impostata una prova di confronto con il metodo standard.

Questo metodo prevede l'impiego di fogli di carta da filtro (metodo noto con il termine di Paper Roll).

I semi da testare vengono posizionati tra due fogli di carta da filtro saturati con acqua.

I fogli vengono poi arrotolati e posizionati in camera di crescita.

I dati mostrati nei Grafici 3 e 4 sono stati ottenuti dalle medie dei valori di germinazione di 100 semi ed elaborati statisticamente con il test chiquadrato di indipendenza.

Nei Grafici 3 e 4 è stata indicata in ordinata la percentuale di germinazione dei semi di mais trattati rispettivamente nel caso di Paper Roll e di Seed Roll, mentre in ascissa con Ctr i semi non trattati, con SR i semi trattati con la soluzione repellente, con SR/FC i semi trattati con soluzione repellente e fibre di cotone.

16

Come mostrato nei Grafici 3 e 4, i risultati ottenuti da questa prova di verifica non mostrano differenze statisticamente significative ($p > 0,05$) tra i valori di germinabilità determinati con i due approcci, ovvero il test di germinazione effettuato con il Seed Roll rispetto al test di germinazione standard tramite Paper Roll.

Si evidenzia inoltre che anche i due trattamenti a confronto (semi trattati con slurry repellente con o senza l'aggiunta di fibre) non determinano variazioni significative ($p > 0,05$) della germinabilità dei semi.

L'impiego del sistema messo a punto per valutare la germinabilità dei semi conciati (Seed Roll) rappresenta pertanto una valida alternativa al test di germinazione standard.

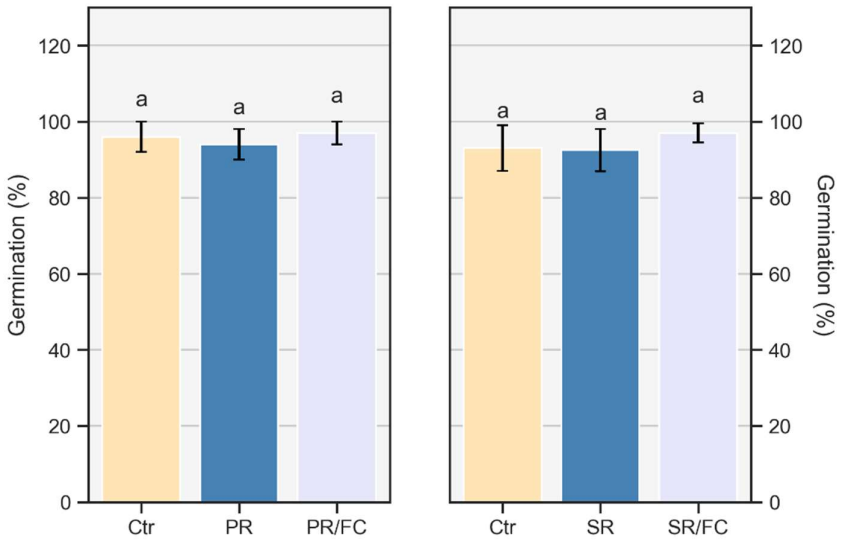

Grafico 3: Germinabilità testata con metodo Paper Roll

Grafico 4: Germinabilità testata con metodo Seed Roll

Questo approccio può essere utilizzato anche per valutare la dose efficace

15 di agrofarmaco affinché quest'ultimo esplichi alla sua funzione. Inoltre, questo metodo permette di lavorare utilizzando bassissime quantità di

principio attivo e riducendo al minimo la sua possibile dispersione nell'ambiente di lavoro.

Le principali caratteristiche dell'invenzione risulteranno maggiormente evidenziate dalla descrizione dettagliata che segue, fatta con riferimento ai

17

5 disegni allegati, che ne rappresentano una forma di realizzazione

puramente esemplificativa e non limitativa, in cui:

[…]

20 - Figura 3 illustra un dispositivo per valutare la germinabilità dei semi

con la presente descrizione;

- Figura 4 illustra un particolare del dispositivo di Figura 3. Con il numero 1 è stato indicato in Figura 1A, un seme rivestito.

[…]

- Figura 3 illustra un dispositivo per valutare la germinabilità dei semi

con la presente descrizione;

- Figura 4 illustra un particolare del dispositivo di Figura 3.

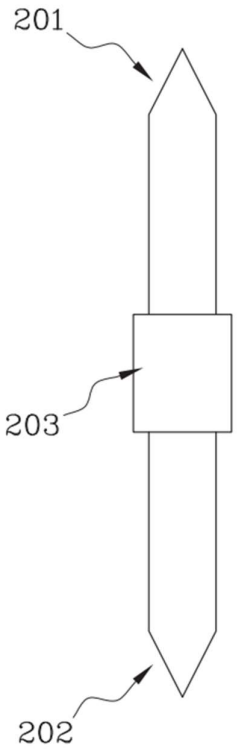

Fig.3

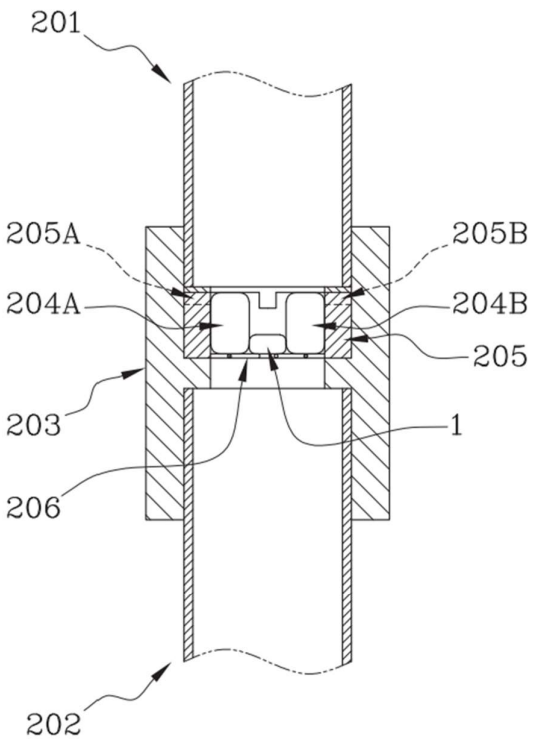

Fig.4

Chapter 2: Seeds and microorganisms

Highlights

- *Certain microorganisms, known as endophytes, can colonize the internal tissues of seeds and remain latent until environmental and physiological conditions trigger their activation.*
- *The developed germination apparatus, combined with Next-Generation Sequencing technologies, enables the detection of endophytic fungi activated during seedling development.*
- *These studies have revealed evidence of induced selection for azole-resistant* Aspergillus flavus *strains as a consequence of azole treatments*.
- *Azoles are a widely used class of fungicides, applied in both seed treatments and medical applications.*

2.1. Seedborne Microorganisms

Many microorganisms (m.o.), such as fungi, bacteria, or viruses, can be associated with seeds (seed born). They may be located on the seed surface, within the seed coat, or in embryo tissue. Some of these m.o. can live and feed in seed parasitically, some of them potentially causing diseases, and are known as endophytes *(endon* Gr., within; *phyton*, plant) (Schulz and Boyle 2005; Black et al. 2006). If an endophyte m.o. is pathogenic, it may directly impact seedling development by causing symptoms like shrinking, abortion, or rot, which can indirectly affect biomass production, photosynthesis, and yield (Black et al. 2006). In contrast, some saprophytic m.o. colonize seeds and complete their life cycle without symptoms, in symbiosis with the host plant. However, some of them can become problematic during storage, leading to spoilage or disease under certain conditions. This overlap between latent pathogens and endophytes is why the distinction between them is often unclear (Schulz and Boyle 2005; Black et al. 2006).

Seedborne microorganisms can infect seeds through direct or indirect mechanisms. Direct infection is most common for viruses, which migrate from the mother plant to the seeds via the xylem. Indirect infection occurs when microorganisms are transported by wind, insects, or pollen, potentially invading the seed through the stigma, penetrating the ovary tissue, or persisting on the seed surface (Black et al. 2006).

The location of the associated m.o. depend on the stage of the seed development at the time of the infection (Barret et al. 2016). Systemic transmission via the xylem or non-vascular tissue of the mother plant happens in the early stage of seed development. This type of direct transmission, also referred to as vertical transmission, is important because allows the transmission of m.o. from one generation to another (Black et al. 2006; Barret et al. 2016). Also, indirect transmission through the floral pathway via the stigma of the mother plant (Barret et al. 2016) results in the colonization of the internal tissues of the seeds, such as embryo and test endophytic m.o. (Black et al. 2006; Barret et al. 2016). If indirect transmission occurs at a later stage of seed maturity, it typically happens through contact with the fruit or during threshing. This usually results in the microorganisms being localized to the seed coat (Black et al. 2006; Barret et al. 2016)

2.2. Endophytic fungi

Fungi associated with agricultural seeds have been well-known since antiquity. For example, some historical texts described rudimentary seed treatments that have been developed to prevent wheat seedborne fungal infections (Buttress and Dennis 1947). Specifically, historical references are cited to the practice of brine and then liming wheat seeds to manage smut or blunt infections (Nattrass 1944; Buttress and Dennis 1947). Like other seed-borne microorganisms, fungi can be found on the seed coat, in cracks, or inside the live tissue (as endophytes) (Copeland and McDonald 2001).

A precise definition of endophytic fungi (e.f.) refers to those that inhabit the plant (or the seed) without causing any visible symptoms, thus remaining asymptomatic at the time of detection (Copeland and McDonald 2001). Among all endophytic m.o., fungi are particularly important for seeds, as many of them can affect seed quality. For example, *Ustilago tritici* (loose smut) and *Puccinia graminis* (wheat rust) are well-known endophytic fungi that affect wheat seedlings (Black et al. 2006). One important characteristic of these fungi is the phase where their growth and colonization temporarily stop. This dormancy is resumed when there is a change in the host seed, which could be a physical change (e.g., germination), an environmental change (e.g., storage conditions), or a physiological change (e.g., change in seed moisture) (Stone et al. 2004).

Endophytic fungi can establish different types of symbiotic relationships with the host, such as parasitism, mutualism, and commensalism (Stone et al. 2004; Schulz and Boyle 2005). Some

species can provide benefits to the host, while others can be considered almost latent pathogens (Stone et al. 2004; Schulz and Boyle 2005).

Fungal endophyte taxa can be classified into three different groups: mycorrhizal fungi, Balansiaceous fungi ('grass endophytes'), and non-Balansiaceous fungi (Schulz and Boyle 2005). The Balansiaceous fungi, belonging to the ascomycetous genera *Epichloë* and *Balansia* (Family Clavicipitaceae), form mutualistic symbioses with their host plants. These fungi are vertically transmitted through the seed and grow systemically and intercellularly throughout the above-ground organs of grasses, rushes, and sedges. The primary benefits for the e.f. include nutritional gains and protection from abiotic stress, such as desiccation, as well as from competing epiphytic organisms. For the plant, the main advantage is likely protection against herbivory, due to the production of toxic alkaloids within the symbiotic association (Schulz and Boyle 2005). Non-Balansiaceous fungal endophytes, which also belong to the Ascomycota, have been isolated from nearly every organ of most plants sampled, colonisation can be inter- or intracellular, localized or systemic (Schulz and Boyle 2005). Although mycorrhizal fungi form symbiotic relationships with plant roots, they can sometimes be found on the seed coat or placed artificially through inoculation. However, they are not considered true endophytes during the seed stage of the plant (Black et al. 2006).

2.3. Seedborn fungi detection

Isolating endophytic microorganisms is a complex and challenging process. Detection of microorganisms is important, not only for a healthy seedling but also because are carriers for the dissemination of pathogens at long distances (Majumder et al. 2013). Seedborn infections could not cause any symptoms on the seed, show no signs of attack, or be inactive and be pathogenic on the seedling or the mature plant (Black et al. 2006).

First of all, it is important to define an efficient protocol to sterilize seed surface, to eliminate environmental and epiphytic m.o. (Stone et al. 2004; Selim 2012). Stone et al. (2004) provide a comprehensive list of commonly used materials and protocols for surface sterilization. Depending on the type of tissue, substances such as ethanol, sodium hypochlorite (NaOCl), and hydrogen peroxide (H_2O_2) are commonly used at varying concentrations and durations. The established practice in our lab for seed surface sterilization involves rinsing the seeds in 1% NaOCl for two minutes under agitation, followed by three thorough washes with sterile water, which has proven to be effective.

One traditional method for the identification of e.f. Involves direct observation using microscopy for the detection of fungi colonization (Stone et al. 2004; Schulz and Boyle 2005; Sun and Guo 2012). This approach, while capable of incorporating more sophisticated tools—such as the insertion of green fluorescent protein genes into the m.o.'s genome or immunoelectron microscopy—requires significant taxonomic expertise. Even for microorganisms that are relatively easy to visualize, identification can be challenging. For instance, while hyphae may be observable, identifying the species is difficult due to the lack of spore-producing structures and sexual or asexual spores (Stone et al. 2004; Sun and Guo 2012). Commonly, traditional methods used cultivation-dependent approaches, an example is shown in Figure 2.3 In this case, after sterilization, seed tissue samples are placed on nutrient agar, allowing fungal growth to be isolated and cultivated under different conditions and on various substrates. Identification is primarily based on their morphological traits (Sun and Guo 2012). The cultivation of fungi to identify them is prone to biases, as some fungi do not sporulate and may therefore be misclassified or remain unidentified. Additionally, fungi with slow or rapid growth rates may go unnoticed on standard growth media or require specific conditions for cultivation. Furthermore, some fungi are unculturable, making identification through this approach impossible (Stone et al. 2004; Sun and Guo 2012).

Control	Disinfected
Wheat	
	

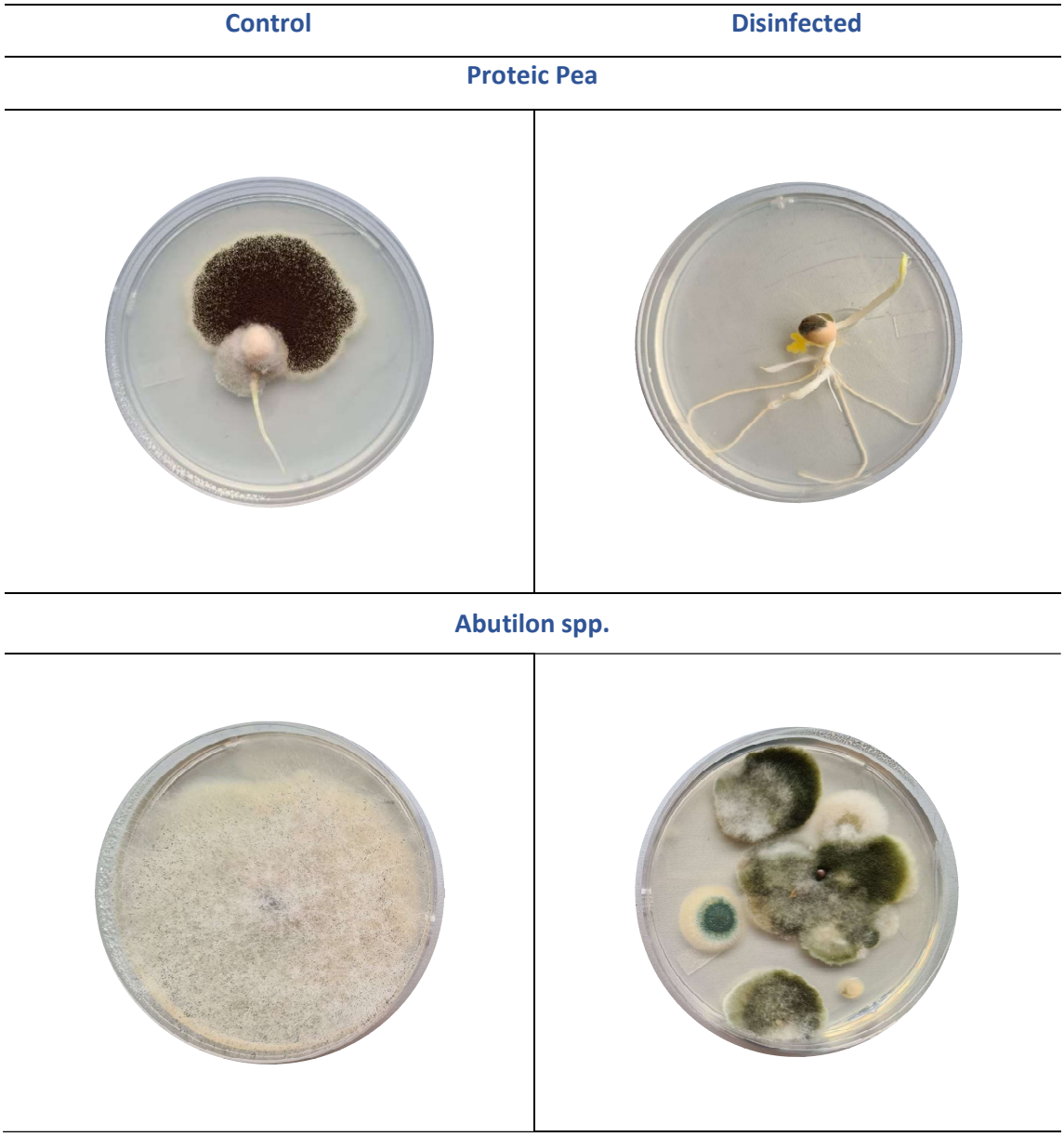

Figure 2.3 Seeds of wheat, proteic pea, and the weed *Abutilon* spp. are placed on Potato Dextrose Agar to observe microbial diversity and abundance. On the right, the seeds were not surface disinfected (Control). On the left, seeds of the same lot were surface-disinfected following standard protocols (Disinfected).

