A Bioinspired Approach to Engineer the Seed Microenvironment

by

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Abstract

Bioinspired by the tardigrade and bombyx mori, we engineer the seed microenvironment to encapsulate, preserve and deliver *Rhizobium tropici*. Scientific discoveries in agriculture and sustainability are at the crossroads of material science, biochemistry, agriculture and biology. They underpin the innovative technological solutions that will impact water, energy and food security (WEFS). These new technologies can then be implemented to address major societal problems that are linked to climate change, soil degradation and increasing population. In particular, our objective is to augment agricultural outputs (*i.e.* crop yield and production) while decreasing inputs (*e.g.* water, energy, fertilizers, land, pesticides) by developing new technology to deploy plant-growth-promoting-bacteria (PGPBs) in the soil to alleviate abiotic plant stressors such as soil salinity and drought. Using PGPBs to reduce and complement the use of synthetic fertilizer, our design approach engineers the seed microenvironment by coating the seeds with PGPBs laden biopolymers. PGPBs are well known to enhance crop production and protect plants from biotic and abiotic stresses, while decreasing the need for water and fertilizers. However, the bacteria's delicate nature has hindered their use in current agricultural practices, due to low survivability. We use a silk and trehalose mixture that is able to encapsulate, protect, preserve and deliver *Rhizobium tropici* to *Phaseolus Vulgaris*, upon sowing. The coated *Phaseolus Vulgaris* seeds are shown to be able to significantly alleviate soil salinity and water stresses in Moroccan soil when compared with uncoated (control) *Phaseolus Vulgaris* seeds.

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Thesis Supervisor: Benedetto Marelli

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My time at MIT has been long, however when I look back it seems just like yesterday when I arrived as a freshman on campus. There were many struggles along the way, joys, friendships built and a lot learnt drinking through the infamous firehose. However, I will spend most of my time reflecting on the last five years I spent in the Marelli Advanced Biopolymers lab. I would like to thank my committee, Dave Des Marais, Katharina Ribbeck and Benedetto Marelli for believing in me and guiding me through my intellectual journey. I would specifically like to thank my advisor for giving me the intellectual freedom to investigate what I believed in and found interesting; Manal Mhada and Professor Kouisini at UM6P for the research support and friendship; the funding agencies ONR, OCP and NSF for supporting our research initiatives.

To my family, my grandmother (passed), my mother and my father who encouraged me to keep going on my lowest days. To my brother and sisters, Francis, Audrey, Primrose and Rosemary who were always a phone call away to cheer me up when I needed it the most. To my lab mates (past and present) Hui Sun, Yunteng Cao and Julie Laurent, who shared this incredible journey with me and have become lifelong friends. And finally, my postdocs Eugene Lim and Doyoon Kim who were instrumental in my development as a scientist and became good friends I would talk to about life and sports. Their mentorship was key to my development. And thank you to everyone else I could not mention friends and interns who worked with me. "Dominus Pastor"

Overview of Thesis Chapters

Delivery of soil microbes is not well studied however it has gained critical importance with the need to grow more food for our growing world population with less resources (water, land and energy), climate change effects (drought, high temperatures and salinity effects) and need for environmental sustainability in agriculture. The dissertation will begin with a review of the challenges and opportunities in the use of microbe based fertilizers. The following chapters 2,3 and 4 will investigate the delivery (storage and administration) of non spore forming rhizobacteria (rhizobium tropici CIAT 899) on a seed surface which encompasses five research tasks 1. Encapsulation 2. Desiccation 3. Preservation 4. Release 5. Colonization. The dissertation will study how we can engineer the seed microenvironment using a protein (silk) and a disaccharide (trehalose) as a seed coating for climate resilient technologies to alleviate stressors such as salinity and drought in plants using Phaseolus Vulgaris as a model.

Chapter 1
Introduction to Engineering Plant Microevironment

The contents of this chapter were published in the American Chemical Society Journal of Agriculture and Food chemistry as: Augustine T. Zvinavashe[1], Ilham Mardad[2], Manal Mhada[2], Lamfeddal Kouisni[2,3], Benedetto Marelli[1,*]

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"Engineering the Plant Microenvironment To Facilitate Plant Growth-Promoting Microbe Association." In press, J. Agric. Food Chem., April 30th 2021

1.1 Abstract

New technologies that enhance soil biodiversity and minimize the use of scarce resources while boosting crop production are highly sought to mitigate the increasing threats that climate change, population growth, and desertification pose on the food infrastructure. In particular, solutions based on plant growth promoting bacteria (PGPBs) bring merits of self-replication, low environmental impact, protection from biotic and abiotic stressors and reduction of inputs such as fertilizers. However, challenges in facilitating PGPBs delivery in the soil still persist and include survival to desiccation, precise delivery, programmable resuscitation, competition with the indigenous rhizosphere and soil structure. These factors play a critical role in microbial root association and development of a beneficial plant microbiome. Engineering the seed microenvironment with protein and polysaccharides is one proposed way to deliver PGPBs precisely and effectively in the seed spermosphere. In this review, we will cover new advancements in the precise and scalable delivery of microbial inoculants, also highlighting the

latest development of multi-functional rhizobacteria solutions that have beneficial impact not only on legumes but also on cereals. To conclude, we will discuss the role that legislators and policymakers play in promoting the adoption of new technologies that can enhance the sustainability of crop production.

1.2 Introduction

Population growth, climate change, desertification and salinization of the earth soils have led to the necessity to build resilient food systems while increasing agricultural output.[1–4] Chemically-derived synthetic fertilizers and pesticides have been used for decades to boost plant growth.[5,6] It is well known that plants primarily require nitrogen, phosphorus and potassium (NPK), for their nutrition. However, these nutrients tend to be the limiting resource in plant growth, thus decreasing the yields.[7] Synthetic fertilizers are responsible for 40 to 60% of the world's food production and are primarily constituted of NPK. Stewart et al [8] reviewed data representing 362 seasons of crop production and reported that a minimum of 30 to 50% of the crop yields can be attributed to synthetic fertilizer use, highlighting the major importance of fertilizer to humanity.[9] Nitrogen based fertilizer production accounts for about 1% of the world's energy consumption while emitting about 1.2% of the global anthropogenic CO_2 emissions that reinforce climate change effects[10,11]. In addition poor fertilizer usage and runoff lead not only to degradation and salinization of soils, but also to eutrophication of our water sources.[11–14] Therefore, upscaling new means to ensure environmentally friendly and sustainable solutions for soil management and agricultural production is required.[15] Furthermore, phosphate is a non-renewable resource[16]. Morocco hosts by far the largest reserve, holding 80% of global rock phosphate[16]. This makes supply a conceivable problem as China, USA and India (the largest food demanders) will runout of phosphate by 2040.[17] Microbes have the potential to increase phosphorus plant intake as most

phosphate is held in inorganic insoluble form [e.g., $Ca_3(PO_4)^2$] and organic insoluble/soluble form (e.g., phytate and nucleic acid) which microbes can make available to plants and therefore limit the synthetic phosphorus fertilizer application.[18] The exploitation of microbes has proven to provide environmentally friendly and sustainable solutions that should be pursued, yet it shows some constraints.[14,19]

Chemical fertilizer attributes such as quick and nonspecific action, low-cost production and ease of storage made them widely acceptable.[20] However, their detrimental effects to soils, plants and animals when they are not used efficiently motivate us to find complementary alternatives to optimize their use and, thereby, lowering their impact on soil fertility and biodiversity.[21–23] Further, pests' resistance and high concentration used/overuse are an unresolved problems that generate an increasing demand for sustainable solutions. Therefore, there is a growing interest in the use of microbial fertilizers as complements to synthetic fertilizers and agrochemicals.[24] Nitrogen and phosphorus are the two most important nutrients to plants and applied nutrients in agriculture. Therefore, to secure food supply and farm sustainability, microbial alternatives are necessary to optimize their use. Nitrogen fixing and phosphate solubilizing microbes can be used in co-inoculations (individually or as consortiums) which result in greater plant growth promotion by providing these essential macronutrients-while lowering our carbon footprint.

Naturally derived nutrients and soil stressor alleviators have existed for centuries for integrated nutrient and disease management and soil biodiversity for rhizobia and now, they are used for other plant growth promoting microbes.[25] Initially, farmers knew that the soil taken from previous legume-sown field to non-legume field often improved the yield. The soil transfer approach was

followed till the end of the nineteenth century for legume seed inoculation.[26] Advances in the understanding of plant-microorganisms interactions are now well-known and have led to the discovery and exploitation of plant growth promoting microorganisms (PGPMs), which include archaea, bacteria and fungi. However, some can be a biohazard.[27] Plant microbes provide the nutrients that plants require and regulate plant growth. PGPMs facilitate this directly through nitrogen fixation, phosphate solubilization and phytohormone production[28] **(Figure 1.1)**, and indirectly by preventing the negative effects of phytopathogenic organisms through the production of antimicrobial compounds or the elicitation of induced systemic resistance.[29] PGPMs pertain to the following classes: the rhizospheric microbes found around the soil in the plants rhizhosphere (root system), phyllosphere (aerial parts of plants), rhizoplane (root surface) and endophytes found inside the plants root, stem and leaf system.[30] Implementing solutions that can be used in agricultural practices is crucial. Our focus in this review will be on bacteria given that archaea are still an under-detected and scarcely studied part of the plant microbiome while fungi (which are eukaryotic) are only able to obtain fixed nitrogen through symbiotic interactions with nitrogen-fixing prokaryotes and we believe cannot fix nitrogen. Nevertheless, a recent study showed potential for nitrogen fixation in the fungus-growing termite gut.[31–33]

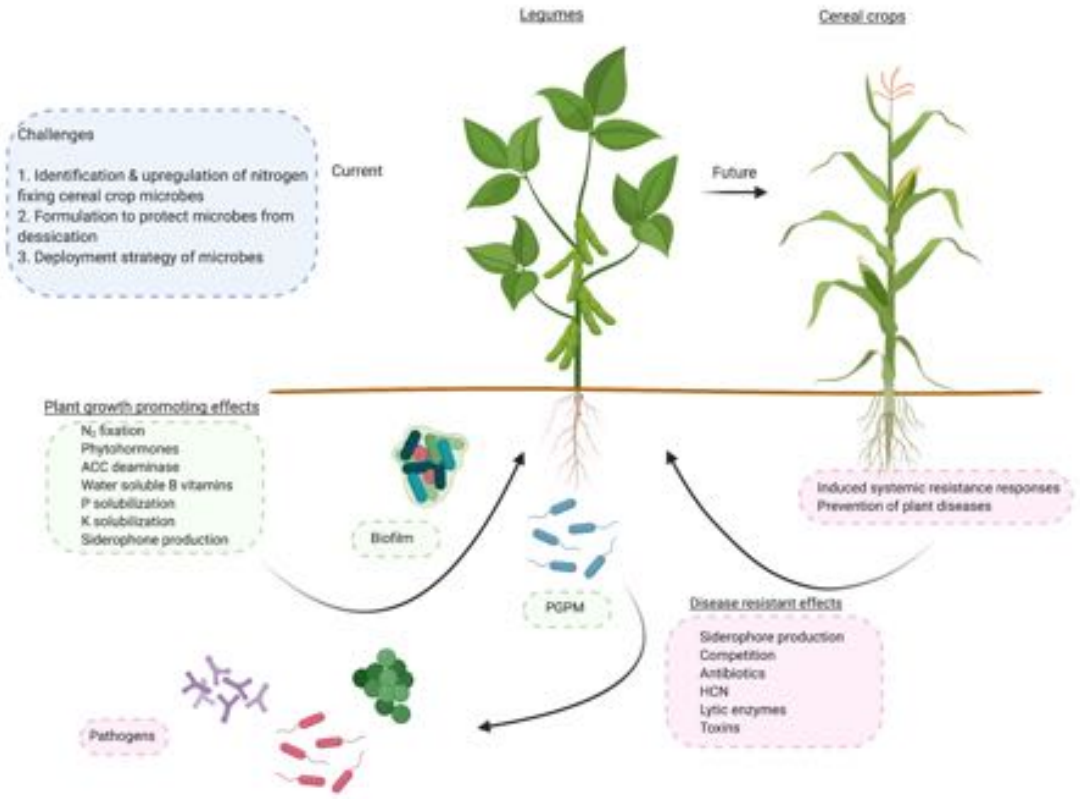

Figure 1.1. Mechanism of plant growth promoting microbes.

Emerging technologies such as proteomics, metabolomics, transcriptomics and next-generation sequencing and data science has made and will make the discovery of useful compounds, microbe interaction understanding and identification and characterization of microbial inoculants fast and easier.[27] Microbes are very specific to the plant and use case. Therefore, the gathering of data on microbial interactions and learning from this data is essential in the use and delivery of plant microbes. Furthermore, the interplay of microbes in a consortium needs to be better understood as some have synergistic effects as singular strains but may have detrimental or beneficial effects when used in a consortium. The inoculation of plants with a microbial consortium provides better benefits to a plant than with a single isolate.[34,35] This could be because microbial consortia may have synergistic interactions to provide nutrients, remove inhibitory products and trigger each

other through biochemical and physical activities that might enhance beneficial effects on plant physiology.[36] Recently, a large-scale genomic comparison of PGPMs discovered that the dominant bacteria associated with plants are Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, which had also been suggested in previous studies.[37,38] Microbiologists are working on better understanding microbial communities and this will be essential in understanding how to deliver microbes in different soils that possess different microbial communities and nutrients. It was suggested that inoculated bacteria are actively influenced by the plant genotype, cropping conditions and by co-inoculated or residing bacterial populations which can considerably influence the resulting PGPB-effects.[39,40]

Microbes can be classified as either gram negative or gram positive. Gram positive bacteria possess a thick (20-80 nm) cell wall as outer shell of the cell. In contrast gram negative bacteria have a relatively thin (<10nm) layer of cell wall, but harbor an additional outer membrane with several pores and appendices.[41] The relatively thin cell wall makes gram negative microbes delicate to dry, handle, resucitate and deliver. Currently, there are several means to deliver microbes in the soil but they are not efficient and lack ease of implementation in remote regions of the world, where agriculture practices cannot account for handling of living bacteria.