Molecular-based methods, mainly based on PCR techniques, can reduce or eliminate the need for fungal culturing and morphological identification (McNeil et al. 2004). Developed in 1986 and widely adopted over the past few decades, these techniques have opened up numerous possibilities for faster, more sensitive more objective fungal detection (Scialpi and Mengoni 2008; Sun and Guo 2012). Molecular techniques can support traditional methods, for

example, DNA isolation and subsequently sequencing ITS genome region of cultured fungi, then blast with public database (ex. Ncbi) allows to identify uncertain fungi or *mycelia sterilia* (McNeil et al. 2004; Naik 2009). Developing a nested PCR approach could increase sensitivity (Sun and Guo 2012), but it may also lead to greater reagent usage and be more time-consuming (Scialpi and Mengoni 2008).

Real-time PCR allows for to quantification of the DNA expression the creation of a standard curve allows for the quantification of fungal inoculum (Accinelli et al. 2023). However, this approach requires development and refinement for each specific matrix and microorganism (Scialpi and Mengoni 2008). Additionally, these molecular approaches do not provide the relative frequency of microorganisms. With endpoint PCR, the results are primarily qualitative, while with Real-time PCR, the results are mostly quantitative (Scialpi and Mengoni 2008). Both methods require the design of specific primers. Nowadays, advancements in sequencing technologies enable the detection of unculturable microorganisms and environmental microbiota, providing insights into the relative frequency of all microorganisms present. The approach developed with the germination apparatus operates in a non-destructive manner, allowing the detection of m.o. active during seedling development.

2.4. Amplicon metagenomics as a tool for detecting microrganisms

The term *Next Generation Sequencing* (NGS) encompasses all sequencing technologies that surpass Sanger's method (Figure 2.4). These technologies enable high-throughput parallel sequencing of DNA and are categorized into second and third-generation sequencing methods (Table or image with sequencing technologies) (Behjati and Tarpey 2013; Slatko et al. 2018). All these technologies, thanks to the possibility to sequence in parallel thousands of sequences, allow the characterization of complex microbial samples without the necessity of previously isolating the single strains on a culture medium (Mayo et al. 2014).

In this work, we focus on Illumina sequencing, as it is the technology we used. At the moment, Illumina is currently considered the most reliable option for our laboratory due to its convenience and cost-effectiveness (35-50 € per sample). In addition, is considered one of the most reliable techniques due to its high accuracy with a typical error rate of 0.1–1% (Quince et al. 2017). However, nanopore sequencing is rapidly advancing and shows great potential for future applications thanks to the possibility to sequence long DNA fragments (reads) (Slatko et al. 2018).

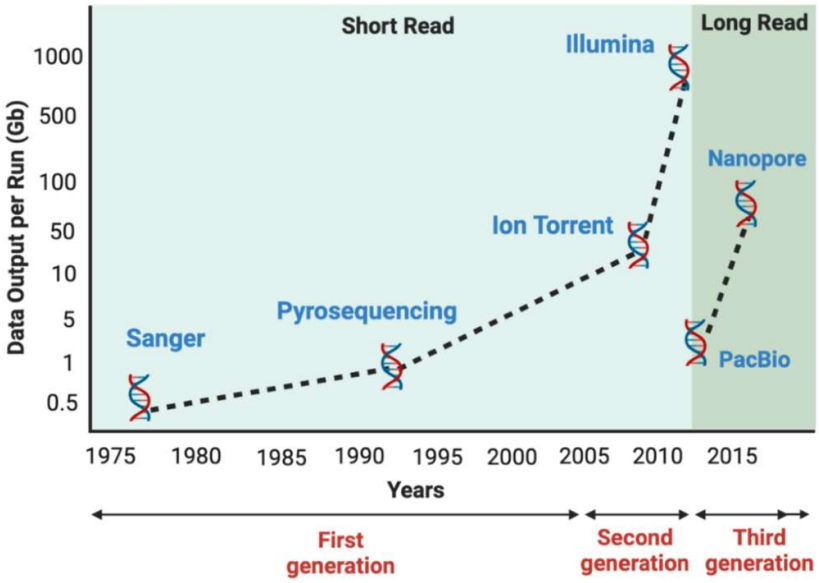

Figure 2.4 The graph illustrates the evolution of sequencing technologies from 1975 to 2015 (Satam et al. 2023).

The application of Illumina and other NGS technologies has revolutionized microbiology in the past few decades (Reis-Filho 2009; Mayo et al. 2014). Key advantages include the possibility to identify taxa, detect the presence of m.o. in small quantities, and provide a comprehensive taxonomic profile (Mayo et al. 2014). These advancements have significantly enhanced research on microbiota and environmental microbial characterization. Specifically, for the identification of endophytic fungi, NGS technologies have enabled the detection of non-culturable fungi and bacteria (Mayo et al. 2014). Sequencing short DNA fragments or even whole small genomes is now fast, reliable, and relatively affordable.

Shotgun sequencing and amplicon-based sequencing, often referred to as metabarcoding, are the two primary techniques used for characterizing total microbial nucleic acids in complex samples, such as those derived from environmental or microbiota studies (Taberlet et al. 2012; Mayo et al. 2014; Shelton et al. 2023). Shotgun sequencing involves the direct sequencing sequencing of all microbial genomes present in a sample (Quince et al. 2017). In contrast, metabarcoding requires an initial amplification step using polymerase chain reaction (PCR) with a single primer set, followed by sequencing that targets only the amplified regions (Mayo et al. 2014; Shelton et al. 2023) which targets a region of DNA shared among a specific taxonomic group (Jovel et al. 2016). For example, the 16S ribosomal RNA (rRNA) gene is commonly amplified for bacterial identification (Jovel et al. 2016), while the internal

transcribed spacers 1 and 2 (ITS1 and ITS2) region is amplified preferred for fungi (Santamaria et al. 2012).

Although shotgun sequencing provides more comprehensive information, such as the number and potential functions of genes within the microbial community, it is more expensive and requires more extensive computational analysis (Mayo et al. 2014; Quince et al. 2017).

2.5. Main steps of bioinformatic analysis for fungi metabarcoding

The result of metabarcoding using Illumina amplification is a dataset of approximately 1 GB of raw data per sample. This data requires quality control, cleaning, and further processing, which is accomplished using bioinformatics tools and software designed to manage and analyze large volumes of information and associated file formats.

For this seed endophytic metabarcoding analysis, the primary software used is QIIME2 (Bolyen et al. 2019). However, quality-checking software like FastQC is essential in the initial processing step to assess data quality and the efficiency of cleaning (e.g., trimming). While QIIME2 includes its own trimming and filtering functions, tools such as Trimmomatic (Bolger et al. 2014) can be used as alternatives for raw data cleaning before analysis.

Typically, analyses must be repeated multiple times, experimenting with different cleaning parameters and filtering tools to determine the most optimal settings. To streamline this iterative process, workflow managers like Snakemake (Köster and Rahmann 2012) or Nextflow (Di Tommaso et al. 2017) are highly useful, allowing for automated and reproducible code execution. All analyses are conducted in a Conda (Anon 2020) environment on a Linux virtual machine.

2.5.1 *Check the quality of the raw data*

Data obtained from Illumina sequencing are stored in a standard file format named FASTQ (.fastq), which contains read data (DNA sequences) and encoded PHRED[3] quality score for each nucleotide (Figure 2.5.1a) (Cock et al. 2010).

```
@SRR27781698.1 M70528:407:000000000-KRHVN:1:1102:16890:1488/1
```

[3] "Phred quality score, or q-score, is a quality measure that estimates the probability that a base was called incorrectly, given on a negative log scale (Q=−log10P(incorrect)) so that a higher q-score indicates a more confident base call." (Hawkins and Yu 2018)

```
CGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGTAACAGGTCTTCGGTCGCTGA
CGAGTGGCGAACGGGTGAGTAATACATCGGAACCTGCCCCGTCGTTGGGGATAACTACTCGAAAGAGTAGCCAATACCGCATA
CGATCTGAGGATGAAAGGGGGGGACCTGCGGGCTTCGCGCGATTGGAGTGGCCGCTGGCTGCATAGGCAGTTTGGGGGGTAAA
AGCGTACCAAGGGGGCCAACTGTAGCTGGGCAATGTGGAGGATCAGGCCAAA
+
@ABBA?CBBCFGGEHHHHHGFFFFFDDCCEEEEHHHHHGFFFFFEEEDDCCCC@CCCCCFFFFGHHHHHHHHHHHHHHHGGGG
GGHHHHHGGGGGGFFFFFFEEEDDDBBBBAAAAAAAAAABBBCCCCCCDDDDDFFFFFGGGGGHHHHHHHHHHHHGGGGFFFFF
FFFEEEEDDCCBBBBBBBCCCCFFFFHHHHGGGGGFFFFFFFFFFFEEDDCCCCCCBBBBBBBBBBCCCCCCDDDDDFFFFFF
FFFFFFFFFFFFFEEDDDDDCCCCCCBBBBAAAA????????
```

Figure 2.5.1a An example of one of the hundreds of reads contained in a .fastq file.

In the case of Illumina sequencing results, each file stores hundreds of different reads, each composed of a header, the sequence itself, and the quality data. Sometimes, the data are in the "paired-end" format, meaning there are two separate files for each sample storing data from forward and reverse sequencing as schematized in Figure 2.5.1b (Masella et al. 2012).

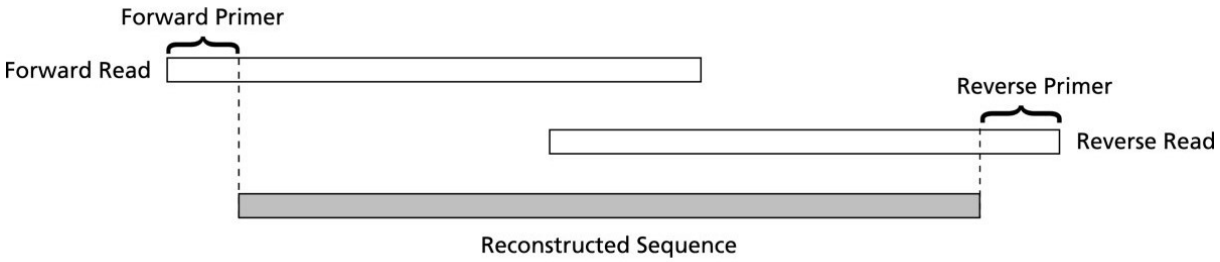

Figure 2.5.1b Schematic of paired-end assembly (Masella et al. 2012)

First of all, it is necessary to check the quality of the reads. If there are two reads for each sample, in the case of paired reads, it is important to ensure that the number of reads is equal for both. FastQC is the software used to assess read quality and provides various information about the reads in graphical form (Andrews 2010). The data obtained from quality checks with FastQC are essential for determining parameters for the trimming and cleaning step.

2.5.2 Import raw data, cleaning and trimming

Main of the analysis can be conducted with QIIME2, this software works only with two specific file formats: artifact files (.qza) and/or visualization files (.qzv). Firstly, it is necessary to import data from sequencing and store their data in a .qza file (Estaki et al. 2020).
There are two ways to clean and trim data: one is to perform it beforehand using the software Trimmomatic (Bolger et al. 2014), and the other is to do it after importing the data into QIIME2

using the DADA2 algorithm (Callahan et al. 2016). Essentially, based on the FastQC analysis, it is necessary to remove poor-quality reads, as they typically have lower quality toward the 3' end, as well as PCR and Illumina primers (technical sequences).

2.5.3 OTUs or ASV clustering, feature table filtering

Algorithms like VSEARCH (Rognes et al. 2016) and DADA2 (Callahan et al. 2016), both supported by QIIME2, are designed to cluster sequences at a defined similarity threshold, usually set at 97%, into a file called a feature table. The results are referred to as OTUs (Operational Taxonomic Units) or ASVs (Amplicon Sequence Variants). In practice, a feature table assigns each OTU or ASV an ID along with its relative abundance in the sample. Subsequently, the table can be filtered to remove sequences that are too short or have a low frequency (Jeske and Gallert 2022). ASVs are preferred because they are easier to replicate across different studies, while the OTU algorithm is specific to a single study (Jeske and Gallert 2022).

2.5.4 Taxonomic Relative Quantification

At this point, QIIME2 allows matching each ASV or OTU to a reference database, which, in the case of fungi, is typically UNITE (Abarenkov et al. 2024). Each element is matched to a specific genus, and further classification can identify it down to the species level. Databases must either be trained using the algorithm or downloaded as pre-trained versions. Afterward, a boxplot can be constructed, as well as a phylogenetic tree or further analyses can be performed on the data. One example is alpha and beta diversity analyses, which assess differences within a sample (alpha diversity) or between samples (beta diversity) (Tuomisto 2010). The bioinformatic pipeline described above is schematize in Figure 2.5.4.

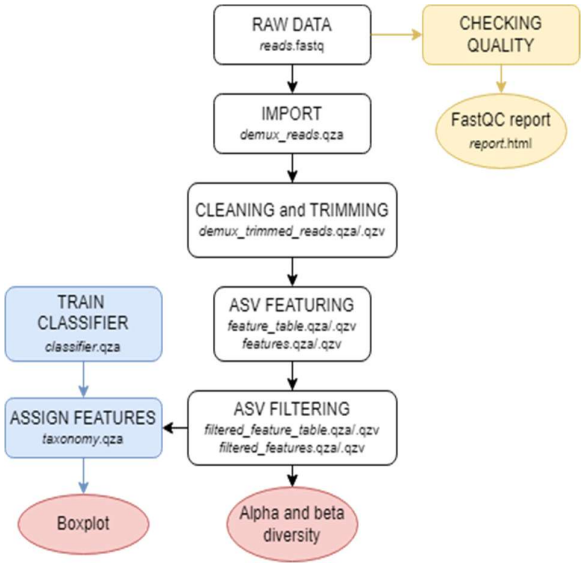

Figure 2.5.4 Bioinformatic pipeline for amplicon metagenomic analysis performed using Qiime2.

2.6. Azole resistance and *Aspergillus*

Azole fungicides are widely used in both medicine and agriculture, representing 20-25% of the total fungicides market (Pérez-Cantero 2020, Jørgensen e Heick 2021). These compounds inhibit the synthesis of ergosterol by targeting Cyp51 enzyme resulting in alterations to fungal cell membranes. This disruption leads to a loss of membrane fluidity and eventual collapse (Pérez-Cantero 2020, Keranova 2023). Key fungicides within the azole class include imidazole prochloraz and triazoles difenoconazole, myclobutanil, propiconazole, tebuconazole (Keranova 2023).