Plant growth promoting bacteria (PGPBs) are endophytic or rhizospheric and are known to associate with a variety of crops in plant root structures, leaves and surrounding soils.[42] In an effort to better understand the microbial delivery tools that are currently used to deliver PGPBs effectively, it is first necessary to take into account the best strain of microbe or a microbial consortium for the intended effect on the target crop. Then, the formulation of the inoculant should

be addressed and, finally, the delivery method (**Figure 1.2**).[43] Currently, delivery happens through biopriming, which is a biological process of seed treatment that mixes seed hydration and seed inoculation with plant beneficial microorganisms in order to improve seed's germination and their protection against soil borne pathogens, achieving seedling and vegetative growth.[44] However, given it is labor intensive nature, this process is mostly appropriate for low-medium volumes of high value crops.[45] Soil inoculation is also used as an alternative. However, it requires high volumes of inoculant and is labor intensive thus expensive and may be restricted by local environmental regulation and health concerns.[46] Seed coating has the potential to be a cost-competitive and time-saving approach for crop production and protection. Nonetheless, microbial seed coating is hindered by low performance and standardization, which limit its broader use.[46]

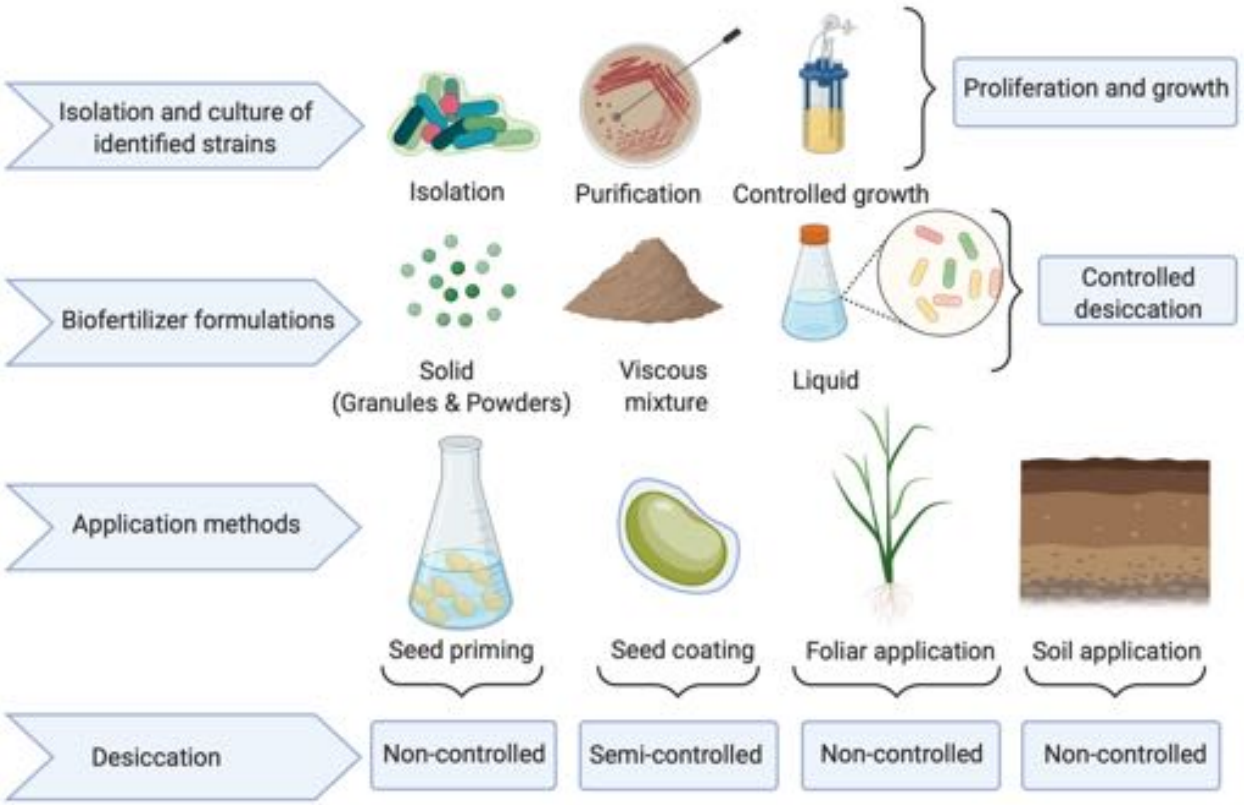

Figure 1.2. From identification to formulation and application of microbial fertilizers. Application procedure and formulation control the desiccation process.

1.3 Challenges

Several challenges such as unpredictability of results, difficulties in the identification and isolation of bacterial strains in field experiments, poor understanding of specific mechanisms that regulate the interplay between microorganisms, plants and soil have limited the use and effectiveness of PGPBs.[47] In this context, two key aspects that dominate the effectiveness of inoculation are the microbial isolation and the application technologies.[43] The design and delivery of microbial consortia through inoculation is challenging and requires the understanding of their modes of interaction, microbial adhesion to seeds, plant root colonization and antagonistic relationship interactions, if present.[48] Differences in root communities have been attributed to plant host effects and microbial host preferences, as well as to factors pertaining to soil conditions, microbial biogeography and the presence of viable microbial propagules.[49] The unprotected, inoculated bacteria must compete with the often better-adapted native microflora and withstand predation by soil microfauna.[43] The environmental conditions also affect the inoculant efficacy and adverse abiotic stresses (hot, dry and saline conditions) can cause rapid decrease in PGPBs populations.[50,51] The following challenges are important in improving PGPBs performance:

Desiccation

Microbial desiccation affects viability of microorganisms. The number of metabolically or physically active microbes is the leading factor towards the efficacy of PGPBs when applied to the seed surface.[52] Desiccation is the process of water removal from (or extreme drying of) an organism, therefore drought stress affects microbial biodiversity in soils. Microbial viability is

important as it increases the effectiveness of microbe infection, permitting PGPBs to induce a positive effect in plants. Therefore, desiccation tolerant microbes are highly desirable because they can remain in soils and inoculant formulations for a longer time than those that are not desiccation tolerant.[34] A recent study reported that 95% of PGPBs does not survive in the time intercurring between inoculation of the seed and planting (considering a 4 hour time window) and that 83% of the surviving microorganisms dyes in soil within 22 hrs.[53] In nature, there are anhydrobiotic organisms that are able to survive desiccation by going into a dormant state in which metabolism is undetected. Once rehydrated, they are able to restore their metabolic processes. Learning anhydrobiosis from such organisms will be a beneficial approach in finding ways to mitigate desiccation stress. Some PGPBs have acquired desiccation tolerant mechanisms such as the production of intrinsic trehalose.[53] The trehalose produced may regulate most of the plant's enzymatic and non-enzymatic responses by supporting the production of the plant's collection of phytohormones.[54] Other organisms, called xero-halophiles, are extremophiles and live in areas where soil is very saline and dry. Desiccation is a topical subject in microbial fertilizers because the efficacy of microbe fertilizer is correlated with viability of the microbes. As the agriculture field looks for opportunities to transition from synthetic fertilizers to microbial ones (also known as biofertilizers), there is an increasing interest in scalable technologies that address desiccation tolerance by providing, for example, a microenvironment that facilitates microbe survival and growth in the form of seed coatings that then degrade in the soil and deliver PGPBs. Alternative technologies to boost PGPBs performance include the selection of desiccation resistant strains, and the use of synthetic biology tools to provide desiccation resistant genes.

Climate Change

Climate change has impacted soil microbial communities resulting in increased atmospheric CO_2 concentration, temperature, precipitation and drought.[55] The effects have been both positive and negative. Numerous studies have showed how elevated CO_2 levels increased the abundance of arbuscular and ectomycorrhizal fungi, whereas the effect on PGPBs and endophytic fungi were more variable. Mostly, PGPBs were beneficial under elevated CO_2,[55] which leads to higher carbon availability in the rhizosphere and may alter root exudation composition. Root exudates play a huge role in the structure and function of microbial communities. This indicates that colonization of plants depends on compounds produced by plants, which are affected by climate change factors such as temperature and drought. In these conditions, different microorganisms show potential for different functional activities that leads to altered community structures and may be used to impart different colonization strategies by inoculating microorganisms such as arbuscular mycorrhizal fungi to change the composition of the microbial community.[56] Further, at elevated CO_2 concentrations, nitrogen becomes a growth-limiting nutrient and as such nitrogen fixing and acquiring microorganisms may gain increasing importance.

Temperature effects are coupled with soil moisture, thus difficult to deduce. Soil microorganisms and the processes they mediate are temperature sensitive. Decomposition of organic soil matter, soil respiration, and growth of microbial biomass increases with temperature. It has been hypothesized that temperature effects are transient; as temperature increases, the soil carbon substrates are quickly depleted by enhanced microbial activity and because of tradeoffs microbial

communities either adjust, shift in composition, or constrain their biomass to respond to altered conditions and substrate availability.[57,58]

Drought leads to soil moisture stress, which impacts the soil microbial community, however it is less investigated than CO_2 or temperature. Drought amplifies the differential temperature sensitivity of fungi and bacteria.[55] Small changes in soil moisture can shift fungal communities from one dominant member to another while bacteria remain constant. Typically, drought reduces fungal colonization, although the outcome can be strain dependent.

Soil pH

Soil pH is one of the most influential factors affecting the soil microbial community.[59] pH greatly affects abiotic factors, such as carbon availability, nutrient availability, and the solubility of metal ions. Furthermore, pH may affect biotic factors, such as biomass composition of fungi and bacteria in both forest and agriculture.[59] The challenge of studying pH effects are its varied effects on multiple factors. Rousk et al showed that as pH drops from 8.3 to pH 4.5, a fivefold decrease in bacterial growth and fivefold increase in fungal growth was measured. Fungi generally exhibit wider pH tolerance when compared to bacteria, which tend to tolerate narrower ranges.[60] The shift in fungal and bacterial importance as pH drops has a direct negative effect on the total carbon mineralization. Below pH 4.5, there is general microbial inhibition, probably due to release of free aluminum and the decrease in plant productivity. Conversely, studies conducted from soils from North and South America have shown that both the relative abundance and diversity of bacteria

increased with soil pH, considering ranges between pH 4 and 8.[60] The relative abundance of fungi was, however, unaffected by pH and fungal diversity was weakly positively related.[60]

Competition in the Soil and Microbe Concentration

Inoculated legume root nodules are mostly formed by indigenous microbes present in the soil.[52] Microbe competition is one of the key determining factors for infection effectiveness. Rhizospheric microorganisms connect plants and soils and together develop an ecosystem that provides nutrient life cycle and soil fertility.[61] Technological advances in DNA sequencing, molecular ecology and data science have provided the tools to study plant-associated and soil microbial diversity and to assess the implication of this diversity on ecosystem functioning.[62] When microorganisms are delivered into the soil, we need to consider the surrounding ecosystem that will be in competition with them. The viability, concentration and delivery method of microbes become vital as a competitive advantage over other microbes as the physiological state of microbes can prevent biomass buildup. Therefore, microbe release mechanism in soil becomes paramount as it affects the concentration and location of delivery that are impacted by rhizospheric microbe competition. A threshold number of cells, which differs among species, is essential to obtain the intended positive plant response. For example, it has been reported that 10^6–10^7 cells · plant^{-1} are necessary for the PGPB Azospirillum brasilense.[63] Oliveira et al, showed that a consortium of microbes improved plant growth more than a singular isolate inoculation.[48] Gottel *et al*. and Shakya *et al*. found that the ecological niche (endosphere vs. root) outperformed other

measured factors (soil properties, season, plant genotype, etc) (upland vs. lowland) in shaping microbial communities.[49,64]

Soil Structure

Soil structure is the arrangement of primary soil particles and the pore spaces between them. Microbe-plant interactions are influenced by the soil type, soils that share a certain set of well-defined properties.[49] Biological linkages between soils, roots and the atmosphere are poorly characterized. However, Bonito et al showed that bacterial communities in the root are more tightly structured by plant host species than by soil origin.[49] Plants, soils and microbiota interact and function in a zone known as the root microbiome,[65] which is characterized by elevated rates of respiration, nutrient turnover, and carbon sequestration, highlighting its importance to the functioning of terrestrial ecosystems.[66] The nutrient concentration, pH and water content play an active role on microbe colonization. Microbes are very specific therefore have differing niche microenvironments that accommodate them best. The distribution of bacterial and fungal communities and their function varies between different aggregate size classes.[67] Further, compaction of soil has detrimental effects as it affects physical properties of soil such as bulk density, soil strength and porosity. Compaction limits the mobility of nutrients, water and air infiltration and root penetration in soil.[68] Juyal et al. have shown how increasing soil bulk density (compaction) significantly reduced the number of microorganisms in soil and their growth rate. Good soil structure provides an array of niches, such as substrate availability and redox potential, which can house diverse microbial communities.[69] Microbes reside in pores and inner surfaces of

aggregates as microcolonies of 2–16 microbes each, and extensive colonization is restricted to microsites with higher carbon availability, e.g., rhizosphere and outer surfaces of freshly formed macroaggregates.[70] Location of aggregates in relation to roots, organic residues, and macropores is more important for determining the microbial community composition and their activity.[69] Understanding the microbes niche environment will help build predictive models and skill us in shaping the rhizosphere of the plant as microbes are very specific with regards to conditions required for colonization.

Perspective

PGPBs are plant and soil specific, which makes them challenging to deploy universally. However, as our understanding of soil structure, soil pH, impact of climate change, soil microbe concentration and desiccation impact plant and soil microbe interaction increases, the efficacy of microbe-based fertilizer can be enhanced by precise microbe selection, developing models based on plant, and investigating microbe and soil interactions. All the extrinsic factors influencing PGPBs growth and metabolism are coupled together and understanding how they all interact will be key to design highly effective techniques to develop and deploy, at scale, biofertilizers.

1.4 Formulations

Rhizobia bioformulations have been on the market for centuries in numerous forms. Commercial biofertilizers can be solid carrier based (organic or inorganic), liquid formulations, synthetic polymer based or metabolite based formulations.[51] The formulation is composed of the microbe,

carrier material, and additives. The first commercial nitrogen biofertilizer of rhizobia, 'Nitragin' was patented by Nobbe and Hiltner.[51] Initially, inoculation procedure entailed transferring soil from legume grown soils to soils that will host plants. Following this first technology, solid based carriers came into use in the early 1900's. Even today, many of the microbial inoculants all over the world are based on solid based carriers, mostly peat formulations. This has been true for well-developed legume inoculants based on selected rhizobial strains, due to peat bacterial protection properties,[71] such as high water holding capacity, chemical and physical evenness, non-toxic and environmentally friendly nature.[72] However, peat is very inconsistent and is a non-renewable resource making it unusable on a large scale.[73] Thus, interest in substitutes grew and alternatives such as lignite, filter mud, coal-bentonite, cellulose, coal, soil, charcoal, manure, compost, powdered coconut shells, ground teak leaves and wheat straw have been used as solid carrier materials.[51] Granular carriers were also developed for direct application to the soil, which made handling, storage and application easier.

Liquid formulations were developed as alternatives to solid carriers due to their limitations such as environmental impact and carbon emissions of peat-made solid carries.[72] Further, liquid formulations are better suited for mechanical sowing in large fields.[43] In 1958, freeze-dried inocula came on to the market, then gel based microbial inoculants that entrapped rhizobia in polymer gels such as polyacrylamide-entrapped *Rhizobium* (PER), alginate-entrapped *Rhizobium* (AER), and xanthan-entrapped *Rhizobium* (XER); which gave satisfactory results in wet conditions.[51,74] In the early 2000's, the modification of liquid formulations by addition of additives and cell protectants were proposed. The additives promote cell survival in storage and after application to seed or soil.[75] Commonly used additives for rhizobial inoculants were polyvinyl pyrrolidone (PVP),

carboxymethyl cellulose (CMC), gum arabic, sodium alginate and glycerol.[51] PVP protects microbes from desiccation and harmful seed exudates and CMC's rheological property increases the gel viscosity of carriers to make it more suitable for viability of rhizobial cells.[51] Further, genetic modification of rhizobia is being developed to improve the efficacy of nitrogen fixation in new formulations, such as upregulating nitrogen fixation.[76] The emerging technique of secondary metabolites addition (flavonoids and phytohormones) to bioformulations increases agricultural productivity by improving the inoculants efficiency.[77] The addition of flavanoids to rhizobial formulations during growth, significantly alleviates the effects of adverse conditions,[78] enhances nitrogen fixation [79], improves the rhizobial competitiveness and nodulation.[51] The cost associated with flavonoids isolation or synthesis is sometimes justified by the low concentrations used in the final formulation.[80,81]

Despite, the abovementioned technologies, bioformulations still face many limitations. Inoculation formulations have improved microbial survival during storage of products, but these efforts have not improved survival on the seed or in soil.[52] Bacterial survival on the seed are mainly affected by three factors: desiccation, the toxic nature of seed coat exudates and high temperatures.[82] Therefore, there is a need to find biomaterials that could provide a microenvironment to protect microbes from desiccation while also having the mechanical properties to conform around a seed (**Figure 1.3**).[83] Biomaterials are biocompatible, biodegradable and abundant, thus have potential in enhancing food security and safety. [84–87]

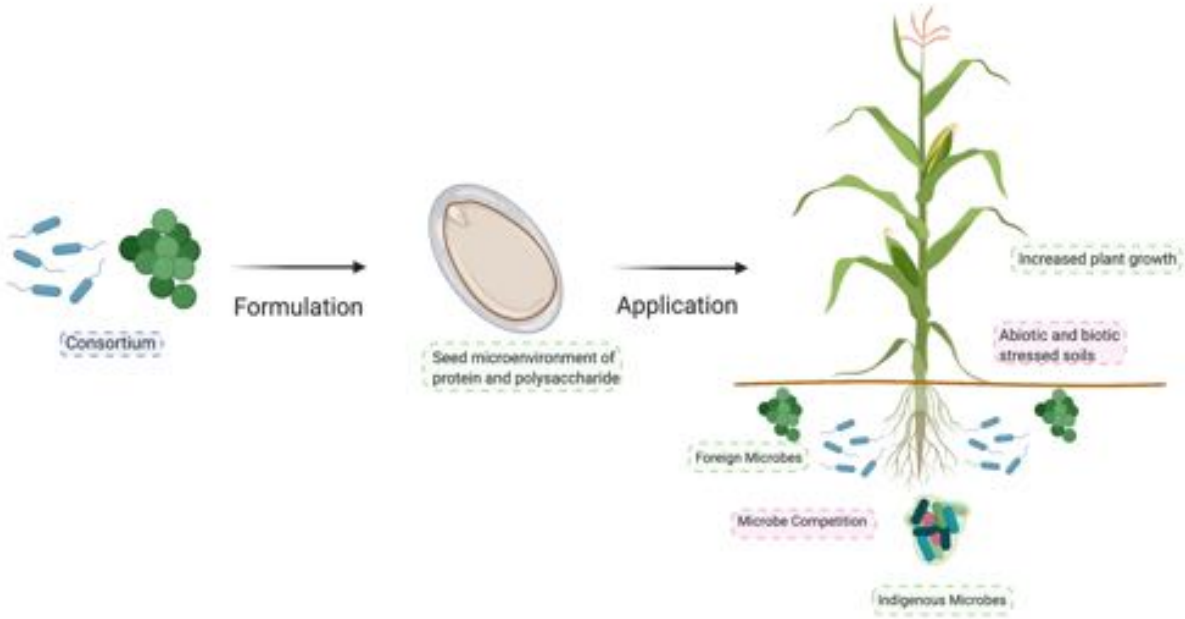

Figure 1.3. Seed coating technology encapsulates and protects microbes while providing a targeted in situ release of payload to be delivered.

Efficacy of formulations depends on their shelf life, which depends on several factors such as production technology, carrier and packing material used, transport activity and farmers' practices to sustain the quality of inoculants.[88] Factors related to production processes (quality and marketing standards) are also important for consistency and user uptake. Currently, the storage, preparation and application of formulations needs special facilities and skills, which most farmers and suppliers do not possess.[89] Therefore, an easy to use alternative is necessary for better adoption. The current problems with most formulations are a lack of robust scientific data. According to Brockwell et al [90], 90% of inoculants have no impact on target crop. Further, Herrmann et al.[91] reported that more than 50% of the inoculants have high levels of contamination. Contaminants have detrimental effects on the quality of rhizobial inoculants and 25% contaminants of the commercial inoculants can be opportunistic human pathogens. Therefore, many inoculants produced globally, because of lack of quality control, tend not to perform well. Thus, there is a requirement for strict regulations for rhizobial bioformulations to overcome the

abovementioned problems related to worldwide production and application of biofertilizers. In the future, emphases should be given to techniques that increase population density and survival of rhizobial strains in inoculants and minimize operator exposure to high dose of PGBPs whether in solution or in water droplets. Additionally, survival of cells is mandatory for better commercialization of rhizobial inoculants in the global market.[92]

Nano-bioformulations of biofertilizers has emerged as one of the most promising techniques to achieve this goal. It comprises nanoparticles made up of organic or inorganic materials, that interact with microorganisms and enhance their survival by providing protection from desiccation, heat, and UV inactivation. Applications of nano-bioformulations also include environmental cleanup strategies.[93] In 2015, PGPBs such as (*Pseudomonas fluorescens*, *B. subtilis* and *Paenibacillus elgii*) treated with silver, aluminium, and gold nanoparticles have been shown to support plant growth and increase pathogen resistance.[94] The release of such nanoencapsulated biofertilizers into target cells is operated in a very controlled manner, free from any harmful effects and increasing the adhesion of beneficial bacteria within the root rhizosphere.[95] Additionally, nanobiofertilizers may be considered as an alternative to chemical pesticides,[96] although the deployment of nanoparticles in the environment needs to satisfy stringent requirements imposed by policymakers.

The application of phyto-nanotechnology on agriculture could change the traditional plant production systems, providing the controlled release of agrochemicals (e.g., pesticides, herbicides, fertilizers) and target-specific transport of biomolecules (e.g., activators, nucleotides, proteins). Nanoencapsulation using biodegradable materials also makes the assembled active elements

straightforward and safe to be handled by the farmers. Advanced understanding of the interactions between nanoparticles and plant responses (uptake, localization, and activity) could transform crop production through improved disease resistance, nutrient use, and crop yield.[97]

The use of polymeric inoculants and alginate beads have already been tested and need more exploration for their future use.[43,51] Furthermore, the use of stress tolerating microbes/rhizobia in inoculations is also thought to be imperative in developing bioformulations that will survive in stress conditions (high temperature, drought, salinity).[98,99]

The use of genetically improved rhizobia as inoculants has some legislative constraints because it requires permission from environmental protection agencies to release into the environment and due to the little understanding of microbial ecology.[100] Further, the majority of microbial seed inoculation involves private companies (agrichemical and seed companies) that rarely disclose their data and formulations[45], although there is a compelling need to develop a more comprehensive knowledge that integrates academic efforts to speed up advancements and the development of disruptive technologies.

Perspective

Peat-based formulations have been traditionally used for the delivery of microbe-based fertilizers. These tend to be good at providing the niche for microbe growth when outside the soil and when inoculated. However, since peat is a non-renewable resource, new formulations are required. Liquid-based formulations have been developed, however performance in microbe preservation

can be improved to ensure high efficacy of the inoculant. As we learn new lessons on how microorganisms survive desiccation, e.g. by looking at tardigrades production of trehalose and intrinsically disorder proteins to promote water substitution and vitrification, new strategies can be designed to engineer formulations that better protect and store microbes outside the cold chain and in operational conditions before deployment in the field.

1.5 Rhizosphere and Endosphere

Rhizobacteria

The rhizosphere is the region of soil directly surrounding the root system that is directly influenced by root secretions and associated soil microorganisms known as the root microbiome.[101,102] Rhizobacteria implies a group of bacteria found in the rhizosphere that can colonize the root system.[103] It has been demonstrated that bacterial cells first colonize the rhizosphere following soil inoculation.[104] Therefore, microorganisms delivered in the soil need to be able to colonize the rhizosphere before they can have an impact on plant health and metabolism. Bacterial cells have been visualized as single cells attached to the root surfaces, and subsequently as doublets on the rhizodermis, forming a string of bacteria.[105] Colonization then occurs on the whole surface of the rhizodermal cells.[106] For microbes to produce plant growth promoting factors, they need to be able to colonize the rhizosphere and/or the rhizoplane during an extended period characterized by strong microbial competition with rhizosphere competent microbes (microorganisms that have the capacity to effectively build a population of microorganisms on plant roots or in the vicinity).[107] Furthermore, root colonization is complex and non-uniform. This can be explained by different factors such as varying root exudation patterns released by plants and containing chemoattractant to promote microbe colonization and growth.[108] Rhizosphere colonization is however a complex system influenced both by microorganisms competition during inoculation and rhizosphere

competence of the microbe. We are yet to fully understand these interactions, which are soil specific as a microbe needs a specific niche to perform optimally.

Endophytes

There are types of microorganisms that do not only colonize the rhizosphere but also enter and colonize plant tissue for beneficial effects, i.e. endophytes.[105] Studies have shown how plants host a diverse group of endophytic microbes and most endophytes are derived from the rhizosphere, e.g. rhizobium.[109,110] Endophytes are a subgroup of rhizobacteria known for entering the endorhiza (the root interior) once the rhizosphere has been colonized. Moreover, they are known to show a plant growth promoting behavior more intense when compared to exclusively rhizospheric colonizing microbes.[111] The penetration process does not involve an active mechanism, but rather a passive one. Passive penetration can take place at cracks, such as those occurring at root emergence sites or created by deleterious microorganisms, as well as by root tips.[112] However, some microorganisms have developed active mechanisms, such as root nodulating rhizobia. The nodulation mechanism is mediated by root release of chemoattractants (e.g. flavonoid exudes) and microbial signals (nod factors) and as such it is specific and specialized. Root invasion can happen through fissures that occur at lateral root base and by cortical intracellular entry.[113,114] Besides, plant-rhizobia endophytic interactions are not well understood. Further, emerging but limited knowledge exists on endophytes colonizing flowers, fruit and seeds.[115] In addition, evidence of endophytic microbes found in plant stems and leaves and not in the rhizosphere highlights other potential colonization mechanisms. Bacterial endophytes are carried inside the seed (vertical

transmission) and can be equally important for the evolution of the microbial community of the seedling.[116,117]

Perspective

Microbe identification remains a very important matter as we search for the best performing microbes with regards to nitrogen fixation and phosphate solubilization. These remain a matter of interest as we search for nitrogen fixing microbes for cereal crops. Cereal crops makeup a considerable percentage of the foods farmed globally. The diversity of our soils has decreased with modern agricultural practices, however PGPBs play a pivotal role in enhancing the sustainability of the agriculture system and may enable the production of better-quality food, thus promoting health and wellness.