However, like other fungicides, the widespread use of azoles has contributed to the emergence of resistant fungal isolates. These resistant strains have acquired the ability to tolerate doses of azole fungicides that would typically inhibit their growth (Pérez-Cantero 2020, Kenarova 2023). In seed technology, azole fungicides are used in seed-coating treatment within other protection agents for corn, wheat, and many important crops (Accinelli 2018).

In recent years, fungi species in the *Aspergillus* genera have raised concern due to the spread of azole-resistant isolates. *Aspergillus* genus comprises species such as *Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus,* and *Aspergillus niger* (Pérez-Cantero 2020). These species are common colonizers of crops like corn, peanuts, cotton, and many other plant species, where they can cause significant yield losses (Bhatnagar-Mathur 2015). Additionally,

there are major concerns about their role as causal agents of invasive aspergillosis (IA) in humans and their production of mycotoxins, carcinogenic secondary metabolites (Bhatnagar-Mathur 2015, Pérez-Cantero 2020).

The aim of the study presented in the next chapter is to investigate the possibility that seed treatment with azoles may increase the incidence of resistant isolates. For this purpose, a germination apparatus was used, and further analyses of microbial diversity were conducted using Next Generation Sequencing (NGS).

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2.7. Induced selection of tebuconazole-resistant *Aspergillus flavus* isolates during germination of treated corn seeds

Chiara Morena, Cesare Accinelli, Veronica Bruno, Hamed K. Abbas, Ryan T. Paulk, W. Thomas Shier (2025). Journal of Agriculture and Food Research, 19(101566):2666-1543 https://doi.org/10.1016/j.jafr.2024.101566.

Highlights

- *Other than in human medicine, azole fungicides are also widely used in agriculture.*
- *The azole fungicide tebuconazole is used in foliar and seed treatment applications.*
- Aspergillus flavus *was recovered from germinating tebuconazole-treated corn seeds.*
- *At the recommended dosage, up to 72 % of isolates were resistant to tebuconazole.*
- *This treatment also increases the percentage of aflatoxin-producing isolates.*

2.7.1 Abstract

Azole fungicides are used for spraying crops and also in seed treatments of corn, wheat, and other important agricultural crops, in which seeds are dressed in a plastic-like coat containing an azole fungicide and other seed and seedling protection agents. In this study, the effect of tebuconazole in corn seed treatment on selecting tebuconazole-resistant *A. flavus* isolates was investigated. Seed-borne *A. flavus* isolates growing during seed germination were tested for tebuconazole resistance. When seeds were treated with increasing dosages of tebuconazole, the relative abundance of resistant isolates increased. At the recommended dosage, up to 72.1 % of the seed-borne *A. flavus* isolates that emerged from germinating seeds were resistant to tebuconazole. Resistance increased to 83.4 and 95.1 % when dosages were doubled or quadrupled, respectively. Application of tebuconazole also increased the abundance of aflatoxin-producing isolates of *A. flavus*, from 32.2 % in untreated seeds to 67.4 % in seeds receiving the highest dosage. Results from this study suggest that seed treatment with tebuconazole should be included in the list of hotspots that induce resistance to azole antifungals and that measures and strategies, such as alternative fungicides with different metabolic targets, should be considered for reducing this risk.

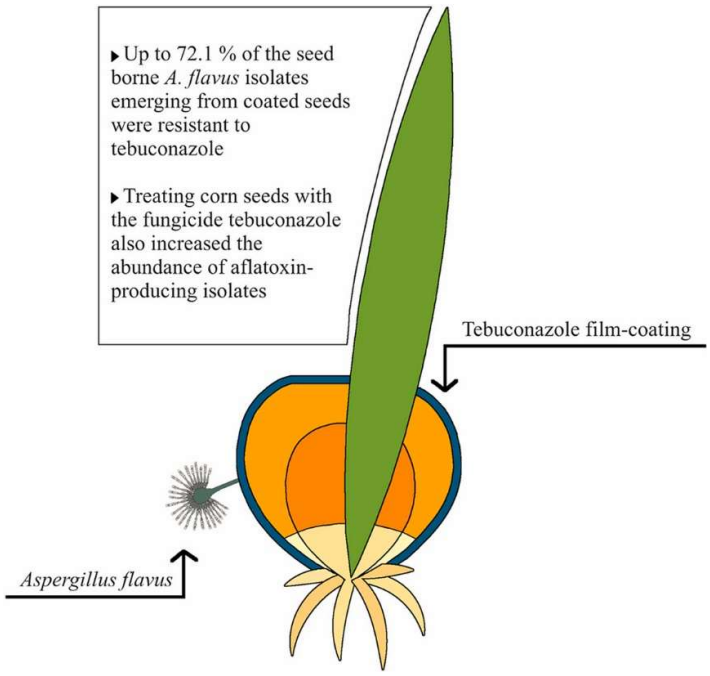

Figure 2.7.1 Graphical abstract

2.7.2 Introduction

Seeds of numerous crop species are routinely treated with pesticides before planting to reduce the risk of pathogen and pest attacks during the vulnerable phases of seed germination and seedling standing (Hitaj et al 2020; Munkvold et al 2002). Among the different techniques and approaches, with seeds of adequate size and uniform shape (i.e., corn, sunflower, cotton, etc.), pesticides are commonly applied by covering their surface with a thin plastic-like film coat containing one or two fungicides, an insecticide, and in most cases, a bird repellant (Accinelli et al, 2021). Additives and dyes are then added to improve mechanical planting and to prevent adulteration of food and feed. In practice, film-coating is achieved by applying a small volume of liquid slurry to seeds which are rotating in a coating pan. Size and shape of film-coated seeds remain unaltered, with only a small increase in mass (Taylor et al, 1991).

A major advantage of film-coating, and in general of seed treatment, with respect to broadcast and in-furrow applications, is that pesticides are precisely placed in strict proximity of the target of protection (i.e., germinating seeds and seedlings). For effective protection against pests and pathogens, slurries are prepared with a high dosage of pesticides, usually in the range of parts per thousand. Beside technical aspects, these elevated dosages could exert negative and/or selective pressure on microorganisms that come in contact with the artificial

seed coat. This is especially the case of filamentous fungi, including soil- and seed borne fungi, and fungi that are located on the seed surface. Resistance development by fungal isolates that are exposed to field crop fungicides is becoming a serious issue, especially when these fungi are also of medical importance (Schoustra et al 2019; Verweij et al 2020). Many agricultural fungicides share the same metabolic target sites and have similar mode of action as medical antifungals (Bastos et al, 2021). An emerging issue regarding this phenomenon is the increasing occurrence of azole-resistant *Aspergillus fumigatus* and *A. flavus* isolates, two fungi that are responsible for invasive aspergillosis and are readily isolated from environmental samples, including soil and seeds (Abbas et al 2009, Snelders et al 2009). Azoles are a group of antifungals that are widely used in medicine and agriculture. They were introduced in the 1970s and are currently the most used fungicides in crop protection for both foliar and seed treatment applications (Price et al 2015). Tebuconazole is a widely used azole fungicide with an aromatic ring that gives it the potential for endocrine-disrupting effects. Despite intense research efforts aimed at developing novel anti-fungal agents, azoles remain the most prescribed antifungals for controlling fungal infections in humans, including invasive aspergillosis. While *A. fumigatus* is the most frequently reported agent causing aspergillosis infections, the incidence of *A. flavus* is increasing, especially in areas with hot and arid climates (e.g., Middle East, Asia and Africa) (Kanaujia et al 2023). Other *Aspergillus* species are also involved in invasive aspergillosis, such as *A. terreus* and *A. nidulans* (Sugui et al 2015). All these filamentous fungi are ubiquitous, living as saprophytes in soil, plant debris, compost and other organic-rich substrates, including seeds (Tekaia et al, 2005; Abbas et al, 2009; Alves et al, 2009). However, differently from the others, *A. flavus* is also an opportunistic plant pathogen, responsible for ear rot in corn and, most importantly, a major producer of aflatoxins which are potent carcinogenic toxins for humans (Abbas et al 2009). *A. flavus* is commonly recovered from corn kernels, and when seeds are improperly stored (e.g., high seed moisture content), the fungus continues to grow and thus negatively impact seed quality in terms of both aflatoxin content and reduced germinability and vigor (Majumdar et al 2021; Owolade et al 2005; Sreenu et al 2019). As stated above, film-coating corn seeds with fungicides serves to prevent the growth of seed-borne fungi than can reduce seedling germination and growth, and for protecting the seeds from being infected by soil-inhabiting pathogenic fungi (Munkvold et al 2002). Consequently, applications of seed film coats containing one or more fungicides could also provide conditions for increasing the frequency of antifungal agent-

resistant isolates within the populations of seed-borne fungi and soil-inhabiting fungi living in proximity to the artificial coat. Recent studies have investigated the role of soil fungicidal residues from foliar applications in promoting development of antifungal agent-resistance, focusing on *A. fumigatus* and azole fungicides. However, a limited number of studies have investigated *A. flavus* and none of these have specifically dealt with *A. flavus* and azoles when applied as a seed treatment (Price et al 2015; Hermida-Alava 2021).

The main objective of the present study was to evaluate the hypothesis that coating corn seeds with tebuconazole, an azole fungicide widely used for treatment of multiple seed species, could induce the selection of tebuconazole-resistant seed-borne *A. flavus* during the seed germination process. The study also focused on the effect of tebuconazole seed treatment on the relative abundance of aflatoxigenic and non-aflatoxigenic *A. flavus* isolates recovered from germinating seeds.

2.7.3 Materials and methods

Seeds and seed film-coating

Seeds of the commercial corn hybrid Kristal (KWS Italia S. p.A., Forlì, Italy) were surface sterilized by washing for 3 min in a 1 % NaClO solution, followed by rinsing four times in ultrapure sterile water. Surface-sterilized seeds were then film coated with an aqueous slurry prepared with the commercial formulation Sepiret® 9290 (Basf Corp., Ludwigshafen, Germany) with the addition of tebuconazole, 1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol (Pestanal®, analytical standard; Merck KGaA, Darmstadt, Germany) at the following dosages: 5, 10, and 20 mg mL^{-1}, corresponding to 17 (1x), 34 (2x), and 68 (4x) µg $seed^{-1}$. Before application, the slurry was sterilized by passing through a 0.2 µm sterile filter. Seeds (50 g) were then transferred into a sterile 50-mL centrifuge tube and coated by adding 0.25 mL of slurry while vortexing the tubes at maximum speed for 2 min using a Vortex- Genie 2 (Scientific Industries Inc., Bohemia, NY, USA). Visual inspection confirmed that seeds were completely coated by the colored coating material. The procedure was repeated using a bio-based coat formulation containing extruded starch (5.5 % w/v), Arabic gum from acacia tree (2.7 % w/v), soybean lecithin (0.3 % w/v) and soy wax (0.05 % w/v), as described in Accinelli et al. (2021).

Recovery of seed-borne microorganisms from germinating seeds

Corn seeds were incubated in single seed germination tubes as shown in Figure 2.7.3a. Seed-borne microorganisms were recovered from germinating corn seeds using germination tubes (2.7.3a) specifically designed to avoid microbial contamination and to exclude any potential effects from external nutrient sources (i.e., agar-based germination substates) (Accinelli et al 2023). Briefly, seeds were placed in the center of a 2-cm diameter plastic support, which was provided with two lateral sterile cotton filters (diameter of 10 mm; length of 18 mm) that served for keeping the seed moistened when they were wetted. The disc was placed between two conical 50-mL centrifuge tubes, connected by a screw cylinder. Except for the centrifuge tubes, all other components were manufactured using a Form 3 L SLA 3D Printer equipped with a 100 µm resolution clear resin. Printer machine and resins were obtained from Formlabs Inc. (Boston, MA, USA). The two filters were moistened with a fixed volume of sterile ultrapure water (1.5 mL each filter plug) and then tubes were incubated in a germination chamber at 25 °C with 12 h of light per day.

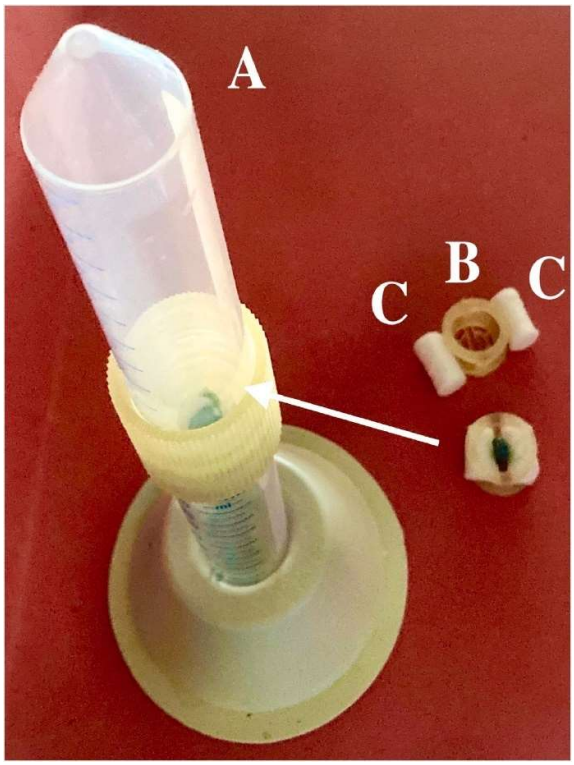

Figure 2.7.3a Seeds were incubated in single-seed germination tubes (A), consisting of two connected centrifuge tubes containing a plastic support (B) provided with two cotton plugs (C) for both moistening the seeds and entrapping seed-borne microbes.

Germination percentage was recorded daily with mean germination time (MGT) calculated as follows: MGT = $(\Sigma n_i \cdot h_i)/\Sigma n_i$ in which n_i is the number of seedlings present on interval i, and h_i is the number of hours since the beginning of the test (Ellis and Roberts 1980). Seeds (100 per treatment) were considered to have germinated after the radicle emerged. After 4 days from the beginning of seed incubation, cotton plugs were aseptically removed and directly used for microbial evaluation.

Aspergillus flavus *isolation and qPCR quantification, and composition of the fungal community*

Cotton plugs recovered from germination tubes were transferred to 15-mL centrifuge tubes containing 19 mL of autoclaved 0.02 % Tween 20 solution and shaken on a horizontal shaker for 30 min at room temperature. Aliquots of 100 µL were spread onto plates of modified 2,6-dichloro-4-nitroaniline Rose Bengal (MDRB) agar medium and incubated at 37 °C for 7 days. Isolates were randomly selected, sub-cultured on PDA and then tested for azole resistance and aflatoxin production. For both analysis a total of 50 randomly selected *A. flavus* isolates were used.

Cotton plugs from incubated samples were processed for quantifying *A. flavus* using a molecular approach. Specifically, quantification was performed by qPCR following the procedure described in Accinelli et al. (2023). The total DNA from 100 aliquots of the above-mentioned microbial dispersion was isolated using the commercial kit DNeasy® UltraClean® Microbial Kit (Qiagen Corp, Hilden, Germany) following the manufacturer's instructions. DNA was quantified using a BioDrop spectrophotometer (BioDrop Ltd, Cambridge, UK) and then amplified with an Open qPCR (ChaiBio, Santa Clara, CA, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each 25 µL of reaction mixture contained 12.5 µL of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each primer (Accinelli et al 2022), and 40 ng of DNA. Samples were amplified on a standard curve (r^2 = 0.92; efficiency = 94 %; slope = - 0.21) generated by plotting cycle threshold values (Ct) against known spore concentration values.