1.6 Application Methods

Soil microbe delivery systems, to be effective for field-scale use, have to be designed to provide a dependable source of bacteria that survives in the soil and becomes available to crops, when needed.[43] Rhizobia application can be performed on the seed surface or directly into the soil or through plant inoculation.[43,46] Seed inoculation outnumbers soil application and depends on the requirement of the type of inoculant, the seed type and inoculant volume. The efficacy of each inoculation technique needs to be taken into account. Effects such as high temperature of a seed coater and an air seeder, high pressure, rapid drying when the inoculant is sprayed into sowing

machinery and when inoculated seeds are sown under hot, dry conditions, or when seeds are treated with fungicides and herbicides potentially have large deleterious effects.[43]

Seed Inoculant: Seed Coating and Bio-priming

There is typically limited success from coating seeds with rhizobia because it is difficult to maintain living and active bacterial cells.[118] Factors such as temperature, humidity, and toxic substances all affect the survival of rhizobia in the seed-coating agent.[82] However, this is the most common and practical seed inoculation procedure. This happens because it is the easiest method to use and it requires considerably small volumes for inoculation.[82] Additionally, the standard seed coating technology has not changed in years.

Seed coating is a technique that entails the covering of a seed with a material laden with microbes to enhance seed performance and plant establishment while reducing cost, to meet the requirements in development for precision agriculture. (**Figure 1.4**). Historically, coating seeds has been broadly used as a cost-effective way to alleviate abiotic and biotic stresses, thus boosting crop growth, yield, and health.[119] The process is very streamlined; seeds are dusted with peat inoculant, with or without water or adhesive. With small seeds, fillers such as limestone are added, with or without adhesive, and allowed to dry.[43] The coated seeds are dried in situ or just before sowing. In situ coating standardizes the delivery and makes the technology easy to use for farmers but tends to lead to lower microbial count than coating before sowing. Seed may be a basic input deciding the fate of productivity of any crop. Commonly, seeds are studied for their germination and distributed to growers. Despite the very fact that the germination percentage registered within

the seed testing laboratory is about 80-90%, these efficiency can hardly be replicated in the field because of the inadequacy or non-availability of sufficient moisture under rain fed systems.[120]

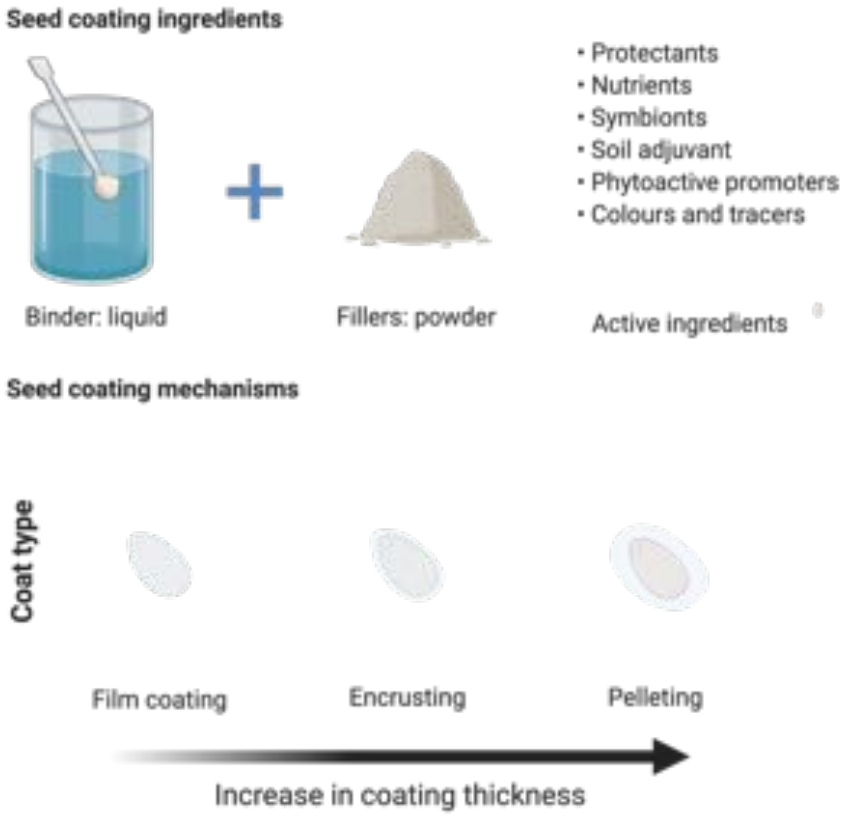

Figure 1.4. Seed coating ingredients, process and types.

One essential condition to seed coating is adding adhesive materials. There is no standardized material used as an adhesive.[121] Adhesives are used to ensure that a threshold of microbes are added and to secure microbes on the seed. Adhesives include gum arabic, carboxy-methyl cellulose, sucrose solutions, vegetable oils, as well as any non-toxic, commercial adhesive that can bind to bacteria and seeds.[43] With regards to seed coating applications, coating is either performed by hand, rotating drums that are cheap to operate, large dough or cement mixers, or mechanical tumbling machines.[122] Liquid inoculants are directly sprayed onto the seed before being sown once

dry. The microbes can be macro or microencapsulated during the process. Microencapsulation leads to smaller particles thus larger surface area, which enhances controlled release.[123] However, seed coating has several disadvantages. Each seed can only contain a restricted amount of inoculant, which may be a limiting factor because a threshold of bacteria may be needed for successful inoculation with most PGPBs.[43] Seed coating process may damage seeds' natural coating and alter the water or oxygen absorption properties of the seed, affecting its germination capabilities.[43] Furthermore, release and degradation properties of microbes from seed coating are important parameters to control to induce microbe colonization and combat desiccation in the soil. Some fungicides and insecticides applied to the seeds before coating may be detrimental to the inoculant, therefore seed treatments need to be carefully streamlined to avoid detrimental effects on the final product.

Bio-priming is a process of biological seed treatment that involves the soaking of seeds in any solution containing required biological compound followed by redrying the seeds, which results into start of germination process except the radicle emergence.[124] It allows the bacterial imbibition into the seed, creating ideal conditions for the bacterial inoculation and colonization in the seed and reduces the chance of desiccation and the amount of pesticide applied to the field.[124] Soaking of seeds initiates the physiological germination processes, where plumule and radicle emergence is prevented, until the seeds are provided with the right temperature and oxygen after being sown. Microbes in the seed keep on multiplying and proliferate in the spermosphere even before sowing.[124] Bio-priming leads to improved germination and seedling establishment, however it has to be done on site and can be labor intensive.[46] Given the effort required for this process, it is most appropriate for low-medium volume high value crops, such as vegetable seed.[45]

Soil Inoculant

Soil inoculation is used to release high volumes of inoculant into the soil but is time intensive, expensive and may be limited by threshold number regulations.[46,125] Soil inoculation can be achieved by adding granules in the seedbed or adding a liquid inoculant into the seedbed.[43] This process ensures that no inoculant is lost during seed planting through sowing machines. Besides, small seeds that have limited surface area can be sufficiently inoculated with enough microbes using this technique.[43] In highly mechanized farming, granular inoculants work well because the machinery for seeding commonly includes accessories for application of fertilizer and pesticide and inoculation is just one additional input during seeding.[43]

Granular forms of soil inoculant include peat, marble combined with peat, perlite, charcoal or soil aggregates. Granular inoculation enhances the chance for the inoculant to be in contact with plant roots which helps with microbe colonization and therefore effectiveness.[43] The method of soil inoculation used depends on the farmer preference. Nonetheless, it always tends to be more expensive than seed coating. The method of application is determined by the seed size, equipment availability, seed fragility, presence of insecticide and fungicide on seed surface and the cost the farmer is willing to pay.[43]

Plant inoculation

The plant microenvironment is naturally colonized by microorganisms. More than 90% are bacteria.[126] Some of them are PGPBs with the ability to enhance plant growth via providing required nutrition or increasing the availability of nutrients in an assimilable form. Plant inoculation involves the inoculation of plants through root dipping or foliar spray.[46] These techniques require large amounts of inoculant, and with regards to root dipping, plant nursery preparation is also required.[46] This highlights that the root dipping process is very time and labor intensive, which makes it unfeasible in large scale agriculture.[45] PGPBs application performed on roots or on cuttings to promote in vitro rhizogenesis is mainly performed in recalcitrant species.[127,128] They can be applied as a dipping solution or can be added to the rooting media just before transferring the shoots.[129,130]

Exogenous application using foliar spraying is conducted using the inoculum alone or in a specific formulations to ensure bacterial cells fixation on the leaves, and also to maintain live bacterial count until colonization through the stomatal apertures.[131] This method of application relies on climatic conditions; increased atmospheric temperature alters plant microbe interaction by reducing the bacterial charge and inducing intrinsic reactions in the plant by water deficits.[132] To overcome this issue, inoculant's screening based on their thermotolerance has shown great efficacy. Current findings in greenhouse studies suggest that co-application with *Bacillus cereus* and humic acid can be used in the mitigation of heat stress damage in tomato seedlings and can be commercialized as a biofertilizer.[133] But, the inoculation is also affected by humidity and rain revealing the unfeasibility of this method in large scale agriculture with certain microbe and plant types.[45] However, Fukami et al,[134] showed that foliar spray in maize and wheat improved

colonization of leaves, while soil inoculations favored root and rhizosphere colonization (**Table 1.1**).

Table 1.1 Comparison table between Biofertilizers application methods

Advantages

Application method	Comparison	References
Seed inoculation		
Seed coating	**Advantages**	
	Seed inoculation is less expensive than in-furrow inoculation, especially for small seeds	[135]
	Can be stored easily	[136]
	Low costs of storage. Easy handling and transportation	[45]
	Used for recalcitrant species multiplied by seeds like Orchids	[137,138]
	Controlled release of microorganisms	[119]
	Increase of the microbial shelf life	[119]
	Limitations	
	Adapted to microbes compatible with dry formulations	[45]
	Non-sporulating bacteria experience large viable cell losses during dry formulation	[75]
	Affected by storage conditions	[139]
	Affected by the abrasion and seed contact	[140]
	Antagonism between the soil microbiome and the inoculated bacteria	[141]
Biopriming	**Advantages**	
	Useful to combat the disease problem	[142,143]
	Improve immediate availability of micronutrients	[144]
	Used for recalcitrant species	[145,146]
	Limitations	
	Immediate application	[147]
	Depend on the interaction time	[147]

Soil inoculation	**Advantages**	
	Increase of the effectiveness by immobilization of inoculant cells and their embodiment in polymers	[148]
	Limitations	
	Antagonism between the soil microbiome and the inoculated bacteria	[141]
Plant inoculation		
Root	**Advantages**	
	Adapted to *in vitro* plants and recalcitrant species	[127,128]
	Facilitate bacterial root adhesion through formation of biofilm on root surface	[149]
	Limitations	
	Requires large amounts of inoculant and the concentration of the bacterial suspension	[150]
	Depend on the exposure time of the root to the bacteria	[150]
Foliar	**Advantages**	
	Passive colonization through to the stomata apertures, plant wounds or insect feeding	[134,151]
	Can be combined to nanoparticles to increase the efficiency and the effectiveness of the inoculation	[152]
	Limitations	
	Unfeasibility in large scale agriculture	[45]
	Spraying equipment can influence the uniformity of foliar spray	[153]
	Depend on droplet size in terms of microbe concentration and leaf coverage	[154]
Seedling pretreatment	**Advantages**	
	Can be used in greenhouse vegetables	[155]
	Limitations	
	Requires a plasma treatment for immediate and effective bacteria activation	[156]

Perspective

Seed coatings provide a targeted, controlled, and low volume way to deliver beneficial microbes to the plant microbiome. An ideal strategy for future technologies consist in the development of seed coating techniques that can be streamlined in seed treatment processed and applied during the seed packaging to ensure standardization of seeds for planting. However, inoculation through seed coating formulations need to reach performances that are comparable to coating on site or soil inoculation, to have an impact in precision agriculture, despite providing an easier technology.

1.7 Legislation and Business Opportunity

Regulation and legislation from production to on field application of microbial fertilizers will play an important role in their use and eventual success.[157,158] Environmental policies regulate the type and quantities of microbes allowed in their environment, but also impose restrictions the type of carrier used and degradation profile permitted for each carrier. In particular, an increasing amount of attention is growing in the use of microplastics in agricultural practices, despite the low quantities involved. One of the toughest challenges for policymakers is the lack of a universally accepted definition for microbial fertilizer. The different types of microbes utilized to improve plant growth (fungi or bacteria) and the different mechanisms they used to obtain this final effect have created some inconsistencies in the definition of biofertilizers. There is then a need to develop adequate standards and legal provisions to support the production and use of biofertilizers at the global level. Globalization of microbial markets and the need for environmentally friendly and sustainable agricultural activities strengthens this need.

Recently, the European Union (EU) came up with a definition for microbial fertilizers. The new regulations will come into effect in 2022. Prior to these new regulations, the European market was segmented and now it will move into a more consolidated one. Further, this type of regulations will reduce costs and administrative burden when launching a product. Europe is the second largest biofertilizer market with 30% of the industry in 2019 and is expected to grow at 10%/year for the next several years.[159] Further, the EU defined biostimulants by what they do, not by what they are. The European Biostimulant Industry Council defines plant biostimulants as substances and/or microorganisms whose function when applied to plants or to soil is to stimulate natural processes to enhance or benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality.[160] It is projected that this new EU regulation will improve transparency, quality and safety. Additionally, the EU set out a new procedure for authorizing biostimulants in agriculture, which will ensure conformity and accreditation in all member states. New regulations are stricter and manufacturers can only declare those benefits derived from their products that have been scientifically proven. These new requirements will provide greater transparency and confidence when defining the limits of the efficacy. However, on the innovation side, only four microorganisms are regulated, meaning any product developed from other microorganisms cannot be marketed in the EU. This highlights the growing need of aligning innovation and regulation.

In the USA, there is no federal law regulating biofertilizers. However, the individual states regulate this type of product through the United States Department of Agriculture.[158] Regulations may differ drastically, where in some states only notification is required and in some other, local efficacy trials are required. The fragmented market makes it costly and bureaucratic to operate in

the US market.[161] Further, in the USA there are currently no legal definitions for the term 'biofertilizer', or specific legal provisions defining their characteristics.[162]

The global biofertilizers market size was USD 1.34 billion in 2018 and is projected to reach USD 3.15 billion by the end of 2026, showing a compound annual growth rate of 11.3% forecast 2019-2026.[163] With regards to application, the global fertilizer industry is segmented into seed treatment, soil treatment and other. Seed treatment has the largest market share [164] (65% in 2014) and is expected to grow by 12.1%/ year between 2019-2026. Therefore, making the seed treatment application a lucrative sector to enter. Further, nitrogen fixing biofertilizers are the leading segment in the market (82%) and is expected to remain the most important biofertilizer segment. North America and Europe account for 55% of the global market revenue. The trade in North America is expanding considerably, due to the growing number of organic farms in prominent economies, such as the U.S., Canada, and Mexico. Novozymes AS, Rizobacter Argentina S.A., Lallemand Inc., and BioWorks Inc. are the key active players in the biofertilizers business. North America is expected to hold the highest market share in the biofertilizers market. The market is highly fragmented, with many small and large players present across different geographical regions. The global biofertilizers commerce being unregulated is the reason why there are many small companies in the market. Once proper regulations are put in place, it is likely that the market will be consolidated among a few companies.

Further, with the recent European Union ban on intentionally added microplastics (IAMPs), agriculture based companies will require to be cognizant on the type of materials manufactured for plant and soil application and thus, microbial fertilizer application tools.[165] Recently, IAMPs have

become an issue of importance because of their ubiquitous presence. However, most research has been focused on the marine environment and not much on soil until of late.[166] Soils may represent a large reservoir of IAMPs, with sources such as sewage sludge applied as fertilizer and fallout from the air. Therefore, IAMPs may pose a threat to soil biodiversity. However, there is still a lack of information.[167] Recent studies, show harmful effects of IAMPs on various groups of soil fauna such as earthworms, snails, collembolans and nematodes.[168] Nevertheless, the impacts of IAMPs on soil microbial communities have led to inconsistent results.[168]

Perspective

Farming is a low margin business thus any new strategy suggested requires to be effective and cheap. Numerous effective techniques have been developed in laboratories across the world. However, collaboration between research and business is required to ensure scalability of these exciting ideas. Thus, startups working to scale up and lower costs of farming techniques will be required to bring some of the new technologies and techniques to the farmer. Also, working with government will be critical to develop supportive legislation for these initiatives.

1.8 Future Perspective

Climate change and rapid population growth combined with the scarcity of resources impose a rapid transformation of agriculture to a more resilient and sustainable infrastructure. Crop production is currently too carbon intensive and lower the carbon footprint of synthetic fertilizers is one of the major goals to enable a more sustainable future for our society. Microbial fertilizers

have shown great potential in solving the environmental challenges we face.[169] Future formulations for microbial inoculants will focus on precise and scalable delivery tools for microbes, while also focusing on developing multi-functional microbe solutions that work for a variety of crops. However, we face a two-pronged challenge for the effective use of biofertilizers that will spur large and small-scale uptake: 1. Effective delivery methods 2a. Microbes for cereal crops 2b. Multi-functional microbe solutions. Furthermore, cost of microbial inoculants will be key to complementing with synthetic fertilizers.

Engineering the seed microenvironment with microbes in silk and trehalose seed coating has recently shown to effectively deliver plant microbial fertilizers.[83] A protein and polysaccharide mixture that encapsulated microbes was shown to be able to protect rhizobium from desiccation for over a month and finally deliver in the soil the microbes for colonization.[83] The bioinspired approach that guided the material formulation imparted the appropriate mechanical properties and preservation capabilities required for an effective microbial delivery tool. This may enable the application of the proposed seed coating technology both for small scale farmers and large-scale farmers, independently from their resources, skills and equipment. Secondly, the ability to preserve microbes at standard conditions suggests that storage costs can be lowered as most microbial fertilizers to be preserved require to be refrigerated. The framework of the technique of engineering the seed microenvironment can be used at large scale to solve the most important challenges faced in making microbial fertilizers ubiquitous in agriculture.

Cereal crop production accounts for a large proportion of agricultural production in the world providing 60% of plant calories for humans.[170,171] Therefore, corn, wheat and rice are some of the most important crops that will be essential in driving uptake of microbial fertilizers. Nitrogen based

fertilizers account for more than two thirds of global revenue.[172] Recently, Pivot Bio commercialized and released nitrogen fixing microbes for corn that can supply cheaply and environmentally the necessary nitrogen in association with synthetic fertilizer, thus lowering environmental impact (**Figure 1.5**). From 2015, several techniques have been explored. One technique mentioned by Geddes [173], is the transfer of nitrogenase and other supporting traits to microorganisms that already closely associate with cereal crops as a logical approach to deliver nitrogen to cereal crops . Ryu et al. [174] show to engineer inducible nitrogenase activity in two cereal endophytes (*Azorhizobium caulinodans* ORS571 and *Rhizobium* sp. IRBG74) and the well-characterized plant epiphyte *Pseudomonas protegens* Pf-5, a maize seed inoculant.[174] Such synthetic biotechnology tools have opened up possibilities for rice and wheat nitrogen fixation in the near future as highlighted by previous literature and Pivot Bio.

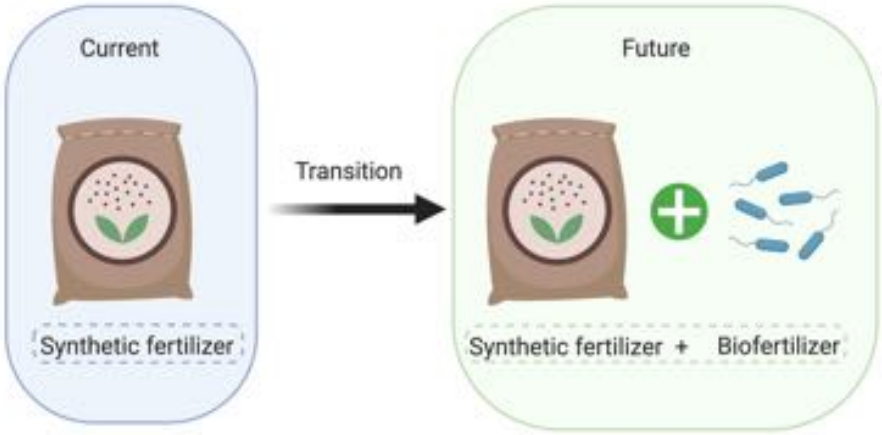

Figure 1.5. Transition from synthetic to microbe-based fertilizers in synergy with synthetic fertilizers to improve soil health and lower environmental impact through increasing fertilizer absorption rates thus minimizing runoff rates, solubilizing phosphates and fixing nitrogen for the plant.

Special attention is increasing for microbial inoculants that have multifunctional properties and contain more than one organism.[172] Most biofertilizers to date consist of one inoculant. However, it has been shown a consortium of microbes confer additional benefits to the plant and soil.

Therefore, the drive to commercialize multifunctional property and consortium microbe fertilizers. Strains of *Rhizobium*, phosphate-solubilizing bacteria and fungi, arbuscular mycorrhizal fungi, and free-living nitrogen-fixing *Azotobacter* strains improve the nodulating ability, nitrogen content and herbage yield (up to two-fold) of subabul seedlings (*Leucaena leucocephala*), in comparison with the independent application of each component of the consortium. This use case has also led to the developing of consortium-based delivery systems, which will be an important technique in enhancing colonization and performance. Further, synthetic biology has led to the development of high-throughput tools to identify elite strains at the single nodule level with the potential to revolutionize the search for elite indigenous rhizobia. [175]

Regulation will also play a huge role in the coming years to ensure standardization of products and easier product market entrance. Since biofertilizers are not yet ubiquitous, innovators will need to work with policy makers worldwide in developing robust policies that encourage product development and protect the environment and farmers.

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Chapter 2

Statement of the problem

Plant growth promoting bacteria (rhizobacteria) are well known to enhance crop production and protect plants from biotic and abiotic stresses, while decreasing the need for water and fertilizers. However, the rhizbacteria's delicate nature and poor delivery technologies has hindered their use in current agricultural practices, due to low survivability. The delivery of rhizobacteria is technologically challenging especially delivery of delicate microbes on a seed. Bioinspired by the tardigrade that uses proteins and polysaccharides to survive desiccation, silk and trehalose are used to engineer the seed microenvironment to deliver fragile (non spore forming) gram negative rhizobacteria. The delivery (storage and administration) of non spore forming rhizobacteria (*rhizobium tropici* CIAT 899) on a seed surface encompasses five research tasks 1. Encapsulation 2. Desiccation 3. Preservation 4. Release 5. Colonization. The dissertation will study how we can engineer the seed microenvironment using a protein (silk) and a disaccharide (trehalose) as a seed coating to alleviate stressors such as salinity and drought in plants using *Phaseolus Vulgaris* as a model. Chapter 3 studies how to improve the storage of rhizobacteria, the first three research questions (encapsulation, desiccation and preservation). Chapter 4 and 5 studies the administration of rhizobacteria, the last two research questions (release and colonization).

Chapter 3

Nonspore forming rhizobacteria encapsulation, desiccation and preservation in silk based materials as a seed coating

The contents of this chapter were published in the American Chemical Society Journal of Agriculture and Food chemistry as: Augustine T. Zvinavashe[1], Eugene Lim[1], Hui Sun[1], Benedetto Marelli[1, *]

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"A Bioinspired approach to Engineer Seed Microenvironment To boost Germination and Mitigate Soil Salinity." In press, PNAS., November 27th 2019

3.1 Abstract

Human population growth, soil degradation and agrochemicals misuse are significant challenges that agriculture must face in the upcoming decades as it pertains to global food production. Seed enhancement technologies will play a pivotal role in supporting food security by enabling germination of seeds in degraded environments, reducing seed germination time, and boosting crop yields. So far, a great effort has been pursued in designing plants that can adapt to different environments and germinate in the presence of abiotic stressors like soil salinity, heat and drought. The technology proposed here seeks a different goal: to engineer the microenvironment of seeds by encapsulation, preservation and precise delivery of biofertilizers that can boost seed germination and mitigate abiotic stressors. In particular, we developed a biomaterial based on silk fibroin and trehalose that can be mixed with rhizobacteria and applied on the surface of seeds, retrofitting currently used techniques for seed coating, i.e. dip coating or spray drying. A micrometer thick, transparent, robust coating is formed by material assembly. The combination

of a polymorphic protein as silk fibroin and of a disaccharide used by living systems to tolerate abiotic stressors provides a beneficial environment for the survival of non-spore forming rhizobacteria outside the soil and in anhydrous conditions. Using *Rhizobium tropici* CIAT 899 and *Phaseolus vulgaris* as working models, we demonstrated that rhizobacteria delivered in the soil after coating dissolution infect seedlings' roots, form root nodules, enhance yield, boost germination and mitigate soil salinity.

3.2 Significance Statement

In a world that strives to accommodate population growth and changes in climate patterns, there is a compelling need to develop new technologies to enhance agricultural output while minimizing inputs and mitigating their effects on the environment. In this study, we describe a biomaterial-based approach to engineer the microenvironment of seeds through the preservation and delivery of plant growth promoting rhizobacteria (PGPRs) that are able to fix nitrogen and mitigate soil salinity. PGPRs are encapsulated in silk-trehalose coatings that achieves bacterial preservation and delivery upon sowing. Biomaterial choice is inspired by a recent finding that a combination of proteins and disaccharides is key for anhydrobiosis. This simple technology is effective to boost seed germination and mitigate soil salinity.

3.3. Introduction

Global food production is projected to rise in the upcoming decades due to 800 million people currently lacking food security and 9.7 billion people projected to inhabit the world by 2050 [176]. Land degradation, excessive freshwater consumption and misuse of agrochemicals cause inefficiencies in agricultural practices, while changes in climate patterns and spread of transboundary pests and diseases require rapid crop adaptation to abiotic and biotic stresses. To address these challenges, precision agriculture has emerged based on advanced technologies designed to make food production more efficient, with the main goal of increasing crop production while minimizing inputs such as water and agrochemicals and mitigating environmental impact [177–179]. As a result, agriculture is becoming more sustainable and technologically driven with big data analysis, geolocalization, modernization of mechanical equipment and sensing systems being the main drivers of innovation in a sector that in the recent years has mostly benefited from improvements in agrochemical formulations, weather prediction, breeding and seed engineering.

Seeds are the agricultural product with the most value added and not only represent a source of food, but also the most important resource in agricultural practices [180]. There are many reasons for seedling suboptimal germination and mortality including diseases, pests, excessive use of fertilizers in the seed row, improper seeding depth, osmotic stress, frost and drought [181]. The use of precision tools to manage seed sowing and to support germination is then paramount to guarantee efficiency in terms of output over space. In the last few years, seed enhancement technologies have emerged to improve seed performance by exposure to specific conditioning and regimes [182]. Seed coatings have been developed to control seed surface properties, locally

enrich the soil with nutrients and influence seed water uptake [82,180]. However, the attention has mostly been focused on the investigation of payloads used to boost seed germination as a function of soil properties and seed type, rather than on the materials used to encapsulate and deliver the payloads. This approach has limited the formulation of seed coatings that encapsulate beneficial but labile compounds such as biofertilizers, i.e. plant growth promoting rhizobacteria (PGPRs) that increase availability of nutrients and phytohormones during interaction with plant roots while mitigating the environmental side-effects of synthetic fertilizers and pesticides [183–185]. The incorporation of inocula in an artificial seed coat can result, in fact, in the loss of microbial viability, with coated seeds unable to be stored for extended periods of time [186]. The synthetic seed coat is usually a hostile environment for PGPRs, mostly due to osmotic and desiccation stress and, when protectant compounds are present, their biological activity could pose a threat to the survival of symbiotic bacteria. Biomaterials that are adopted from the field of drug delivery represent a technological opportunity to develop an advanced seed coating technology that combines biodegradation with encapsulation, preservation and controlled release of payloads that can boost seed germination and mitigate stressors.