DNA samples that were recovered from cotton plugs were then used for next-generation sequencing (NGS) analysis of the seed-borne fungal community. DNA samples were sequenced by BMR Genomics S. r.l. (Padova, Italy) with Illumina high-throughput sequencing methodology. The fungal ribosomal ITS2 region was amplified with ITS3_KYO2 (GATGAAGAACGYAGYRAA) and ITS4r (TCCTCCGCTTATTGATATGC) primers. A total of

1,481,944 paired-end reads were produced by the amplification, with an average of 82,330 paired-end reads per sample. Microbiome bioinformatics were performed using an adapted Snakemake pipeline (Mohsen et al 2022, Mölder et al 2021). Raw sequence data were quality checked with FastQC. Sequence quality was improved using the Trimmomatic tool (Bolger et al 2014) by clipping Illumina adapters, removing bases with a Phred quality score lower than 15, and, sequences shorter than 36 bp. OTUs clustering was performed with DADA2 (Callahan et al 2016), such as the construction of their relative frequency table. Taxonomy was assigned with QIIME 2 2017.4 (Bolyen et al., 2019) using the q2-feature-classifier (Bokulich et al 2018) classify-sklearn naïve Bayes taxonomy classifier against the UNITE (Abarenkov et al 2024) reference sequences database (97 % similarity). Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI (accession number PRJEB72729).

Tebuconazole susceptibility and aflatoxin production of recovered A. flavus isolates

Isolates of *A. flavus* that were recovered from germinating seeds were evaluated for their susceptibility to the fungicide tebuconazole and their ability to produce aflatoxins.

Tebuconazole susceptibility test was performed following the microbroth dilution method outlined by the Committee for Antimicrobial Susceptibility Testing (CAST), reference method version 9.3.1 (Arendupt et al 2016). Fungal isolates were sub-cultured on potato dextrose agar (PDA) and spores were collected as described above. Spore dispersions were adjusted to 3 x 10^5 spores mL^{-1} and aliquots of 100 µL were used to inoculate an equivalent volume of 2 x RPMI 1640 medium with 2 % glucose (ThermoFisher Scientific Inc., Waltham, MA, USA), contained in each single well of 96-well microplates. Tebuconazole solutions (30 µL) were added to the wells to achieve final concentrations of 0.03, 8 and 16 mg L^{-1}. Control wells prepared with no tebuconazole and uninoculated wells were included. Two *A. flavus* isolates, NRRL 30796 and NRRL 30797, were included as quality control. After incubation for 48 h at 37 °C, wells were visually inspected and the minimal inhibitory concentration (MIC) of tebuconazole was determined as the lowest concentration of the antifungal that completely inhibited fungal growth. Isolates with MIC above 16 mg L^{-1} were considered resistant to the fungicide tebuconazole. Analytical grade (>98 % purity) tebuconazole was purchased from Merck KGaA (Darmstadt, Germany).

For assessing the capability of *A. flavus* isolates to produce aflatoxin B1, plugs from PDA plates with active fungal growth were transferred to test tubes containing 5 mL of yeast extract

sucrose broth and incubated without shaking for 7 days in the dark at 30 °C. Samples were then extracted with chloroform (2 mL) by shacking for 1 min and extracts evaporated to dryness in vacuo. Residues were dissolved in methanol/H_2O (70:30, v:v) and aflatoxins concentration determined by HPLC, following the method of Accinelli et al. (2019). A calibration curve was obtained using solutions with known concentration of analytical grade aflatoxin B1 (purity ≥98.0 %; Merck KGaA, Darmstadt, Germany). The limit of detection was 0.1 ng g^{-1}. Dry weight of mycelial mats was determined after air drying for 48 h at 70 °C.

Statistical analysis

Experimental data were processed by one-way analysis of variance ANOVA, using the software SPSS ver. 29 (SPSS Inc., Chicago, IL, USA). Means were compared by Fisher's least significant difference (LSD), and P values < 0.05 were considered statistically significant.

2.7.4 Results and discussion

Recovery of seed-borne A. flavus isolates from germinating seeds

Seed germination data are summarized in Table 2.7.4a Application of the commercial or the bio-based formulations to non-sterile or surface-disinfected seeds did not affect the germination percent or the mean germination time. These two parameters were also unaffected when the two film-coat slurries were applied with the addition of tebuconazole. Although, under some circumstances (e.g., chilling conditions), tebuconazole can affect corn seed germination, in general, when applied at recommended doses, germination and seedling growth are not influenced by this fungicide (Yang et al 2016).

Table 2.7.4a Germination, mean germination time and *A. flavus* contamination of corn seeds. The following treatment of corn seeds were studied: uncoated and non-disinfected seeds; uncoated surface disinfected seeds; and surface disinfected seeds that were then coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage. Data are presented as mean ± standard deviation. Values followed by the same letter in the same column are not significantly different (P > 0.05).

Seeds	Germination	Mean germination time	*A. flavus*-contamination
	(%)	(h)	(%)
Uncoated	99.9 ± 1.0 a	49.0 ± 2.1 a	87.3 ± 5.1 a
Uncoated/Disinfected	98.9 ± 1.1 a	48.5 ± 1.7 a	57.4 ± 2.4 b
Commercial Coat			
0x	99.7 ± 1.1 a	44.2 ± 1.1 a	51.2 ± 3.1 c
1x	100.0 ± 1.3 a	50.9 ± 1.8 a	12.4 ± 2.7 d
2x	97.8 ± 1.4 a	44.0 ± 1.1 a	8.1 ± 2.2 e
4x	99.8 ± 1.1 a	44.5 ± 1.3 a	3.3 ± 1.4 f
Bio-Based Coat			
0x	99.3 ± 0.9 a	44.0 ± 1.1 a	56.0 ± 3.5 b
1x	99.9 ± 1.3 a	43.7 ± 1.3 a	25.2 ± 2.2 g
2x	98.8 ± 1.7 a	44.2 ± 1.0 a	17.3 ± 3.1 d
4x	99.5 ± 1.0 a	43.9 ± 1.1 a	15.1 ± 2.9 f

As summarized in Table 2.7.4a, *A. flavus* was detected in 57.4 % of the untreated and surface-disinfected germinating seeds, confirming that this fungus is a common colonizer of corn kernels and not only one of the numerous soil-borne microorganisms that remain on the seed surface after being transported by wind, insects or other vectors (Abbas et al 2009; Accinelli et al 2023). As expected, the percentage of *A. flavus*-contaminated seeds was significantly higher in germinating seeds that were not surface-disinfected, with values of contaminated samples up to 87.3 %. Fewer *A. flavus* isolates were recovered from germinating seeds that

were coated with the commercial formulation, while the bio-based formulation did not affect the number of isolates.

Seeds are infected by various microorganisms, including pathogen and endophytic species (Nelson 2018; Zhang et al 2019). However, and for different reasons (e.g., competition among species, diversity in terms of growth condition requirements), some seed-infecting microorganisms can grow and proliferate during seed germination and seedling elongation while others do not (Alves et al 2009). A common approach used to investigate seed-borne microorganisms is based on recovering microbial propagules or microbial DNA from seeds after crushing the entire seeds. In other methods, seeds are incubated on agar-based nutrient media and then growing microorganisms are picked using inoculation loops or pipette tips (Verma 2017; Sharma et al 2019). While DNA-based approaches do not effectively discriminate among seed-borne microorganisms that are either actively growing on germinating seeds, dead or not growing, cultural methods are limited to culturable microorganisms (Kumar et al 2022). These investigations are even more complicated when seeds are treated with pesticides. In the case of corn, and as described above, seed treatment is achieved by covering seeds with a self-adhering plastic-like coat containing formulants (e.g., binders, fillers, dyes, etc.) and pesticides (e.g., fungicides, insecticides, bird repellents, etc.). When seed treatment is prepared with one or more fungicidal active ingredients, the artificial coat surrounding the seed is expected to act as a shield against unwanted fungi coming from the soil, trying to pass through and infect the seeds. While this practical aspect has been described in the technical and scientific literature (i.e., effectiveness of seed treatment to control fungal infestation), the effects of fungicidal seed treatment on selection and fitness of seed-borne fungi have remained surprisingly unexplored (Accinelli et al 2019; Accinelli et al 2021).

When seeds were film-coated using the commercial polymeric coat, the estimated number of *A. flavus* propagules that were recovered from the two cotton plugs showed a 10.8 % reduction with respect to uncoated seeds (Table 2.7.4a). This was not observed with the bio-based coating. Application of the synthetic polymer-based formulation likely resulted in the formation of a more compact and less penetrable layer than that obtained with the bio-based slurry. More specifically, at the end of the 4-day incubation period, no visible cracks or fissures were observed in the synthetic coat. In contrast, the bio-based coat began to lose consistency,

with visible small fissures. In the present experiment, this rapidly degradable coat was specifically included to compare with the more persistent and firmly adherent synthetic coat. As expected, treating the seeds with the commercial formulation containing tebuconazole at the recommended dose (17 µg active ingredient seed^{-1}) resulted in a significant reduction in the number of recovered *A. flavus* propagules from germinating seeds. More specifically, the number of propagules decreased to 78.4 %. A further decrease was observed when tebuconazole was applied at the 2x dosage. Only a reduced number of *A. flavus* isolates were capable of crossing the tebuconazole-containing coat when the antifungal dosage was quadrupled. Comparable results were observed when the synthetic polymeric coat was replaced with a bio-based and less persistent coat, except that the effect of tebuconazole was less pronounced. For instance, at the 1x, 2x and 4x dosage, the percent of recovered *A. flavus* propagules were of 25.2, 17.3, and 15.1 %, respectively (Table 2.7.4a). As discussed above, this was likely due to the more porous and fissured structure of the bio-based coat. The use of the qPCR approach was chosen for estimating the potentiality of tebuconazole-treated coat to select *A. flavus* isolate capable to cross this selective barrier. Coupled to the tebuconazole susceptibility test, this provide information on the extend of this phenomena.

Various seed-borne fungal species were recovered during seed germination using next-generation sequencing (NGS) techniques (EMBL-EBI accession number PRJEB72729; Table 2.7.4b). The dominant fungal class was *Mucoromycetes*, followed by *Dothideomycetes*, and *Sordariomycetes*. *Rhizopus arrhizus* was the most abundant species, followed by *Alternaria alternata*, and *A. flavus*. These findings are consistent with those discussed above, thus confirming that *A. flavus* is readily isolated from corn kernels. While *A. flavus* was detected in control and treated samples, *A. neoniger* was detected in treated samples, but not in the untreated control. *A. sydowii* was only detected (<0.01 %) in samples receiving the 2x tebuconazole dosage. Application of tebuconazole at the dosage of 2x and 4x, increased the relative abundance of *A. flavus*. These differences are likely due to the selective effect of the fungicide tebuconazole and the occurrence of seed-borne fungal isolate that have acquired tolerance to this fungicide.

Table 2.7.4b Relative frequency of more representative fungal classes and species detected from germinating seeds using the next-generation sequencing (NGS) approach. Seeds were surface-disinfected then coated with a commercial formulation containing tebuconazole at the recommended dosage of the fungicide, and at multiples of that dosage.

Class *Species*	Frequency (%)					
	Uncoated	Uncoated/disinfected	0x	1x	2x	4x
Mucoromycetes	–	92.03	65.12	56.98	92.08	78.88
Rhizopus arrhizus	–	92.03	65.12	55	92.08	78.88
Dothideomycetes	86.67	2.75	18.04	26.77	4.87	11.60
Alternaria alternata	25.56	2.30	9.56	21.80	1.29	9.32
Sordariomycetes	–	3.45	10.13	2.95	<1	6.51
Eurotiomycetes	–	<1	3.02	10.94	<1	1.65
Aspergillus flavus	–	<1	2.18	10.58	<1	1.56
Aspergillus neoniger	–	–	<1	<1	<1	<1
Aspergillus sydowii	–	–	–	–	<1	–

Even at elevated tebuconazole doses, the artificial coat surrounding seeds was only partially effective in inhibiting the growth of seed-borne fungi, presumable because the fungicidal barrier was not impenetrable. Importantly, observed differences in the composition of the fungal community between untreated and tebuconazole-treated seeds indicated that when applied as a seed treatment, tebuconazole affects the number and species of seed-borne fungi that can be dispersed from the seeds into the soil and the environment.

Susceptibility to tebuconazole and aflatoxigenicity of recovered A. flavus isolates

Seed-borne *A. flavus* isolates that were recovered from germinating seeds were evaluated for their susceptibility to tebuconazole using the CAST protocol and their potential to produce aflatoxins. As summarized in Figure 2.7.4b, up to 7.1 % of those recovered from untreated seeds showed resistance to the fungicide tebuconazole, thus confirming that resistance to this class of chemicals is widely spread in environmental *A. flavus* isolates (Hoda et al 2019; Monpierre et al 2021). While most of the studies concerning resistance of aspergillosis-causing

fungi to azole fungicides have focused on the primary agent of invasive aspergillosis, *A. fumigatus*, only limited information is available for the closely related fungus, *A. flavus*. Recent investigations have indicated a major role for agricultural applications of azole fungicides in the development of selective resistance in these two species (Bosetti et al 2023; Doung et 2020).

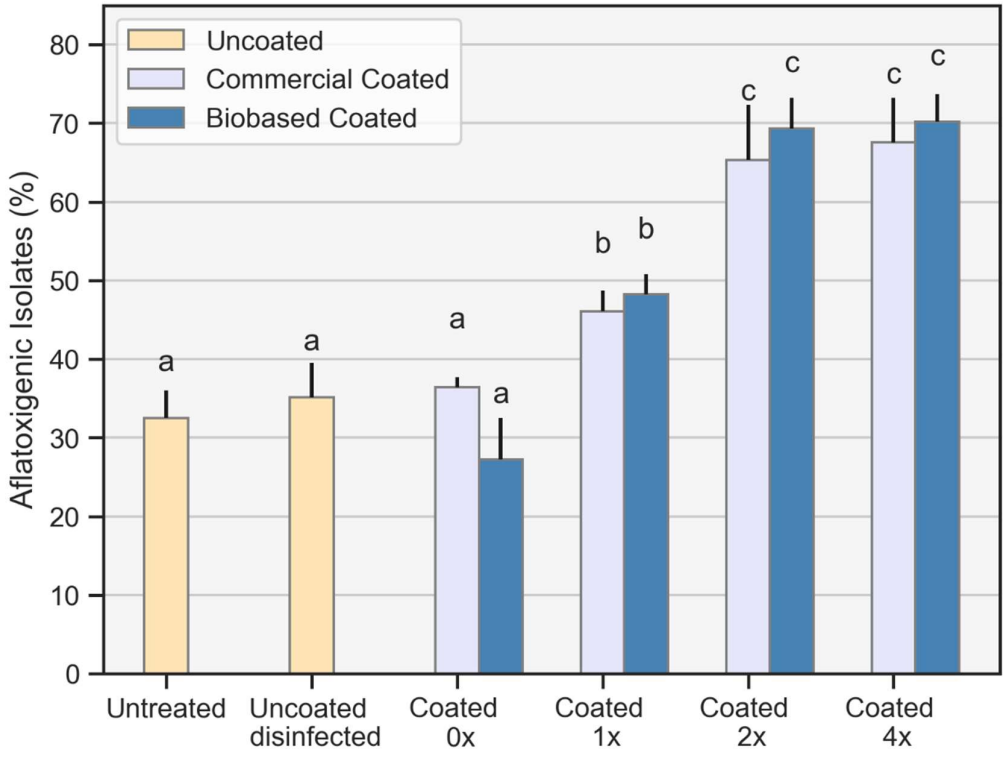

Figure 2.7.4a Percentage of seed-borne *A. flavus* isolates showing resistance to the fungicide tebuconazole. Isolates were recovered from germinating seeds that were coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage of 17 µg seed^{-1}. Before coating, seeds were surface disinfected. Data were calculated with respect to the total recovered seed-borne *A. flavus* isolates. Each bar is presented as mean ± STD. Bars with the same letters are not significantly different ($P > 0.05$).

Being an opportunistic plant pathogen infecting oil-rich seeds, such as peanuts, cottonseeds, corn kernels, and others, seeds can easily spread spores and other propagules of environmental *A. flavus* isolates, including azole fungicide-resistant isolates. Since seeds of these species are routinely treated with fungicides, including azole fungicides, the potential effect of tebuconazole, an azole fungicide widely used in seed treatment, on selecting resistant seed-borne *A. flavus* isolates was evaluated in this study. As discussed above,

application of tebuconazole at the recommended dosage blocked and/or deactivated most of the seed-borne *A. flavus* isolates (Table 2.7.4a). However, when seeds were coated with a commercial polymer slurry containing tebuconazole, the percentage of resistant isolates increased to 72.1 %. This percentage further increased when the tebuconazole dosage was doubled. At the 4x dosage, 95.1 % of the recovered isolates were resistant to tebuconazole. When the commercial polymer slurry was replaced with a bio-based and rapidly degradable but more permeable coating, this phenomenon was less pronounced. More specifically, with the 1x, 2x, and 4x tebuconazole dosage, the percent of resistant isolates was 58.1, 70.0, and 68.9 %, respectively (Figure 2.7.4b). This may have been due to some isolates growing through fissures and pores in the tebuconazole-containing bio-based coating, which was not observed with the commercial coat.