In this study, we developed a biomaterial-based approach to engineer seed coatings that can boost germination and mitigate abiotic stressors like soil salinity. In particular, we designed a biomaterial based on silk fibroin extracted from *Bombyx mori* cocoons and trehalose. The mixture can be mixed with rhizobacteria and applied on the surface of seeds, retrofitting currently used techniques for seed coating, such as dip coating or spray drying. Silk fibroin is a structural protein that is well known for its application in textile and that has been reinvented as a naturally-derived technical material with applications in regenerative medicine, drug delivery, implantable optoelectronics and food coating [187,188]. The structural protein is purified from

cocoons into a water suspension using a water-based process that uses chaotropic agents as LiBr to break the inter- and intra-molecular hydrogen bonds that crosslink silk fibroin molecules into fibers [189]. Upon removal of the ions via dialysis, silk fibroin has the form of nanomicelles in water suspension that are stable for a period of time that ranges from days to months, depending on concentration, pH and molecular weight [190,191]. Material assembly is driven by water removal and formation of new intra- and inter-molecular hydrogen bonds. This process can be engineered to obtain several materials formats, including transparent, robust membranes that have been used to extend the shelf-life of perishable crops [192]. The combination of a diblock copolymer-like structure with hydrophobic repetitive amino acid sequences spaced out by hydrophilic, negatively-charged non-repetitive sequences make silk fibroin polymorphic as the protein can be obtained in random coil or beta-sheet rich structures, enabling the fabrication of silk materials that are water soluble or water-insoluble, respectively [193,194]. Silk fibroin structure also provides a distinct environment that can preserve labile compounds ranging from antibiotics to growth factors, enzymes and viruses by mitigating oxidative stress, providing sufficient hydration and maintaining biomolecules configuration in anhydrous conditions [195]. Trehalose is a non-reducing disaccharide in which the two glucose units are linked via an α,α-(1,1)-glycosidic bond. This disaccharide has been isolated from all domains of life including plants, animals, fungi, yeast, archaea, and bacteria [196]. Trehalose is also industrially produced as it is used in the food, cosmetics, and pharmaceutical industries. This disaccharide can serve as a signaling molecule, as a reserve carbohydrate, and as a stress protectant (e.g., drought, cold, and salt stress) [197]. Accumulation of trehalose occurs both intra- and extra- cellularly [196,198,199]. There are two competing, but not mutually exclusive, hypotheses about the mechanism of trehalose-driven cellular protection; (i) the vitrification hypothesis suggests that trehalose forms a glass-like

matrix within cells, physically preventing protein denaturation, protein aggregation, and membrane fusion, (ii) the water replacement hypothesis posits that hydrogen bonds between water and cellular components are replaced by trehalose as cells dry, which would also prevent protein denaturation, aggregation, and membrane fusion [200]. Recently, it has been shown that a particular class of proteins known as intrinsically disordered proteins (IDPs) also contributes to anhydrobiosis. For example, a mixture of water-soluble proteins rich in hydrogen bonds and disaccharides is a successful strategy that anhydrobiotic organisms such as tardigrades have developed to survive desiccation [201]. Inspired by these recent findings, we have investigated a biomaterial formulation that synergistically use the coating-forming, payload encapsulation, preservation and biodegradation capabilities of silk fibroin with the ability of trehalose to offer protection from osmotic and desiccation stresses in rhizobacteria to develop a seed coating technology that can boost germination and mitigate abiotic stressors like soil salinity.

3.4 Results and Discussion

3.4.1 Formulation of silk fibroin – trehalose biomaterials

Li and co-workers have recently reported how the preservation of biomolecules in silk fibroin formulations correlates with matrix β-relaxation, as it does in sugar-based dry formulations [202]. It was also found that inclusion of sugars like sucrose in silk fibroin-based materials enhances the protein stabilizing performance as they can act as antiplasticizers that suppress β-relaxation and decelerate degradation rates. In Fig. 3.1, we report the effects of trehalose on silk fibroin matrices. Molecular dynamic simulations were used to investigate the molecular mobility of a silk fibroin-like system made by 18 $(GAGSGA)_2$ peptides organized in a beta sheet configuration when suspended in water or in a water-trehalose mixture (Fig. 3.1A). Time evaluation of the

root-mean-square deviation (RMSD) of atomic position from the original conformation indicated that trehalose reduces the dynamics of the eighteen peptides systems and correlates with the general knowledge that sugars form a matrix around proteins that lock them in the original conformation by slowing down protein dynamics. Experimentally, we have demonstrated that trehalose does not interfere with silk fibroin assembly as its addition to silk fibroin suspensions does not impart any modification to the random coil structure assumed by silk fibroin nor does it drive protein assembly. Circular dichroism (CD) spectra of silk fibroin (S) and silk fibroin – trehalose (ST) water suspensions depicted that the protein maintained a random coil structure (indicated by the negative bands near 195 nm and low ellipticity above 210 nm) [203] when exposed to increasing concentrations of trehalose, up to 75 dry wt% (Fig. 3.1B). Dynamic light scattering (DLS) was used to measure the hydrodynamic radius of silk fibroin nanomicelles in S and ST water suspensions (Fig. 3.1C). No statistically significant difference ($p>0.05$) was found in the measured nanomicelles diameters at increasing trehalose concentrations, indicating that trehalose does not influence assembly of silk fibroin molecules in water. Attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR) was used to evaluate the effects of trehalose on silk fibroin polymorphism upon drying (Fig. 3.1D). All the spectra of silk fibroin films obtained using an increasing concentration of trehalose had an Amide I resonance centered at 1647 cm^{-1}, indicating that the structure of silk fibroin molecules was not affected by trehalose and possessed a random coil configuration[204]. Interestingly, methods such as water annealing that are commonly used to drive the random coil to beta-sheet transition in assembled silk fibroin molecules were still effective in crystallizing silk fibroin at high concentrations of trehalose. This phenomenon suggests that the replacement of hydrogen bonds between silk fibroin molecules and water with inter- and intra-molecular hydrogen bonds may be

thermodynamically favorable even in the presence of trehalose and that the protein can undergo structural reconfiguration, water loss and volume change despite the trehalose-induced vitrification of the protein. Nanoindentation mechanical tests conducted on silk fibroin films containing increasing concentrations of trehalose showed that films' hardness and Young modulus increased as the trehalose concentration increased. The inclusion of large quantities (up to 75 wt%) of the disaccharide imparted an antiplasticizing effect, which followed the rule of mixture and resulted in a more brittle final material, especially at trehalose concentrations >50 dry wt% (Fig. 3.1E). However, when water annealing post-processing was applied to enhance films' beta-sheets content, the hardness and Young modulus of ST materials did not follow the rule of mixture. Hardness increased for trehalose concentrations up to 25 dry wt% (i.e. ST3:1) and then decreased in films with a trehalose content of 50 and 75 dry wt% (i.e. ST1:1 and ST1:3, respectively). Young modulus of ST materials increased for ST3:1 films and then plateaued for larger trehalose contents. In SI Appendix, Fig. S3.1 we report characterization of films obtained by mixing silk fibroin with sucrose, a disaccharide that was not considered in this study for seed coating applications given its large use as food ingredient. CD, DLS and ATR-FTIR analyses showed that sucrose did not modify silk fibroin folding and assembly behavior, similarly to what found for trehalose (SI Appendix, Fig. S3.1A-D). However, nanoindentation measurements showed that rule of mixture can predict mechanical properties when sucrose is incorporated in silk fibroin materials, even when water annealing is applied. These data suggest a difference in the effects of vitrification imparted by trehalose and sucrose on silk materials; trehalose possesses higher glass transition temperature ($T_g \approx 393$ K) when compared to sucrose ($T_g \approx 348$ K) and can form a more homogenous network with proteins [200,205]. As a result, the trehalose brittle matrix is disrupted by silk fibroin random coil to beta sheets structural changes during

water annealing, yielding a weaker material for trehalose concentrations > 25 dry wt%. Nonetheless, ST materials showed mechanical properties in the order of currently available seed coatings (Young modulus of 10^{-1}-10^{1}GPa) [206].

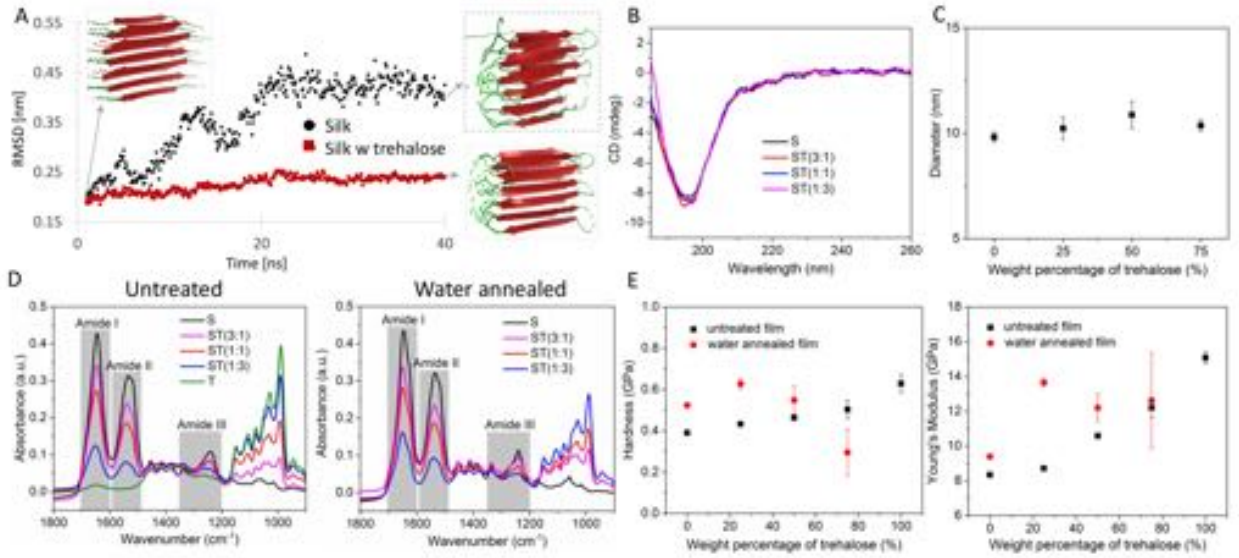


Figure 3.1. Characterization of silk fibroin, trehalose and their mixtures used to manufacture films for seed coating. (A) MD simulation of silk fibroin when exposed to water (black dots) and to a water and trehalose solvent-matrix (red dots); root mean squared deviation (RMSD) measures average distance between the atoms. The three inserts depict silk fibroin structure after relaxation and at the end of the simulation (40 ns) in the different media. (B) Circular dichroism (CD) of suspensions of silk fibroin (S) and mixtures of silk fibroin and trehalose (ST). The disaccharide has no impact on the protein random coil conformation in water suspension, as indicated by the negative bands near 195 nm and low ellipticity above 210 nm. (C) Dynamic light scattering (DLS) analysis of silk fibroin nanomicelles aggregation in water as a function of trehalose concentration. The average diameter of silk nanomicelles was not affected by trehalose concentration in the water suspension. Error bars indicate standard deviation. (D) FTIR spectra of films obtained by drop casting of silk fibroin (S), trehalose (T) and their mixtures (ST). The random coil-dominated resonance peaks of the amide bonds in the Amide I and Amide III regions were not affected by the presence of trehalose, indicating that the disaccharide has no effects on the protein polymorphism during the sol-gel-solid transition. In the right panel, FTIR spectra show that a polymorphic random coil to beta sheet transition of silk fibroin can be induced post film formation with water annealing, as evidenced by the appearance of a peak at 1621 cm^{-1}. (E) Mechanical properties of films composed by silk fibroin, trehalose and their mixtures investigated with nanoindentation. Trehalose concentration and water-annealing post-process resulted in an increase in hardness and apparent modulus and in the formation of more brittle films. Error bars indicate standard deviation.

3.4.2 Coating assembly and biofertilizer encapsulation and release performance

Silk fibroin assembly is driven by water evaporation and results in a sol-gel-solid transition process that yields a transparent material. The resulting film has a roughness of few nanometers (measured by AFM on flat films) and a thickness that can be controlled by modifying solution rheological parameters [207]. At constant solid matter content, inclusion of trehalose in silk fibroin suspensions decreases solution viscosity (SI Appendix, Fig. S3.2), which however remains in the order of 10^{-3} Pa·s, thereby enabling the application of ST suspensions on complex geometries such as spheroids by retrofitting existing technologies commonly used for seed coating. Contact angle (θ) measurements also showed that θ decreases at higher trehalose concentration, given the higher hydrophilicty of the disaccharide when compared to silk fibroin (SI Appendix, Fig. S3.2). When using borosilicate glass beads with a diameter of 5 mm as model for seeds, dip coating and spry drying of ST solutions enabled the encapsulation and delivery of payloads such as PGPRs via formation of a micrometer thick coatings that biodegrade when exposed to water (Fig. 3.2). Given the transparency of silk materials, we used green fluorescent protein (GFP)-producing PGPRs such as GFP-modified *Rhizobiun tropici* CIAT 899 (GFP-CIAT 899) to evaluate the encapsulation, preservation and delivery of rhizobacteria. CIAT 899 is a broad host-range rhizobial strain and the most successful symbiont of *Phaseolus vulgaris* [208,209]. CIAT 899 provides high tolerance to environmental stresses such as high temperature, acidity and salinity and its potential use as a biofertilizer is highly desirable but hindered by the low survivability of gram-negative bacteria during the desiccation and rehydration steps required for coating formation and inoculation [186,210]. In Fig. 3.2A we report fluorescent images of glass beads coated with ST materials using dip coating and spray drying techniques. When compared with the

negative controls, it is possible to see how the GFP-CIAT 899 were successfully encapsulated in the coating materials as glass beads fluoresced when excited with a blue light. Spray-coated beads exhibited brighter fluorescence, which suggests the achievement of an enhanced encapsulation of GFP-CIAT 899. However, dip-coating methods are often preferable due to the easier implementation at scale. Scanning electron microscopy (SEM) was used to evaluate the thickness of the coatings obtained as a function of increasing relative concentration of trehalose in silk fibroin matrices (i.e. dry mass remained constant). SEM micrographs revealed that coatings thickness was on the order of few micrometers (5±2 μm) and depicted the presence of bacteria in the vitrified polymer matrix (Fig. 3.2B). Successful encapsulation and release of GFP-CIAT 899 on glass beads as a function of ST mixture coating material (i.e. increasing relative content of trehalose) was then evaluated via streaking of resuscitated bacteria on an agarose gel using a plate count method (Fig. 3.2C). Given the coating thickness (t), the spherical geometry of the substrate (r), the known concentration of bacteria in the coating solution (C_b) and assuming an homogenous dispersion of bacteria and the formation of an homogenous coating, it is possible to estimate the number of bacteria encapsulated in the coating (N) by multiplying C_b times the volume of the coating spherical shell (V):

$$N = C_b \cdot V = C_b \cdot \left(\frac{4}{3}\pi R^3 - \frac{4}{3}\pi r^3\right) \approx C_b \cdot (4\pi r^2 t) \qquad \text{(Eq. 1)}$$

Where $R = (r + t)$ and $4\pi r^2 t$ is an approximation for the volume of a thin spherical shell obtained as the surface area of the inner sphere multiplied by the thickness t of the shell. Using r = 0.25 cm, t = 0.0005 cm and $C_b = 10^{10}/cm^3$, then $N \approx 3.9 \cdot 10^6$, which indicated a 1 log reduction of GFP-CIAT 899 culturability during the coating and resuscitation procedures (circa $3 \cdot 10^5$

CIAT 899 were resuscitated from ST coatings, as shown in Fig. 3.2C). Using Phytagel as a model for soil moisture content, we investigated ST films biodegradation and release of GFP-CIAT 899 using a ChemiDoc MP Imaging System. Time-lapse images of the materials indicated that an increasing relative content of trehalose accelerated material reswelling such that structural integrity was lost within 10 minutes. Additionally, fluorescence microscopy images taken on glass beads coated with ST materials encapsulating GFP-CIAT 899 showed bacteria release in Phytagel a few minutes after materials were in contact with the artificial soil (SI Appendix, Fig. S3.3 and Video S3.1).

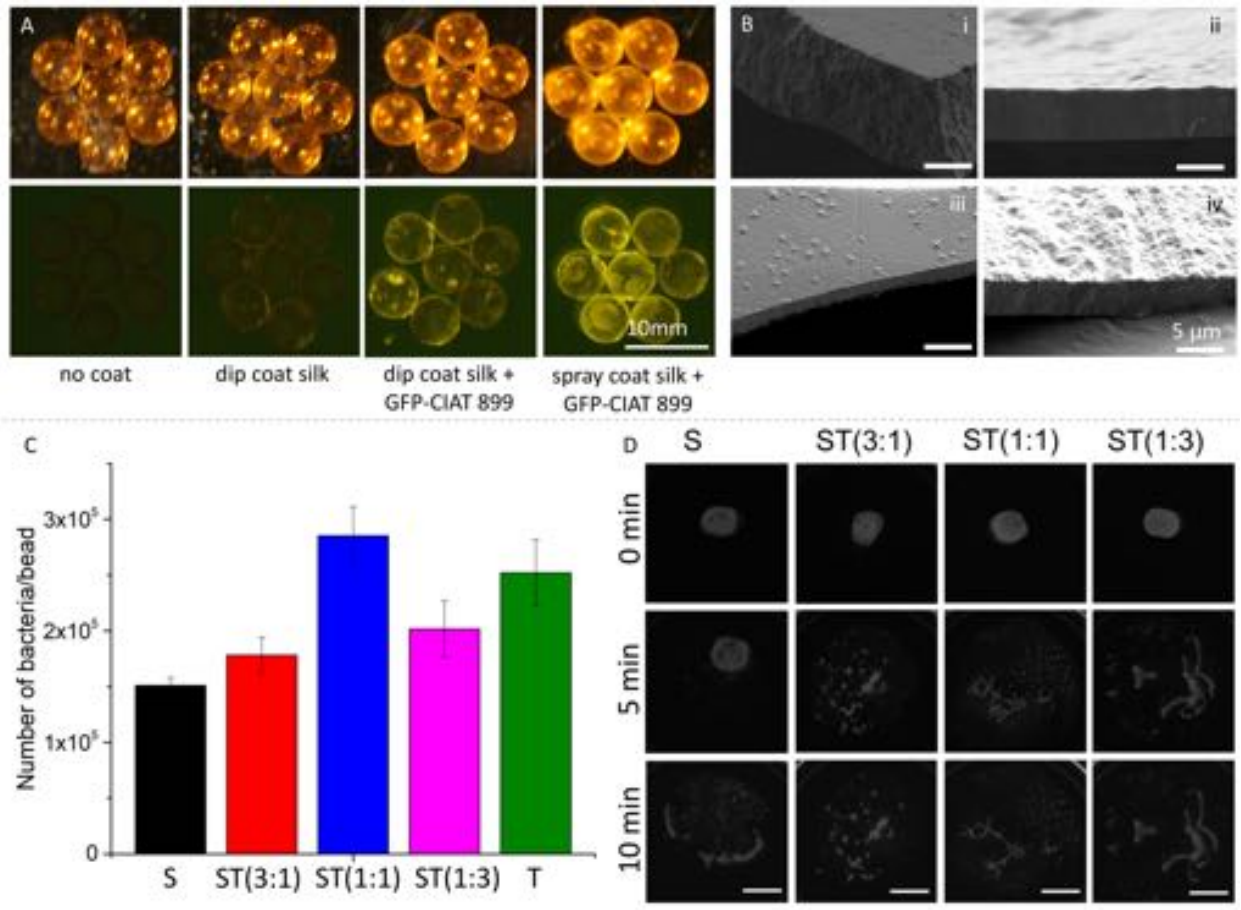


Figure 3.2. Coating manufacturing, inoculant encapsulation and material degradation. (A) Single-pot coating of glass beads used as seeds phantom with dip coating and spray drying techniques. GFP-modified CIAT 899 allowed to visualize bacteria distribution on the glass beads. (B) SEM micrographs depicting the cross section of (i) silk (S), (ii) silk-trehalose 3:1 ST(3:1), (iii) ST(1:1), and (iv) ST(1:3) coatings obtained by dip coating. The resulting film

thickness was of circa 5 µm. The four micrographs have been taken at the same magnification. (C) Investigation of bacteria encapsulation efficacy during dip coating process. The quantity of bacteria per bead after dip coating was quantified by colony counting. All coating solutions had similar dry matter concentration, number of bacteria and viscosity. Error bars indicate standard deviation. (D) Degradation of coating films encapsulating GFP-CIAT 899. Films were positioned on thin phytagels, which were used as transparent artificial soil. Film degradation was investigated as a function of time. Top views of films transilluminated with fluorescence light were taken using a ChemiDoc MP imaging. Scale bar is 1cm.

3.4.3 Preservation of CIAT 899 in silk fibroin – trehalose coatings

PGPRs like CIAT 899 are non-spore forming bacteria with limited viability outside the soil and poor survival post-desiccation [211]. Long-term storage of rhizobacteria in seed coating is one of the major bottlenecks that hinders a large-scale use of these biofertilizers in agricultural practice [212,213]. Application of PGPRs directly in soil and handling of living bacteria require tools and expertise that are not largely available, and thereby the successful encapsulation of PGPRs in seed coatings is seen as a key step to translating the beneficial effects of biofertilizers from bench to field. To assess the potential use of ST materials as seed coating technology to encapsulate, preserve and deliver PGPRs, viability and culturability studies were conducted on CIAT 899 embedded in silk, trehalose and their mixtures at T = 23°C and relative humidity (RH) of 25% and 50% for up to 4 weeks. GFP-CIAT 899 preserved in sodium chloride and polymers (e.g. methylcellulose (MC) and polyvinylpyrrolidone (PVP)) found in commercially available seed coatings were used as controls. Fig. 2.3 shows that ST materials outperformed silk, trehalose, MC and PVP in preserving GFP-CIAT 899. Interestingly enough, water annealing of silk and ST materials (labelled with A at the end of the samples name in Fig. 3.3) did not enhance preservation, as previously reported for biomolecules such as antibiotics, enzymes and growth factors [214,215], but appeared to be detrimental. Silk fibroin films anneal into a water-insoluble material when left at room temperature and very high RH, as the random coil to beta-sheet

transition is thermodynamically favored [216]. This process, commonly named water annealing, causes a partial re-hydration and a second drying of the materials which may have stressed and damaged GFP-CIAT899. Viability measurements (Fig. 3.3, top row) obtained with alamarBlue staining showed that ST(1:3) provided the best environment for GFP-CIAT 899 preservation at both RH levels considered. At week 4 post film formation, more than 25% of GFP-CIAT 899 encapsulated in ST(1:3) films were found to be metabolically active when preserved at RH=25%. Higher humidity levels decreased viability to ~5% at week 4, indicating that the coating performance suffers from the hygroscopic nature of the materials used. alamarBlue was indicative of GFP-CIAT 899 bacteria that were alive (i.e. active metabolic state and intact membrane) post-resuscitation. However, in order to survive in a competitive environment like the rhizosphere and to form nodules with the host plants, PGPRs need to be able to form colonies. GFP-CIAT 899 re-culturability was investigated by streaking resuscitated bacteria on agarose gels as a function of storage material, time and RH (Fig. 3.3, bottom row and SI Appendix, Fig. S3.4 and Fig. S3.5). Culture media was not added to resuscitate bacteria in order to better simulate soil conditions, where no recovery time would occur. GFP-CIAT 899 colony counting indicated lower viability levels when compared to results obtained with alamarBlue metabolic activity assay, suggesting that a large quantity of GFP-CIAT 899 were viable but non-culturable (VBNC). VBNC state in PGPRs was previously described as a side effect of desiccation using several encapsulation matrices, including nitrocellulose filters, where viability dropped to 4.0% after one week and to less than 2% after 4 weeks at RH=22% [217]. In our experiments, silk, trehalose and ST mixtures produced a statistically significant increase in viability relative to PVP and MC, which are commercially used in seed coating formulations. Additionally, ST(1:3) preserved GFP-CIAT 899 better than other ST mixtures and with similar

performance to pure trehalose, indicating that the disaccharide is the key ingredient in the silk fibroin-trehalose mixture to achieve bacterial re-culturability post-desiccation. ST(1:3) was then chosen as the best performing coating because it integrates beneficial trehalose vitrification with the ability of silk fibroin to provide sufficient mechanical robustness, adhesion and controllable degradation to the end-material. Annealing of silk fibroin at the point of material fabrication may also be used in the future to control (in a time-dependent manner) the coating biodegradation and consequent release of PGPRs in the surrounding environment.

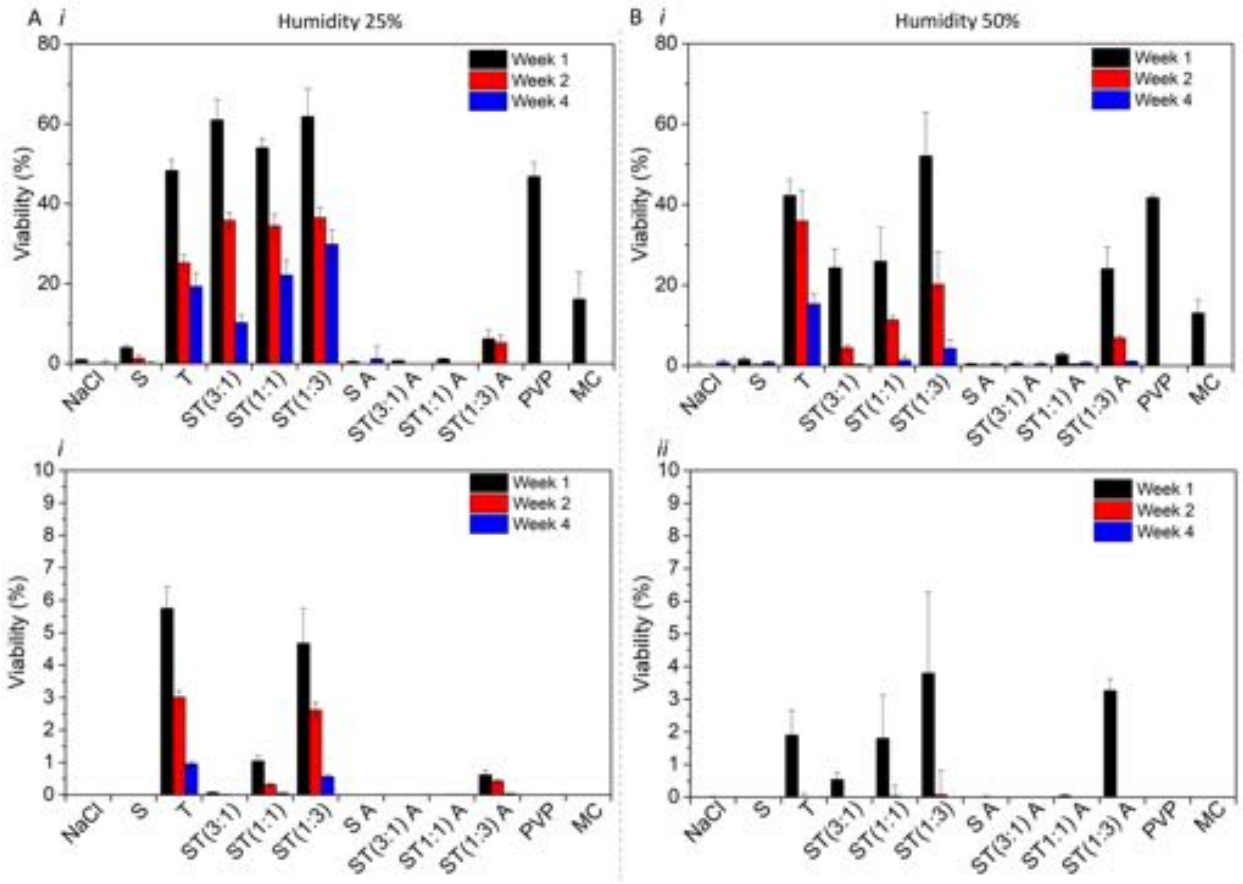


Figure 3.3. Preservation of CIAT 899 in silk, trehalose and their mixtures. Data were collected at weeks 1, 2 and 4 for samples stored at 23℃ and at (A) 25% (B) 50% relative humidity. In the top panels, viability indicates the percentage of bacteria that were metabolically active and had an intact membrane, as investigated by Alamar blue analysis. In the bottom panels, viability was measured as the percentage of bacteria that were culturable into colonies (colony counting analysis). Data are a pooled average ± standard deviation of $n = 7$ replicates across ten samples and single factor Anova test. S=silk, T=trehalose, ST=silk:trehalose, x:x

indicates the relative weight ratio between the two biopolymers, A=annealed 6 hours, MC=methyl cellulose, PVP= polyvinylpyrrolidone.