In addition to soil, particularly the organic debris in it, other hotspots for fungicide resistance development in *A. fumigatus* that have been mentioned include wastes from azole fungicide-treated vegetative propagules (e.g., flower bulbs), industrial and home composting heaps, and treated seeds (Schoustra et al 2019; Verweij et al 2020). Surprisingly, considering the importance of the application of azole fungicides in the treatment of crop seeds, none of the reported studies have specifically focused on the role of treated seeds on the selection of azole fungicide resistance in the genus *A. flavus*.

Application of tebuconazole as a seed treatment also affected the ratio of aflatoxigenic to non-aflatoxigenic isolates (Figure 2.7.4a). In untreated germinating seeds, 32.2 % of *A. flavus* isolates were able to complete aflatoxin B1 biosynthesis and surface disinfection had little but not significant effect for seeds coated with commercial or bio-based formulations with no added tebuconazole. In contrast, when tebuconazole was incorporated into the coat at the suggested dosage (1x dosage), 45.6 % of isolates produced aflatoxin B1, and the percentage increased to 65.3 and 67.4 % when the dose was doubled and quadrupled (2x and 4x dosages), respectively. No significant differences were observed between commercial and bio-based formulations.

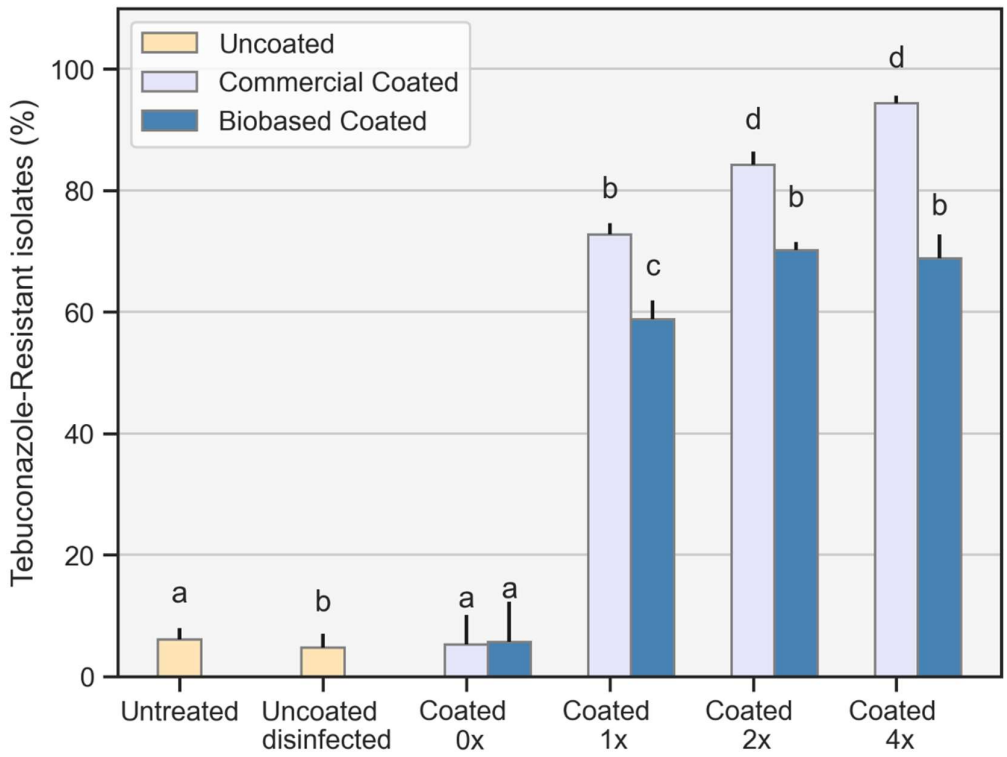

Figure 2.7.4b Percentage of seed-borne *A. flavus* isolates able to produce aflatoxins. Isolates were recovered from germinating seeds that were coated with commercial or bio-based formulations containing the fungicide tebuconazole at 0, 1, 2, and 4 times the recommended dosage (17 µg seed^{-1}). Before coating, seeds were surface disinfected. Data were calculated with respect to the total recovered seed-borne *A. flavus* isolates. Each bar is presented as mean ± STD. Bars with the same letters are not significantly different (P > 0.05).

Recent investigations have demonstrated that the capability of *A. flavus* isolates to produce aflatoxins results in a fitness advantage, especially when the fungus competes with other organisms, such as bacteria and insects, for food resources in nutrient-rich substates, as corn kernels (Drott et al 2019). Although the ecological role of a large number of toxins and other secondary metabolites is unclear, in some circumstances, toxins such as aflatoxins, may play a role in competition among microbial species by alteration of nutrient sources (Janzen 1977). It is not clear how an effective fungicide such as tebuconazole could reduce the nutrient value of corn seeds to seed-borne fungi, including *A. flavus*, thus giving an advantage to aflatoxin-producing isolates. An alternate explanation is that the characteristics of microbes that enable them to acquire mycotoxin biosynthetic cassette genes also enable the acquisition of genetic elements that confer azole fungicide resistance. It has been proposed that mycotoxin and antibiotic biosynthetic cassette genes are acquired using genome mining capabilities

possessed by certain types of microbes found in soil (Shier 2011). These genome mining capabilities are presumed to be based on several better understood processes such as natural competence and transposons. If this proposal is true, when tebuconazole was used to select for fungi on corn seeds that were capable of acquiring azole fungicide resistance genes from their environment, it was in practice selecting for microbes with functional genome mining capability and aflatoxin-producing *A. flavus* isolates would be expected to be among the types of microbes selected for.

2.7.5 Conclusion

This series of experiments using specifically designed single seed test tubes indicated that application of the agricultural fungicide tebuconazole as a seed treatment should be included in the list of hotspots that select for *A. flavus* isolates resistant to the fungicide. In addition, the ratio of aflatoxigenic to non-aflatoxigenic *A. flavus* isolates also increased in tebuconazole-treated seeds. Considering the importance of azole fungicide resistance in *Aspergillus* species, and the widespread use of azole-class fungicides in agriculture, the effects of fungicide-containing seed treatments of agricultural crops on driving antifungal resistance should be included in the evaluation of these risks, and more studies are necessary to better elucidate this aspect.

CRediT authorship contribution statement

Chiara Morena: Software, Investigation, Conceptualization. **Cesare Accinelli:** Writing – original draft, Conceptualization. **Veronica Bruno:** Writing – review & editing, Resources. **Hamed K. Abbas:** Supervision. **Ryan T. Paulk:** Validation. **W. Thomas Shier:** Writing – review & editing.

Research data

Sequences were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI (accession number PRJEB72729), other data are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Chapter 3: Bio-based seed technology

Highlights

- *The development of bio-based solutions in the treatment of seeds of crop species addresses the market and public demand for reducing the environmental impact of this technology.*
- *Nowadays, major companies offer few solutions with a limited number of active ingredients, some of which have shown uncertain results.*
- *Recent and novel solutions that have been developed are consequently required, but a pluriannual research program is needed for their evaluation.*
- *In this experiment, biochar was evaluated as a material for coating of corns seeds*
- *A series of laboratory and field experiments were carried out to evaluate the technical feasibility of this bio-based material and to exclude the risk to promote the growth of the fungus* Aspergillus flavus*.*

3.1. Bio-based seed technology

Bio-based seed treatments are increasingly available on the market, even though there is no strict definition for this category. These treatments comprise all techniques designed to protect seeds from biotic and abiotic stresses, using only renewable resources or, more broadly, avoiding synthetic chemicals.

It is estimated that biotic and abiotic stress could lead to more than a 26% loss of agricultural production, with a predicted annual economic loss of $470 billion worldwide (Bamisile et al. 2021). Since the Green Revolution, due to technological and chemical improvements, the mitigation of crop damages relies on using chemicals (Popp et al. 2013). Pesticides allowed the double of agricultural yields and, as a result, a considerable increase in population, which went from 2.7 billion to 6.8 billion between 1950 and 2010 (Popp et al. 2013). Nowadays, the human population reaches 8 billion people (https://www.worldometers.info/world-population/), and it is estimated that it will hit 9 billion people in 2050 (Popp et al. 2013). For this reason, there is a necessity for a new technological advance to improve food production. This time, in contrast to the massive use of chemicals of the last revolution, the new demand is for “sustainable” ways of food production, where the concept of sustainability is linked to

the idea of a production that does not use more land or water, reduces environmental negative impacts, and can give benefits to society and environment (Firbank et al. 2018).

In this scenario, there is an increasing interest in the use of bio-based products as substitutes for agrochemicals. This is particularly true in the European Union, where due to the Green Deals and strategies many active ingredients were banned, without valid alternatives (Storck et al. 2017). Weiss et al. (2012) have defined bio-based materials as:

"*Materials that are produced partially or entirely from biomass, that is, from terrestrial and marine plants, parts thereof, as well as biogenic residues and waste. Bio-based materials include traditional wood, paper, and textile materials, as well as novel bio-based plastics, resins, lubricants, composites, pharmaceuticals, and cosmetics. The manufacturing processes of bio-based materials range from extraction and simple mechanical processing of natural fibers to fermentation and advanced enzymatic or catalytic conversions*."

In traditional seed technology, the use of chemicals involves not only the use of pesticides as active ingredients but also the composition of the coating, sticking, and binding agents (Accinelli et al. 2018). The application of chemical substances directly to seed surfaces minimizes their use, compared to spray field applications, although some negative aspects require further exploration (Nuyttens et al. 2013). The main issues can be summarized as follows: 1) Seed dust-off and environmental safety; 2) Microplastics dispersion; 3) Operator safety.

Regarding the first point, seed dust-off occurs when dressing, coating, or pelleting is abraded during storing, handling, and drilling operation (Nuyttens et al. 2013; Accinelli et al. 2021). The consequent dispersion of this particle containing varying amounts of chemicals in the environment, with negative consequences for non-target organisms (Nuyttens et al. 2013; Accinelli et al. 2021). Furthermore, dust-off generated from coated or pelleted seeds is directly related to the dispersion of microplastic fragments. These treatments are typically carried out with liquid formulation (or slurry) containing polymers, binders, and plasticizer agents (Accinelli et al. 2021). Lastly, the dispersion of coating fragments and chemicals into the environment is directly associated with an increased risk for operators, as many of these fragments are released during handling and storage (Jovasevic-Stojanovi et al 2004; Sohail et al. 2022).

3.2. Market solutions

While the growing concern for problems related to traditional seed treatments, agrochemical companies are showing interest in the commercialization of seed treatment formulations that include biopesticides and bio-stimulants as active ingredients (Table 3.2). This is also probably due to the growing market interest in these molecules. These products represent 10% of the global pesticide market with an income of USD 5 billion, which is expected to rise to $29 billion USD by 2029 (Marrone 2024).

Table 3.2 summarizes the bio-based solutions commercialized by the main agrochemical companies on the global market. The research was conducted by examining their international websites. In addition, querying the database Fitogest (https://fitogest.imagelinenetwork.com/it/) allowed for flagging (*) the products approved for the Italian market. Overall, a total of 17 products were examined, the majority of which, 7 out of 17, contain one or more microorganism stains as active ingredients.

Table 3.2 A summary of the commercial offerings from the main agrochemicals companies. (*substances authorized for the use in Italy).

Bayer				
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
Ibisio®	Bird Repellent	-Black Pepper Oleoresin	-Corn -Sweetcorn -Sunflower	Provides seeds and seedlings with an unappealing taste and aroma to deter bird predation.
ProStablish®WT	Supports mycorrhization and root development	-Lipo -chitooligosaccharide SP 104 (LCO)	-Winter wheat	LCO enhances the germination of mycorrhizal fungi spores and facilitates root colonization.

BASF				
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
*Votivo ® Prime	Contact nematicide	-*Bacillus firmus*	-Soybeans -Cotton -Maize -Rice -Sugarbeet	Toxins produced by the microorganism act as nematicides.
Nodulator ® Duo SCG	Biostimulant	-*Rhizobium spp.* -*Bacillus subtilis* -Palygorskite -Plaster of Paris	-Pea -Lentil	Promotes nitrogen fixation and forms a root biofilm.
Vault® IP Plus	Biostimulant	-*Rhizobium spp.*	-Soybean	Prevents soil-born diseases and enhances nodule formation.
Integral ® Pro	Biostimulant	-*Bacillus amyloquefaciens*	-Oilseed rape	Enhances the formation of root biofilm.
Corteva				
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
Ympact ®	Biostimulant	-Zinc -Manganese -Copper	-Winter and Spring wheat -Winter and Spring barley -Winter rye -Winter and Spring triticale -Durum wheat -Spelt -Oat	It benefits soil microorganisms and optimizes nutrient bioavailability for seed metabolism.

			-Rice	
Lumiverd ™	Natural insecticide against seedcorn (*Delia platura*) and onion maggots (*Delia antigua*)	-Spinosad	-Onion	The active ingredient is an allosteric activator of nicotinic acetylcholine receptors, leading to hyper excitation of the nervous system and eventually causing insect death.
		KWS		
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
INITIO BIRD PROTECT	Fungicide, Biostimulan, Bird repellent	-Zinc -Manganese -Umic acids -Bird repellent (tannin)	-Corn -Rapeseed -Wheat -Sugarbeet	Improves germination speed and vigor, while deterring bird predation.
		Serbios		
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
*Cedomon Plus	Bio-fungicide	-*Pseudomonas chlororaphis*, MA342 strain	-Winter and Spring wheat -Triticale -Rye	Create a biofilm around the seed and compete with other pathogenic bacteria.
*LALSTOP K61 WP	Bio-fungicide	-*Streptomyces griseoviride* K61	-Many ornamentals and horticultural seeds	Acts as an antagonist deterring the development of pathogenic fungi.

Syngenta				
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
Releaseed ®	Biostimulant	-GEA074 -Molybdenum -Mn EDTA	-Soybean -Corn -Wheat	Improve germination and increase nitrogen fixation.
Valent BioSciences				
Name	**Use**	**Active ingredient**	**Crops**	**Mode of action**
Symvado® Ultrafine	Biostimulants	*-Funneliformis mosseae* *-Rhizophagus intraradices* *-Claroideoglomus etunicatum* *-Claroideoglomus claroideum*	Many	Contains a consortium of arbuscular mycorrhizal fungi to promote root mass expansion, Nutrient use efficiency, Drought tolerance
Symvado™ ST	Biostimulants	*-Glomus intraradices* *-Glomus mosseae* *-Glomus aggregatum* *-Glomus etunicatum*	Many	

SuperSede® Soybean	Biostimulants	-Boron -Cobalt -Molybdenum -Zinc	Soybean	Mitigates early season abiotic stress, improves root and nodulation development, improves uniformity and stand count
SuperSede™ Small Grains	Biostimulants	-Sulfur -Manganese -Zinc	Many	Improves germination and seedling vigor, increases uniformity and stand count, and helps grow a more robust root system

3.2.1 Microrganisms

More specifically, two out of four BASF products, Votivo ® Prime and Integral ® Pro, include species of the genus *Bacillus*, specifically *B. firmus* Bredemann and Werner 1933 and *B. amyloliquefaciens* Priest et al. 1987. It is demonstrated that toxins produced by *B. firmus* acts as nematicides, damaging the gall of several species (Arakere et al. 2022). In addition, this microorganism, together with *B. amyloliquefaciens,* it is considered a plant growth-promoting rhizobacteria (PGPR). These bacteria, colonizing plant roots, promote their growth protecting the plant against pathogens and other pests (Mendis et al. 2018; Paravar et al. 2023).

Vault ® IP Plus and Nodulator ® Duo SCG are biostimulants based on *Rhizobium spp.*, the latter contains in addition *Bacillus subtilis* Cohn 1872**.** Like others PGPRs, *B. subtilis* promotes plant growth competing with others microbes, activating the plant host defense system, and making some nutrients available. It is demonstrated that it is effective under greenhouse conditions

in protecting wheat against *Fusarium graminearum* Schwabe, 1839 (Moussa et al. 2013) and tomato seedling damping-off (Jayaraj et al. 2005; Szczech and Shoda 2006).

Some studies underline that the specific strain used in the formulation can impact its effectiveness (Zhang et al. 2009; Hu et al. 2019), in fact, many commercial formulations contain patented strains. In *Arachis hypogaea* L. (peanut), field studies have demonstrated that *B. subtilis* colonizes roots at a concentration of 10^4 c.f.u./g of root tissue, 120 days after planting (Turner and Backman 1991; Tonelli et al. 2011).

Regarding *Rhizobium* spp., it is a genus of bacteria commonly found in the root nodules of *Fabaceae*, where they fix atmospheric nitrogen in a symbiotic relationship with the plant (Hernandez-Lucas et al. 1995; Husssain et al. 2009). For this reason, it is commonly used for inoculation of *Fabaceae* seeds (Stacey and Upchurch 1984), such as *Cicer arietinum* L. (Kyei-Boahen et al. 2002), *Phaseolus vulgaris* L. (Vargas et al. 2000), *Vigna unguiculata* L. (Kandil and Özdamar Ünlü 2023), *Glycine max* L. (Tahir et al. 2009). In addition, numerous studies suggest that treating *Poaceae* seeds with different *Rhizobium* strains can improve plant growth through mechanisms similar to those of *Bacillus*, such as increasing nutrient uptake efficiency, producing phytohormone and siderophores and inducing systemic disease resistance (Höflich et al. 1995; Biswas et al. 2000; Peng et al. 2002; Husssain et al. 2009).

According to Colla et al. (2015) the application of *Glomus interadicens* (*Rhizophagus intraradices)* and *G. mossae* (*Funneliformis mosseae*) to wheat, together with *Trichoderma atroviride,* increases the yield and quality of the kernels, both in greenhouse and in field studies. The inoculation of *G. etunicatum* (*Claroideoglomus etunicatum*) in maize seeds coating shows a greater plant root development, while better results were achieved when, in addition to the seed treatment, bacteria were inoculated into the soil (Hussain et al. 2021).

3.2.2 Bird repellents

In contrast to the disruptive approach of the past, modern predator management is based on the minimizing of damages (Shivik 2004). The only two bio-based products commercialized as bird repellent are Ibisio ® and INITIO BIRD PROTECT, which are based, respectively, on black pepper oleoresin and tannins. Black pepper oleoresin is a substance, mainly used in the food industry, extracted from black pepper powder (Induruwa Vidana Arachchige Don et al. 2024). In the scientific literature, there are no field studies on its effectiveness against bird damage. However, another oleoresin compound, extracted from *capsicum*, has been tested as a rodent

and elephant repellent (Osborn 2002; Stefanini et al. 2020). Although Conditioned Taste Aversion is a common practice in wildlife management, it is not considered effective in the field (Shivik 2004). Additionally, there is no evidence of the repellent effect of tannins on birds. In general, there are relatively few studies on non-lethal bio-based bird repellents.

3.2.3 Others

Lumiverd ™, produced by Corteva, is a seed treatment based on Spinosad. This molecule, classified as a secondary metabolite, is produced by the aerobic fermentation of *Saccharopolyspora spinosa* Mertz and Yao 1990, a soil actinomycete (Thompson et al. 2000; Hertlein et al. 2011). Its action is based on its neurotoxicity (Vieira Santos and Barbosa Pereira 2019), and its main application is for the effective control of beetle and moth pests in stored grain, covering a wide range of species (Hertlein et al. 2011). Rather than in seed treatment, this substance has been tested in spray or dried foliar formulations for numerous crops (Cisneros et al. 2002). In addition, some studies raise concerns about its safety for non-target organisms and natural enemies (Williams et al. 2003).

Lipo-chitooligosaccharide (LCO) is a molecule produced by *Rhizobium spp.* bacteria and arbuscular mycorrhizal fungi as signals for starting symbiosis with the host plant, briefly allowing the microorganism to be recognized as non-pathogenic by the plant (Maillet et al. 2011; Limpens et al. 2015). The application of this molecule to winter wheat seed is expected to improve the proliferation of mycorrhizal fungi (ProStablish® WT), consequently supporting root development. It has been demonstrated that greenhouse applications can improve tomato growth through foliar spray (John McIver et al. 2007), and increase root length in *Arabidopsis thaliana L.* in vitro experiments (Khan et al. 2011), but it does not appear to be effective in defending soybeans against fungal pathogens such as *Fusarium virguliforme* and *Sclerotinia sclerotiorum* (Marburger et al. 2018). Application in seed treatment has been successfully tested for *Brassica napus* L. (Schwinghamer et al. 2015), *Zea mays* L. (Tanaka et al. 2015), and, *Glycine max* L. exposed to salt stress (Subramanian et al. 2016).

Lastly, the application in seed coating of micronutrients such as zinc, boron, manganese, and others listed in Table 3.1, could be a less expensive alternative to soil or foliar application (Farooq et al. 2012; Afzal et al. 2020). The success of the treatment depends on many factors, first of all on the nutrient used and secondly on the rate of application, as these substances could have a phytotoxic effect or either retained by the coating (Farley and Draycott 1978;

Farooq et al. 2012), for this reason, type of treatment and rate of application are important. According to Farooq et al. (2012) zinc oxide and zinc sulfate is a coating solution with great potential for coating most cereal crops and pulses. Some studies indicate that the application of zinc and boron to seeds led to improvements in growth and yield for *Vigna unguiculata* (L.) Walp., *Helianthus annuus* L., *Zea mays* L., *Triticum aestivum* L., *Glycine max* (L.) Merr., *Arachis hypogaea* L., as well as coating *Oryza sativa* L. seeds with boron (Farooq et al. 2012; Rehman and Faooq 2016; Batista et al. 2022). On the other hand, applying molybdenum to seeds could result in toxicity for nitrogen-fixing and sulfate-reducing bacteria (Farooq et al. 2012; Hara 2017).

Regarding manganese, there is evidence that its application in seed pelleting and coating prevents deficiency in sugar beet and wheat seedlings (Farley and Draycott 1978; Ullah et al. 2018), while it does not affect lupin (Longnecker et al. 1996). Direct application of cobalt to seed has been primarily studied on *Fabaceae*, with good responses observed in *Lupinus angustifolius* L. (Robson and Mead 1980), *Glygine max* L. (Aguiar et al. 2024) and *Phaseolus vulgaris* L. (Mohandas 1985), due to its importance in microbial nitrogen-fixing processes (Lana et al. 2009). Finally, wheat can benefit from sulfur and copper seed treatment (Wiatrak 2013; Kaya et al. 2018).

3.3. Research advancement

In a recent review, Sohail and coworkers (2022) identified six key advancements in the field of seed treatment: inoculation of plant growth-promoting microorganisms (PGPM)[4] in seed coatings; development of polysaccharide-based seed coatings; application of electro-spun nanofibrous seed coatings; implementation of hydrogel-based seed coatings; nanomaterial-based seed coatings; and multilayered seed coatings.

The use of plant growth-promoting microorganisms (PGPM) in seed treatments is one of the most studied applications of bio-based seed treatments. Many studies highlight their roles in biocontrol, biofertilization, enhancing nitrogen fixation, and promoting phosphate solubilization (Sohail et al. 2022). Despite the significant attention from the scientific community, there is a lack of field research data; such as only 3% of the research on

[4] The term PGPM comprise plant growth promoting bacteria (PGPB), rhizobia and arbuscular mycorrhizal fungi (AMF) (Ma 2019).

the use of PGPM integrate laboratory, greenhouse, and field studies (Rocha et al. 2019). One of the main concerns in the practical application of microorganisms in seed coating is the shelf life of the fungi or bacteria adopted (Ma 2019), for example, relative humidity is one of the major factors affecting rhizobia survival during and after coating (Deaker et al. 2012).

The development of different media for coating, for example, the use of polysaccharide, nanofibrous, hydrogel, or nanomaterials, regards the application on a large scale and the sustainability of the production process of these materials (Sohail et al. 2022).

Although field studies have shown uncertain results, the multilayered seed coating technique appears promising due to its easy scalability, as it involves repeated application of coatings to form different layers. One example is the application of PGPM followed by a layer of nutritive substrate, or a protective seed layer followed by a layer incorporating an active ingredient (Sohail et al. 2022).

3.4. Development of a biochar-based seed treatment

The use of biochar as a bio-based seed coat material has been successfully tested for the treatment of rice seeds sown under drought conditions (Zhang et al. 2024), as well as for the inoculation of corn seeds with *Pseudomonas libanensis* (Głodowska et al. 2016) and *Bacillus* sp. MN-54 (Hayat et al. 2023). In addition, coating soybean seeds with a mixture of biochar, liquid culture of *Bradyrhizobium japonicum,* guar gum, and water showed improved seedling growth under greenhouse conditions (Głodowska et al. 2017). Biochar seed coating appears to provide benefits when sowing native species in degraded lands (Shiu et al. 2024) and under arid conditions (Williams et al. 2016).

In the study (Accinelli et al. 2023), conducted through in vitro and field tests, we investigated the performance of corn seeds treated with a biochar-based coating. The aim was to determine the effect of the coating on *A. flavus* infection and aflatoxin contamination.

Figure 3.4 Different treatments tested in the study A) Control, B) Dewaxed, C) Commercial treatment, D) Biochar coating.

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3.5. Use of a Biochar-Based Formulation for Coating Corn Seeds

Accinelli Cesare, Hamed K. Abbas, Chiara Morena, Veronica Bruno, Vivek H. Khambhati, Ryan T. Paulk, Nathan S. Little, Nacer Bellaloui, Walker Forbes, and W. Thomas Shier (2023) Cogent Food & Agriculture 9 (2). https://doi.org/10.1080/23311932.2023.2283956

Highlights

- *Treating seeds with pest control agents or other substances improves seed germination and seedling establishment.*
- *The need for novel bio-based fillers arises from the microplastic pollution caused by traditional polymeric coating treatments.*
- *Biochar, due to its high water retention capacity, improves seed water imbibition and germination.*
- *The study evaluates the use of biochar in seed coating on corn yield, kernel size, population and composition of soil A. flavus, and aflatoxin contamination in corn kernels.*
- *The novel germination apparatus has been used to test seed germination and A. flavus infection in biochar-treated kernels*.

3.5.1. Abstract

The series of experiments summarized here were conducted with the objective to evaluate the benefits of using biochar for coating corn seeds. Seeds coated with a slurry containing bio-based ingredients and biochar were tested for germination and vigor, and for their potential to being infected by the fungus *Aspergillus flavus*, using a novel single seed incubator specifically designed for these purposes. Biochar-treated seeds were also planted for two years in experimental fields in the Mississippi Delta to evaluate their effect on corn yield and aflatoxin contamination of kernels. Experiments were conducted with two types of commercial biochar; one was obtained from hardwood residues and the other from coconut shells. Application of both types of biochar for coating the seeds did not affect seed germination and vigor. However, treated seeds showed increased wettability and a more rapid water uptake. This resulted in a 8.5% shortening of germination time. Microbiological analysis using plate culturing and qPCR methods showed that biochar was not conducive to the growth of *A. flavus*. This was also confirmed by analyzing soil samples that were collected

from experimental fields located in the Mississippi Delta. Most importantly, although aflatoxin contamination was different in the two experimental years, aflatoxin contamination of corn kernels was not affected by biochar-based formulations.

3.5.2 Introduction

Rapid, uniform, and successful seed germination and seedling stand establishment are basic requirements for satisfactory crop production. After planting, seeds and seedlings are exposed to a variable and unpredictable number of biotic and abiotic stress factors that can reduce, delay, or even compromise their germination and seedling stand establishment. Seeds of many agricultural crops are thus routinely treated with pest control agents and other substances to reduce these risks and improve plantability (e.g., better flowability, seed singulation, reduced seed skip) (Hitaj et al 2020; McGee 1988). Among the different treatment approaches, seeds with regular shape and adequate size (e.g., corn, soybean, cotton, sunflower, etc.) are film-coated by covering the seeds with a liquid slurry containing plastic-forming polymers, pesticides, and pigments, using rotating drum or pan coating machines. For rapid drying of the treated seeds, fillers are routinely added to the slurry. Common fillers that are used in the seed treatment include clay minerals such as talc and bentonite, calcium carbonate, gypsum, zeolite, peat, etc (Accinelli et al 2021; Taylor et al 1991).

Since the introduction of industrial seed film-coating in the 1980s, agrochemical companies have developed their own proprietary formulations with a variety of synthetic polymers to uniformly cover seed surfaces with adherent plastic-like coats. However, significant but inconsistent amounts of coating fragments can become detached by mechanical abrasion during seed handling and planting operations, even when effective coating formulations are used. These coating fragments then become dispersed in the environment (Accinelli et al 2018, 2020; Nuyttens et al 2013). These detached seed coat fragments can retain pesticide residues, and studies have indicated that these fragments can have a detrimental effect on bees, terrestrial birds, and other organisms and can contaminate the soil with persistent microplastic-like particles (Krupke et al 2012). For this reason, there is increasing interest in developing biodegradable and bio-based film coat formulations, especially when they are to be combined with biopesticides. As with all film coat formulations, the germination and vigor of treated seeds should not be adversely affected by the surface-applied substances. In addition, the risk of promoting the growth of fungi capable of infecting the root after seed

germination, other plant pathogens, and other harmful microorganisms should be also accurately evaluated. The soil-borne filamentous and aflatoxin-producing fungus *Aspergillus flavus* is included among these microorganisms.

Aflatoxins are carcinogenic secondary metabolites produced by *A. flavus* and related species, including *A. parasiticus* and *A. nominus.* These fungi can infect many crops, such as corn, peanuts, cotton, and others, resulting in direct and indirect losses in many agricultural areas worldwide (Abbas et al 2006). *A. flavus* is a ubiquitous fungus, which is readily isolated from soil and crop residues remaining in the upper soil. In the soil, the fungus plays an important role in the decomposition of organic matter and humus formation. Spores that are produced during the saprophytic life phase of this fungus are transported by air, raindrops, insects, and other vectors and can thus infect susceptible crops (Scheidegger and Payne 2003*). A. flavus* is ubiquitous in the soil microbial community, and its relative level of soil inoculum is correlated with the risk of aflatoxin contamination of susceptible crops in combination with other factors (e.g., air temperature and relative humidity, etc.) (Abbas et al 2009; Fouché et al 2020; Juraschek et al 2022).

In recent years, an increasing number of seed and agrochemical companies have proposed coating solutions containing natural ingredients mixed with biodegradable and renewable polymers instead of conventional oil-based and persistent polymers. Although these solutions are environmentally preferable, in that no release of persistent microplastic fragments into the soil environment occurs, their effects on the soil microbial community have not been clearly elucidated, particularly effects on proliferation of plant pathogenic and/or mycotoxin-producing fungi (i.e., *A. flavus*).

Biochar, a soil amendment that is obtained from thermal decomposition of organic materials under limited oxygen conditions (Das et al 2021; Lehmann 2007), is a potential candidate to be used as a filler for bio-based coating solutions. Due to its high water retention capability, biochar is expected to facilitate water imbibition by seeds, resulting in improved seedling establishment. However, very little information is available on the use of biochar as a material for the treatment of seeds (Głodowska et al 2016; Zhang et al 2022).

The main objective of the present study was to evaluate the benefits of a formulation containing biodegradable polymers and biochar powder for coating corn (*Zea mays*) seeds. Since no specific tests for evaluation of seed germinability of coated seeds are currently available, another objective of the present study was to design a novel tool for assessing this

important parameter. Finally, the study also investigated the effects of this bio-based formulation on corn yield and kernel size, population and composition of soil *A. flavus*, and aflatoxin contamination of corn kernels.

3.5.3 Methods and materials

Seed coating

Seeds of the corn hybrid VT Double Pro (Bayer CropScience, St. Louis, MO, USA) were coated with a two-step procedure using a Hege 11 liquid seed treater (Wintersteiger Inc., Salt Lake City, UT, USA). First, approximately 0.9 kg of seeds were placed in the liquid seed treater spinning plate. While spinning the seeds, 40 mL of 7.5% pre-gelatinized starch solution was slowly added using a 50-mL syringe to coat the seeds. Next, 40 g of biochar powder was added to the sticky seeds and mixed until the seeds were completely covered. The coated seeds were air dried for 24 h at room temperature using a box fan. Two types of biochar were selected for this study, a hardwood biochar (HB; Rockwood Sustainable Solutions, Lebanon, Tennessee, USA) and a coconut hull-based biochar (CN; Cool Planet, Greenwood Village, Colorado, USA). For both biochar types, the application rate was 45 g·kg−1 seeds. The chemical composition and additional properties of the two biochar types are summarized in Table 3.5.3.

Table 3.5.3 Selected properties od the two biochar types used in the study.

Property	Biochar	
	Coconut	Hardwood
Organic carbon (% of total dry mass)	78.0	86.6
Hydrogen/carbon ratio	0.50	0.30
Total nitrogen (% of total dry mass)	0.54	0.91
Total ash (% of total dry mass)	3.3	5.1
pH	6.49	8.86
Electrical conductivity (dS/m)	0.35	0.37

Seed wettability, water uptake, and seed germination

Wettability of treated seeds was evaluated by the sessile drop method using a Theta Lite optical tensiometer (Biolin Scientific AB, Västra Frölunda, Sweden). The apparent contact angle was measured during a 10 s interval after dropping a 10 µL drop of double distilled water on the seed surface. For each treatment, a total of 10 seeds were examined. Measurements also included seed samples that were coated with the commercial polymer formulation Sepiret (BASF, Ludwigshafen, Germany) and dewaxed seeds. The latter seeds were obtained by the removal of the external waxy layer as described in Accinelli et al. (2021).

Water uptake and germination ability were evaluated using a novel seed germination tube specifically designed for testing treated seeds (Figure 3.5.3). As shown in Figure 3.5.3, a single seed was placed in the center of a 2-cm diameter cylinder provided with two lateral cotton filters (diameter of 10 mm; length of 18 mm). The disc was then secured at the connection plane between two conical tubes, which were then connected using a screw cylinder. With the exception of the two 50 mL centrifuge tubes, all remaining parts of the test were designed and manufactured using a Form 3 L SLA 3D Printer equipped with a 100 µm resolution clear resin. Printer and resin were purchased from Formlabs Inc. (Boston, MA, USA). The two filters were moistened with ultrapure water to achieve a total volume of 3 mL. Tubes were then incubated in a germination chamber at 25 °C with 12 h of light per day. For water uptake measurements, seeds were removed at selected intervals (5, 10, 20, 30 and 40 h) and weighed before and after drying at 105 °C for 48 h. Germination, expressed as percent of normal seedlings, was recorded daily with mean germination time (MGT) calculated as follows: $MGT = \Sigma(n_i\, h_i)/\Sigma n_i$, in which n_i is the number of seedlings present on interval i, and h_i is the number of hours since the beginning of the test (Ellis and Roberts 1980). Seeds were considered to have germinated after the radicle emerged. For each treatment, seed water uptake and germination data were obtained from 50 and 100 seeds, respectively.

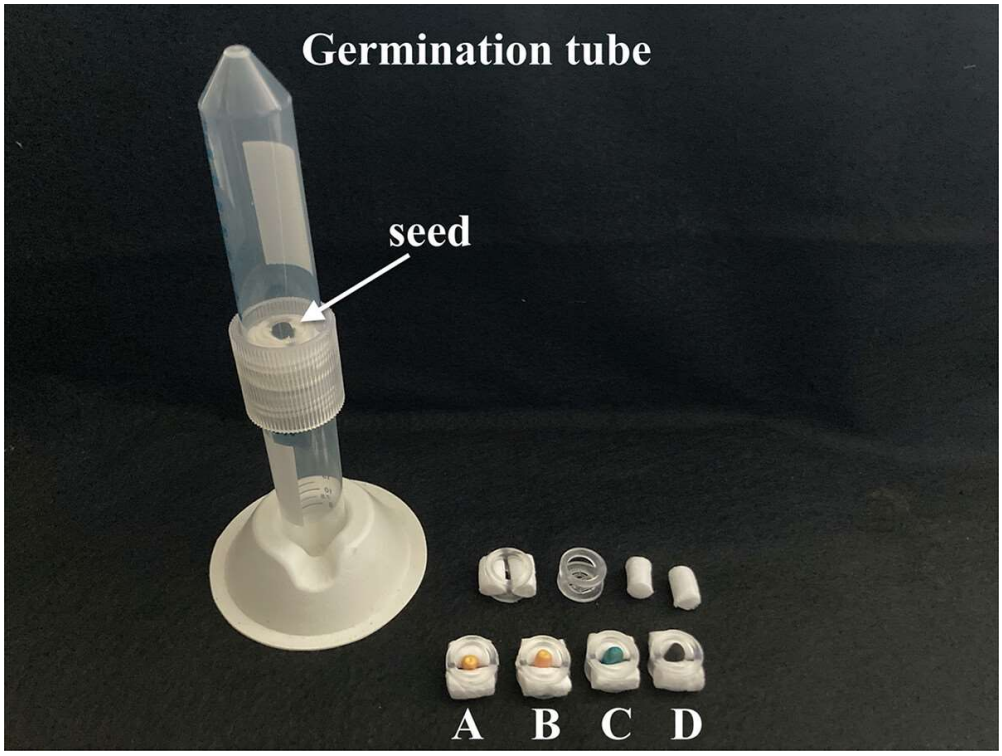

Figure 3.5.3 Corn seed germination and seed water uptake studies were carried out using a specifically designed test tube system (left). Seeds (A: control; B: dewaxed; C: commercially treated; D: biochar treated) were secured between two cotton plugs at the junction of two test tubes held together by a threaded sleeve created with a 3D printer.

Field experiments

Biochar-coated seeds were also evaluated in a field experiment conducted at the Mississippi State University Delta Research and Extension Center (Stoneville, MS, USA). Experiments were carried out in 2020 and 2021 in two 2-ha corn fields (33.4240° N, 90.9151° W) that were managed according to conventional no-till practices of the region. Each plot (15 m long and 10 m wide) was separated by a 1 m wide buffer area. Corn was planted on May 6th and April 6th in 2020 and 2021, respectively, using a MaxEmerge 5 Planter (John Deere Co, Moline, IL, USA) at the rate of 84,000 seeds per ha. The soil had a textural composition of 331, 418, and 252 g·kg−1 of sand, silt and clay, respectively, a pH of 6.2 and total carbon and nitrogen of 13.1 and 1.4 g·kg−1, respectively. Corn was harvested on October 11th and September 1st in 2020 and 2021, respectively, using a Kincade 8-XP plot combine (Kincaid Equipment Manufacturing, Haven, KS, USA) equipped with H2 grain gauge and Mirus software (Harvest Master, Logan, UT, USA). Corn samples were collected in unsealed bags and allowed to dry at 45 °C in a

ventilated oven for two to three days to ensure the moisture content was below 14% to prevent clumping during grinding for analyses.

Aflatoxin analysis

Corn samples were prepared for extraction by grinding approximately 500 g to a cornmeal consistency. Fifty grams of the ground sample was subsequently combined with 70% methanol at a 1:5 ratio (w/v). The resulting mixture was shaken for 30 minutes on a reciprocal shaker at the lowest setting. After resting, the solution was strained through Whatman #1 filter paper to remove large particulates. Twenty milliliters of the filtered solution were collected in scintillation vials, which were stored at − 20 °C until analyzed.

For aflatoxin analysis by high-performance liquid chromatography (HPLC), 500 µL of sample was diluted with 500 µL 100% acetonitrile in 2 mL microcentrifuge tubes. A volume of 800 µL of the mixture was further purified through solid phase extraction using a homemade clean-up column (3 ml syringe) packed with alumina basic powder (200 g) and a glass wool filter under high-pressure vacuum. Purified samples were transferred to 700 µL HPLC vials (Waters Corp., Milford, MA, USA). Five standards were created by serial dilution of a working stock solution and injection solution. The working stock solution was derived from Aflatoxin Mix 4 solution (Sigma-Aldrich, St Louis, MO, USA). Blanks were loaded before and after the standards and after every 10 samples. Waters' Empower software directed the Waters 717 Autosampler to extract 20 µL of sample and flow through a Waters Nova-Pak® C18 4 µm 3.9 × 150 mm column at 30 °C by a Waters HPLC 515 pump at 0.9 mL·min−1 and approximately 3600 psi. Post-column derivatization was performed by a Photochemical Reactor for Enhanced Detection (Aura Inc., San Diego, CA, USA), and aflatoxin was detected with a Waters 2475 Fluorescence Detector set at 365 nm (excitation) and 440 nm (emission). Mobile phase was a solution of water, methanol, and 1-butanol (700:360:12.5) while blanks and injection solutions were composed of methanol, water, and glacial acetic acid (600:380:1). Data were expressed as the total of aflatoxins B1, B2, G1 and G2.

Fungal isolations

Samples from the seed germination study were also analyzed for *A. flavus* infection during the process. Microbial DNA that was trapped by the pair of cotton plugs was isolated using the DNeasy UltraClean Microbial Kit (Qiagen Corp, Hilden, Germany) following the manufacturer's

instructions. DNA was quantified using a BioDrop spectrophotometer (BioDrop Ltd, Cambridge, UK) and then amplified with an Open qPCR (ChaiBio, Santa Clara, CA, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each 25 µL of reaction mixture contained 12.5 µL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.2 µM of each primer (Accinelli et al 2022), and 40 ng of DNA. Samples were amplified on a standard curve ($r2 = 0.92$; efficiency = 94%; slope = − 0.21) generated by plotting cycle threshold values (Ct) against known spore concentration values.

Aspergillus populations within individual plots were assessed during the season for changes over time by culturing soil samples. Soil samples were collected prior to planting and post-harvest. From the center of each plot, three subsamples were taken from the top 4–6 cm of soil with a sterile trowel at random intervals. The subsamples were combined in a 1-quart Ziploc® bag to achieve approximately 1 kg samples. Soil isolations were performed as described by Abbas et al. (2004). In brief, 10 g soil was added to a screw cap bottle containing 90 mL of potassium phosphate buffer and shaken on a reciprocal shaker for 30 minutes at high speed. Using a wide bore pipette tip, 200 µL of the mixture was transferred onto modified Dichloran Rose Bengal (mDRB) agar and spread across the surface using a sterile cell spreader. Four replicates were made for each sample. Plates were then incubated upside down for five days at 37 °C. Propagule density of *A. flavus* was determined on selective media as described by Horn and Dorner (1998). Ten colonies from each plate were transferred to potato dextrose agar plates amended with β-cyclodextrin and evaluated for percent aflatoxigenicity according to Abbas et al. (2004).

Statistical analysis

Data from field experiments were subjected to the analysis of variance ANOVA. Experimental plots consisted of four 15-m rows, which were arranged in a randomized complete block design with six replicates. Mean values from field and laboratory studies were separated by Fisher's least significant difference (LSD), and P values < 0.05 were considered significant. All the data were processed using the software SPSS ver. 27.0.1.0 (SPSS Ltd., Chicago, IL, USA).

3.5.4 Results and discussion

Effect of biochar on seed wettability, water uptake and germination

Seed surface wettability, as measured by the apparent contact angle between seed surface and sessile water drops, is shown in Figure 3.5.4a As indicated by these measurements, the surface of untreated seeds showed low affinity to water. More precisely, with an apparent contact angle higher than 90°, the external layer of seed corn pericarp can be categorized as hydrophobic. Removal of this waxy layer (e.g., dewaxed seeds) increased its wettability. Also, seeds coated with a commercial polymer mixture were more wettable than untreated seeds (Table 3.5.4a). The main role of the outer surface of the seed pericarp, the cuticle, is to protect seeds from pathogens and desiccation (Jeffree, Citation2006). Maintaining an adequate level of moisture inside the seed is directly connected to embryo longevity and vitality (Cordova-Tellez and Burris, 2002; Zhang et al 2010). Application of the biochar-based formulation to the surface of corn seeds resulted in a significant increase in their wettability. With average wettability values of 24.3° to 25.5°, the external layer of these treated seeds turned hydrophilic, with no significant differences between the two biochar types. Increased surface area and occurrence of hydrophilic functional groups are some of the noted benefits from different agricultural applications of biochar (Hill et al 2019). As a soil amendment, biochar has been used for reducing the phytotoxic effect of residual herbicides and other xenobiotics in soil (Marris 2006; Rogovska et al 2012; Wang et al 2022). More recently, there has been increasing interest in achieving similar effects from the application of biochar by seed treatment (Ajeng et al 2020). To date, most applications of biochar as a seed treatment have used it for pelleting seeds of native species to facilitate planting in vegetation restoration operations. This application is especially important for plant species that have small and irregularly shaped seeds (Ma et al 2022). In these cases, biochar is combined with other organic or mineral materials (i.e., peat and clay minerals) using pelleting or extruder machines, to obtain seeds with uniform, spherical shapes and increased mass. With regular shapes and increased size, the plantability of biochar-pelleted seeds is consequently improved (Law et al 2023). In the present experiment, the effects of biochar-coated seed on water uptake and germination were evaluated using a single seed test tube specifically designed for assessing these properties. Consistent with observations in soil amendment studies, water uptake was found to be more rapid in biochar-coated than in untreated control seeds. As indicated in

Figure 3.5.4b, after 10 h from the beginning of the water uptake study, the water contents of biochar-coated seed and control seeds were 27% and 38%, respectively. At the end of the 40-h experiment, moisture of both seed types was approximately 45%, with no significant differences among treated and untreated seeds. However, this plateau was reached significantly earlier in the treated than in the untreated seeds. Imbibition capability of biochar-coated seeds was compared with that of commercially coated and dewaxed seeds. Seed moisture measurements showed that water uptake was more rapid in the biochar-treated seeds than in others tested (Figure 3.5.4b). Application of the two different biochar powders did not affect seed germination (Table 3.5.4b). As expected, the more rapid seed water uptake also resulted in a shortening of the mean germination time of biochar-treated seeds (Table 3.5.4b). The wettability assay used in this study differed from other available approaches (i.e., rolled towel and sand germination test) in that samples remained undisturbed over the entire experimental period and physically separated from other seeds, thus avoiding potential interactions. In addition, this study showed that seed water uptake was similar in seeds that were coated with two different types of biochar. This is consistent with the wettability of this material, as a results of its chemical nature and high specific surface.

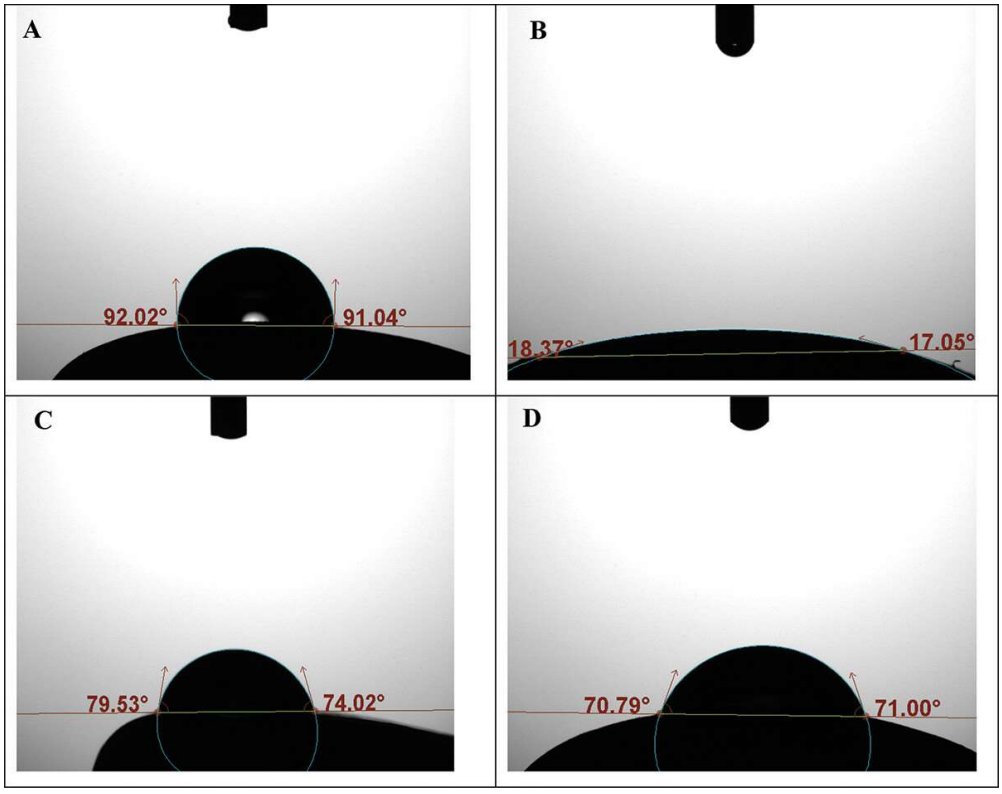

Figure 3.5.4a Examples of measurement of the apparent contact angles of sessile water drops on the surface of untreated corn seeds (A) and seeds that were coated with the hardwood biochar (B). Measurements were also conducted on samples of corn seed that were coated with a commercial polymer (C) and seed which were subjected to the removal of the external waxy layer (D, dewaxed seeds).

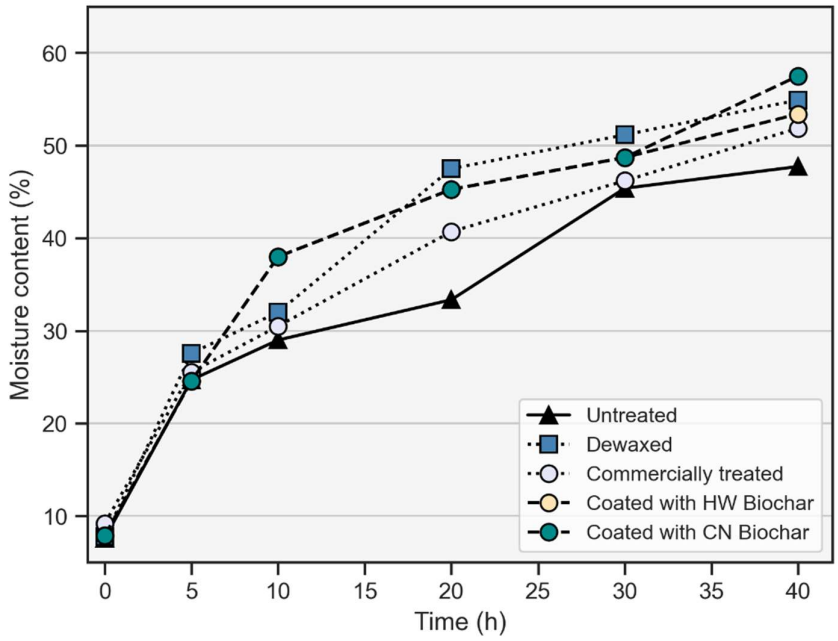

Figure 3.5.4b Water uptake by untreated corn seeds (control) and by seeds that were coated with hardwood (HW) or coconut (CN) biochar. Measurements are also included of samples of seed that were coated with a commercial polymer and seeds that were subjected to the removal of the external waxy layer (dewaxed seeds). Each point represents mean ± STD (n = 3).

Table 3.5.4a Wettability of corn seeds was measured as reduced apparent contact angle of sessile water drops on the surface of seeds that were either untreated (control) or subjected to treatments with two types of biochar (hardwood, HW, and coconut, CN, biochar). Measurements included samples of seeds that were coated with a commercial polymer and seeds that were subjected to the removal of the external waxy layer (dewaxed seeds). Data are presented as mean ± STD. Values followed by the same letter are not significantly different ($P < 0.05$; LSD).

Seeds	Apparent contact angle (°)
Untreated	90.67 ± 3.28 a
Dewaxed	64.45 ± 10.14 b
Commercially coated	74.02 ± 7.26 c
Coated with HW Biochar	25.51 ± 6.77 d
Coated with CN Biochar	24.33 ± 5.01 d

Table 3.5.4b Germination and mean germination time of untreated seeds (control) compared with seeds that were coated with hardwood (HW), coconut (CN) biochar, a commercial polymer or seeds which were subjected to removal of the external waxy layer (dewaxed seeds). Data are presented as mean ± STD. Values followed by the same letter in the same column are not significantly different ($P < 0.05$; LSD)

Seeds	Germination (%)	Mean Germination Time (h)
Untreated	99.2 ± 2.2 a	44.1 ± 0.9 a
Commercially coated	98.9 ± 1.2 a	43.0 ± 1.2 a
Dewaxed	99.0 ± 2.0 a	40.1 ± 0.8 b
Coated with HW biochar	99.2 ± 1.5 a	37.0 ± 0.7 c
Coated with CN biochar	99.5 ± 2.2 a	36.4 ± 0.5 c

Microbiological aspects and aflatoxin contamination of corn kernels

In addition to evaluating seed germination, the test tube-based system described in this study, was also designed to allow for the rapid and effective recovery of microbial DNA from germinating seeds. As shown in Figure 3.5.3, the test tube was provided with two cotton plugs, which were placed on the opposite sides of each germinating seed. Cotton swabs are routinely used for recovering microbial DNA from various sources (Gray et al 2023). In this study they were also used as a germination substrate. Preliminary investigations confirmed that using cotton instead of a polyester plug did not affect the amount of *A. flavus* and other microbial DNA recovered. Cotton plugs were also more effective in providing water to germinating seeds than those manufactured with polyester (data not shown). The cotton plugs were analyzed for DNA content using qPCR and summarized in Table 3.5.4c.

Table 3.5.4c *Aspergillus flavus* propagules recovered during the germination process from untreated (control) seeds, and from seeds coated with hardwood (HW) biochar, coconut (CN) biochar, commercial polymer or subjected to the removal of the external waxy layer (dewaxed seeds). Data are presented as mean ± STD. Values followed by a letter are not significantly ($P < 0.05$) different from other values followed by the same letter (ANOVA).

	A. flavus (number of propagules per seed)		
	2 d	4 d	7 d
Untreated	25.2 ± 2.9 a	32.3 ± 1.7 b	42.1 ± 3.2 c
Commercially coated	26.1 ± 3.1 a	39.4 ± 2.8 b	44.0 ± 2.8 c
Dewaxed	24.9 ± 2.9 a	29.1 ± 2.0 b	39.9 ± 1.8 c
Coated with HW biochar	23.5 ± 3.0 a	29.6 ± 2.6 b	40.5 ± 3.3 c
Coated with CN biochar	21.2 ± 2.9 a	31.1 ± 1.5 b	42.0 ± 3.0 c

In many corn growing areas, aflatoxin contamination of kernels is a serious threat. In addition to the application of specific crop management practices (i.e., avoiding crop water stress, effective insect control, etc.) and control strategies (i.e., field application of non-toxigenic *A. flavus* isolates), planting healthy seeds coated with materials not conducive to the growth of the fungus *A. flavus* is a basic prerequisite for reducing the risk of aflatoxin contamination of corn (Accinelli et al 2019). Considering the increasing interest in using bio-based and biodegradable materials in seed coating, this aspect should be evaluated in the process of developing novel seed treatment formulations. As expected, the number of *A. flavus* propagules recovered from untreated seeds increased during the germination process. This was also observed with biochar-treated seeds and those coated with a commercial polymer or dewaxed. However, the initial level of *A. flavus* was slightly reduced by seed coating with biochar. Because *A. flavus* is a saprophyte and opportunistic plant pathogen, the highly oxidated and slowly degradable organic materials, such as biochar, do not likely promote fungal growth. Studies have shown that starchy and oil-rich substrates (i.e., corn endosperm, peanut cotyledons, etc.) are very conducive to the growth of *A. flavus.* When applied as a soil amendment, biochar is expected to improve soil aeration and water retention, creating more suitable conditions in the soil for the growth of soil microorganisms (Hammerschmiedt et al 2021; Wei et al 2021). However, data from this study indicated that application of the two types of biochar to seeds did not increase the growth of the fungus *A. flavus*, thus confirming the high resistance to microbial degradation of this material.

The size of *A. flavus* populations in field soil planted corn seed coated with biochar followed similar trends during the two years studied in Mississippi (Table 3.5.4d). In both years, the number of soil *A. flavus* propagules increased during the growing season, with a percentage

of aflatoxin-producing isolates ranging from 36% to 44%. Comparable results were observed in soil planted with biochar treated seeds. These data are consistent with those from the in vitro experiment. This was further supported by chemical analysis of aflatoxin levels in harvested corn kernels (Table 3.5.4d). Aflatoxin contamination of harvested corn kernels depends on a variety of factors, including day and night air temperatures, soil moisture, insect damage to ears, etc (Abbas et al 2009; Fouché et al 2020). Although aflatoxin contamination varied among experimental plots, the effect of the biochar seed treatment was not significant. Aflatoxin contamination was higher in 2021 than in 2020. During the kernel filling period in these two years, average high temperatures were 36.70 °C in 2020 and 36.70 °C in 2021 and average low temperatures were 35.60 °C in 2020 and 36.70 °C in 2021 (Figure 3.4.5c). Total rainfall was similar in the two years, with values of 714 mm and 753 mm, in 2020 and 2021, respectively (Figure 3.5.4c). Similar environmental conditions resulted in similar grain yields, with average values of 117 q·ha−1 and no significant differences among treated and untreated seeds (Table 3.5.4d). Several studies have shown that aflatoxin contamination of corn is highly variable between years and difficult to predict (Abbas et al 2017; Accinelli et al 2019). The present experiment reinforced this concept, but more importantly, it demonstrated that coating corn seeds with biochar did not affect aflatoxin contamination of harvested corn kernels

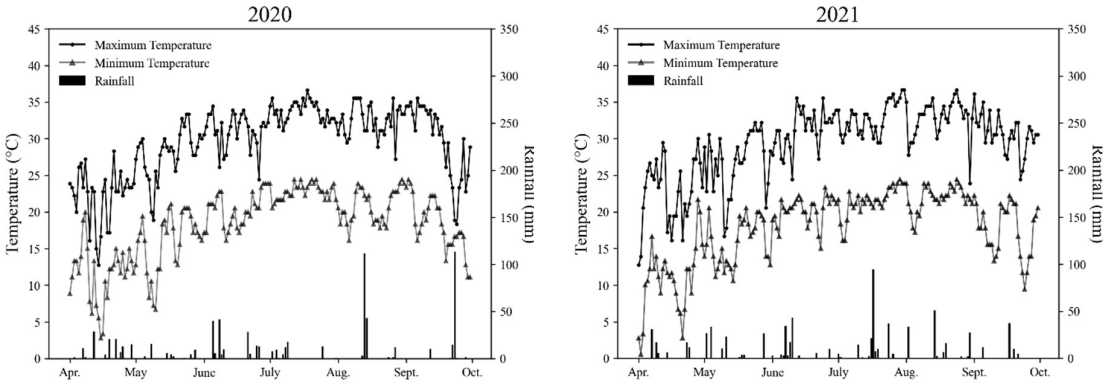

Figure 3.5.4c Rainfall and temperature trends during 2020 and 2021.

Table 3.5.4d Size of the soil *Aspergillus flavus* population and percent aflatoxigenicity of isolates recovered from plots planted with untreated corn seeds and corn seeds coated with one of two types of biochar (hardwood, HW, and coconut, CN). In both years, samples were collected at the beginning (Apr.) and at the end (Sept.) at the end of the corn growing season. Aflatoxin contamination of harvested corn kernels and yield are reported. Data are presented as mean ± STD. For each year, values followed by a letter are not significantly ($P < 0.05$) different from other values followed by the same letter (ANOVA).

	Propagule density (log 10 g^{-1})		Aflatoxigenic isolates (%)		Aflatoxins (ng·g−1)	Yield (q·ha−1)
	Apr.	Sept.	Apr.	Sept.		
			2020			
Untreated	3.9 ± 0.2 a	4.1 ± 0.8 b	36.3 ± 0.1 a	39.2 ± 0.4 b	0.8 ± 0.1 a	12.3 ± 1.4 a
Coated with HW biochar	3.4 ± 0.1 a	4.0 ± 0.4 b	30.2 ± 0.3 a	38.0 ± 0.2 b	0.6 ± 0.1 a	11.3 ± 0.9 a
Coated with CN biochar	3.5 ± 0.2 a	3.9 ± 0.3 b	32.2 ± 0.4 a	35.9 ± 0.5 b	0.4 ± 0.1 a	11.8 ± 0.8 a
			2021			
Untreated	3.7 ± 0.3 a	4.4 ± 0.5 b	35.3 ± 0.2 a	37.1 ± 0.1 a	3.3 ± 0.2 a	11.2 ± 0.8 a
Coated with HW biochar	3.6 ± 0.5 a	4.4 ± 0.8 b	38.8 ± 0.1 a	34.3 ± 0.2 a	3.6 ± 0.2 a	10.2 ± 2.4 a
Coated with CN biochar	3.2 ± 0.7 a	4.1 ± 0.4 b	37.9 ± 0.1 a	38.7 ± 0.3 a	3.0 ± 0.4 a	10.8 ± 0.7 a

3.5.5 Conclusions

The increasing interest in removing synthetic substances from seed treatment formulations has prompted research into novel bio-based and renewable materials to use in their place. Biochar possesses interesting properties that are useful for these purposes, including its high-water retention capability and low cost. However, the use of this material should not interfere with seed quality parameters, such as seed germination and vigor, or promotes the growth of the fungus *A. flavus*, which would increase the risk of aflatoxin contamination of harvested corn kernels, a serious threat to corn production. Laboratory and field experiments conducted for two years in the Mississippi Delta confirmed that biochar-treated seeds did not affect the size of the soil *A. flavus* population and the level of aflatoxin contamination of corn kernels. These results indicate that this renewable material can be used for coating corn seeds without increased concern regarding aflatoxin contamination.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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4. General Conclusion

In conclusion, this research aimed to explore the interactions between seed-borne microorganisms and treated seeds, seeking innovative alternatives to traditional seed quality tests. To this end, a 3D-printed germination apparatus was designed and developed as a cost-effective and user-friendly tool. This tool wants to address the needs of the modern seed industry for testing seed quality.

The first step involved the prototyping and testing of the germination apparatus, leading to a patent application. Subsequently, the tool was employed in multiple research projects that investigated seed germination, growth of seed-borne microorganisms, and interactions between bio-based treatments. This led to the publication of two research papers.

In the first paper presented, this apparatus was integrated with traditional microbiological techniques and advanced Next-Generation Sequencing (NGS) metabarcoding analysis. This approach enabled the identification of the entire fungal community and supported the investigation of azole-resistant isolates.

In the second paper presented, the germination apparatus was used to test seed germination treated with a novel biochar-based treatment. Additionally, it supported molecular analysis for *Aspergillus flavus* kernel infection.

This research work contributes to formulating a hypothesis for a new approach to seed studies. The employment of molecular analyses and the development of new protocols for seed quality testing can give a new insight into understanding microbial interactions in seed treatments. This is particularly significant due to the growing need for environmentally sustainable seed coatings and treatments, development following a bio-based approach. At the same time, understanding microbe-seed interactions is equally important, especially in the context of the rising threat of antibiotic resistance.

As a result of this study, further research could explore other agronomically significant species and their interactions with fungi or bacteria of interest. This work offers an opportunity to rethink the concept of seed quality and to develop new approaches tailored to modern seed technologies, particularly as innovative treatments are developed.

From my perspective, working in seed technology has given me the opportunity to explore this advanced field of agronomy, discovering new perspectives on the application of

microorganisms in agriculture. It has also enabled me to learn and apply advanced research methods and techniques.

Note on the use of generative AI: for the preparation of this thesis, generative artificial intelligence tools were used, specifically ChatGPT based on the GPT-4-turbo model (released on March 2023), for the revision and optimization of the text to improve clarity and coherence, with usage from July to December 2024

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