3.4.4 Intrinsic vs extrinsic trehalose

Several rhizobium species like *Rhizobium Etli* are reported to synthesize, uptake and degrade trehalose [218]. The disaccharide accumulates in the cells as an osmoprotectant in response to increasing osmotic pressure of the medium through the otsAB, treS, and treZY synthetic pathways while internal translocation is regulated by permease proteins like trehalose-maltose ABC transporter, encoded by the trehalose transport and utilization (thu) operon (thuEFGK) [218]. For CIAT 899, it has been reported that trehalose synthesis is osmoregulated [219], suggesting the involvement of trehalose in the osmotolerance of this strain. However, it is still unknown if CIAT 899 has ABC transporter proteins capable of translocating trehalose, as only evidence for a sorbitol/mannitol ABC transporter have been reported [219]. To further investigate the mechanism that underpins stabilization of CIAT 899 in ST materials, we measured intrinsic trehalose content for CIAT 899 incubated in a 1 dry wt% trehalose solution and 0.09 dry wt% NaCl solution for 1-hour (1h). The 1h time point was used to mimic the amount of time CIAT 899 are in contact with ST materials during solution handling and coating formation. The study showed that within 1h, CIAT 899 intrinsic trehalose concentration was not affected by extrinsic trehalose present in the forming ST materials (Fig. 3.4A). This finding suggests that the stabilization process induced by ST coatings leverages extracellular phenomena such as vitrification rather than being driven by intracellular translocation of trehalose to provide intrinsic osmotic protection. To further investigate the interplay between extrinsic and intrinsic trehalose in CIAT 899, we measured the ability to translocate and metabolize trehalose when compared to a rhizobium strain as *Rhizobium etli* CNF42, which is well known to possess the thu operon that can translocate and

utilize trehalose (Fig. 3.4B) [220]. The study was conducted by culturing CIAT 899 and CNF42 in minimal media using trehalose as carbon source and sucrose as a positive control. Optical measurements (OD600) showed that CIAT 899 could proliferate in trehalose minimal media as well as CNF42, indicating the ability of CIAT899 to translocate and metabolize trehalose and suggesting that in the future longer pre-exposure to trehalose may lead to enhance preservation performance.

Figure Legends

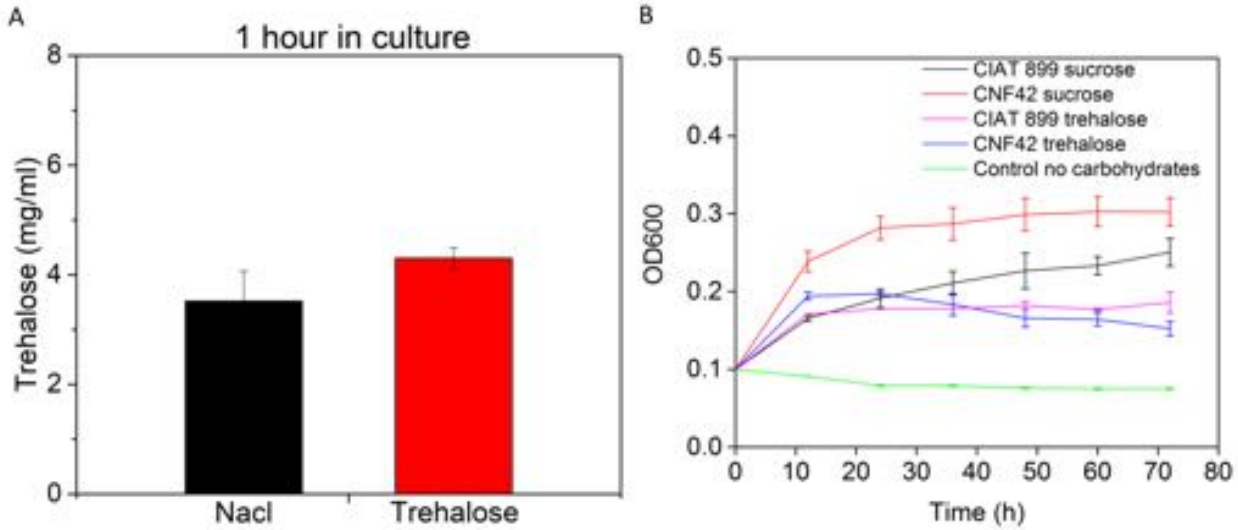


Figure 3.4. Interplay between trehalose and CIAT 899. (A) Bacteria were cultured for 1 hour in 1% dry wt% trehalose solution to measure cellular uptake of extrinsic trehalose. Intrinsic levels of the disaccharide were found to be not statistically significantly different ($p>0.05$) when compared to the control (0.09 dry wt% NaCl solution). Data are a pooled average ± SD of $n = 7$ and single factor Anova test was used. (B) CIAT 899 and CNF42 were cultured in 0.4% minimal sucrose solution and 0.4% minimal trehalose solution. Growth profiles of CIAT 899 shows the ability to translocate and metabolize trehalose and to use it as a carbon source. Data are a pooled average ± standard deviation of $n = 7$.

3.4.5 *Phaseolus vulgaris* germination boost and mitigation of saline soil conditions

P. vulgaris seeds were dip coated with ST(1:3) encapsulating CIAT 899, dried and stored for 24 hours before planting (Fig. 3.5A). Dip coating was used as it is a cheap, high throughput and low technology method easily accessible to all farmers [221]. Among all the materials investigated, ST(1:3) mixture ratio was used given its superior performance in terms of mechanical properties,

solution viscosity and CIAT 899 preservation. Coating processing was designed to coat each seed with 10^7 CIAT 899 bacteria, following requirements generally imposed by policy makers for biofertilizers [162]. CIAT 899-coated *P. vulgaris* were grown over a two week period of time in saline (8 ds/m) and non-saline (4 ds/m) soil, using ST(1:3)-coated seeds with no CIAT 899 as control. Saline soil was established by adding NaCl to topsoil. The CIAT 899-coated *P. vulgaris* seeds exhibited a statistically significant improvement in germination rate for 4 ds/m and 8 ds/m soils in comparison to the control seeds. Over the two-week investigation period, CIAT 899-coated *P. vulgaris* seeds grew into seedlings that were taller and possessed longer and more articulated roots in comparison to the control seeds (Fig. 3.5B). Visual inspection and fluorescence microscopy were used to assess nodule formation. The right panel of Fig. 2.5B depicts how the GFP-CIAT 899-coated *P. vulgaris* seeds germinated into plants that were colonized by GFP-CIAT 899, as indicated by the presence of nodules that exhibited a strong GFP-induced fluorescence. Interestingly enough, the effectiveness of the CIAT 899-ST (1:3) coating in boosting seed germination and producing stronger seedlings was more evident in the high-salinity 8 ds/m soil.

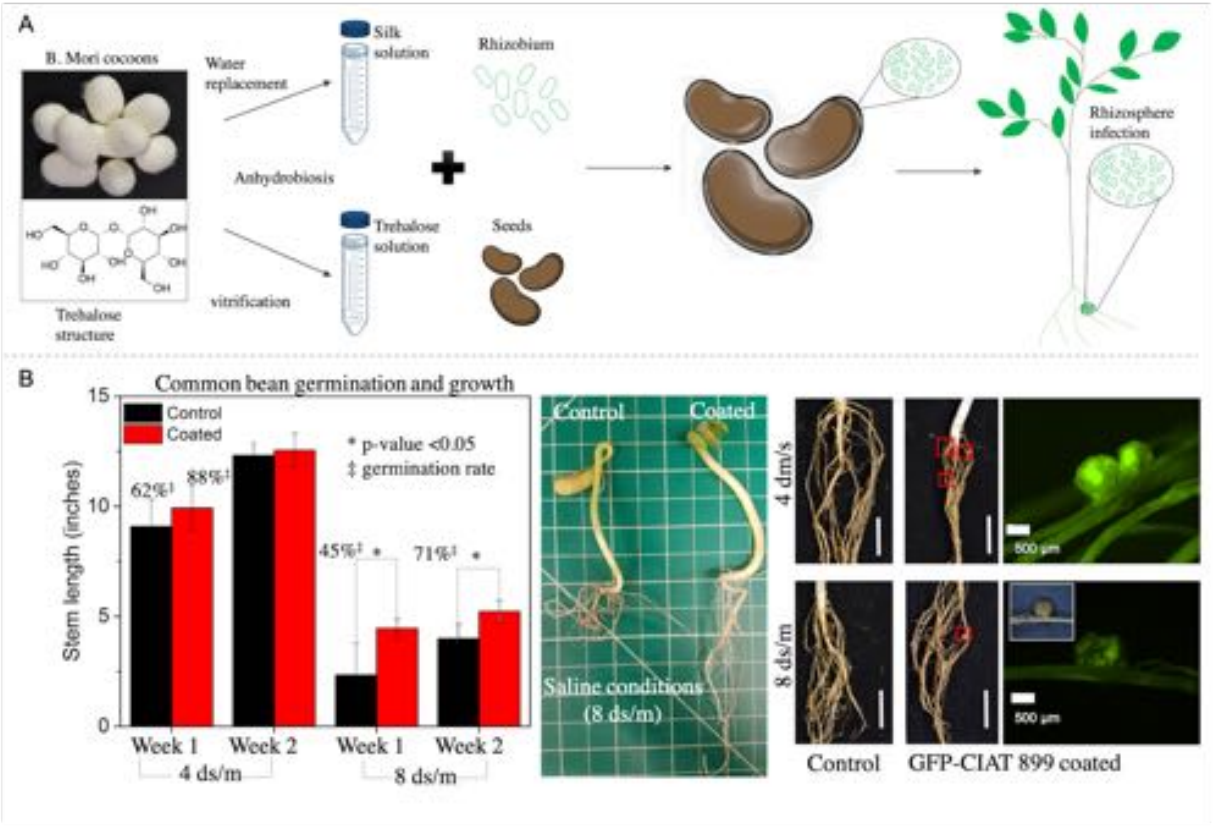


Figure 3.5. Seed coating, plant root colonization and mitigation of saline soil environment. (A) Schematic of the strategy used to preserve and deliver CIAT 899 to induce roots infection through inoculation by seed coating of common beans. (B) Germination rate and stem growth over a 2-week period in non-saline (4ds/m) and saline (8 ds/m) conditions. Error bars indicate standard deviation. (C) Macroscopic pictures and fluorescence microscope images of root nodulation confirmed root colonization by GFP-CIAT 899. Scale bar in root images is 1cm. *P. vulgaris* planted per condition, $n = 15$

3.5 Conclusion

In summary, we developed a new biomaterial formulation capable of precisely coating seeds with biofertilizers and releasing them in the soil to boost seed germination and mitigate soil salinity. The bioinspired approach that we describe combines a disaccharide well-known for its key role in anhydrobiosis with a structural protein that imparts mechanical robustness, ease of fabrication, adhesiveness, conformability and controlled biodegradation. The rhizobium strain used in this manuscript survived encapsulation in the biomaterial coating, was preserved over

time and was successfully released in the soil to form symbiotic nodules with the host roots. ST-coated seeds yielded plants that grew faster and stronger in the presence of saline soil. More broadly, our study opens the door to the application of advanced materials to precision agriculture, introducing concepts that are germane to drug delivery and biomaterials design to a field that needs to implement innovative technologies to enhance food production while minimizing inputs and mitigating environmental impacts. Using this approach, it is now possible to define new applications where biomaterials can be used to engineer seed microenvironment to precisely deliver nutrients, hormones and beneficial biomolecules to seedlings, paving the way for a more sustainable and effective delivery of fertilizers and pesticides.

3.6 Data availability statement

Data Availability Statement: SI Appendix details methods and further experimental results. Correspondence and requests for materials and data should be addressed to B.M.

3.7 Acknowledgments

We thank Jaime Cheah for helpful technical assistance and discussions; Miguel Lara for GFP Rhizobium tropici CIAT 899 from Universidad Nacional Autonoma de Mexico. This work was partially supported by MIT Paul M. Career Professorship, Office of the Dean for Graduate Fellowship and Research Assistantship Fellowship, Office of Naval Research Young Investigator Program (N000141812258) and Université Mohammed VI Polytechnique-MIT Research Program.

3.8 Supplementary

Materials and Methods

MD simulation – The initial model for the (GAGSGA)2 oligomer was constructed by threading the dodecapeptide sequence onto the structure of a poly-(Gly-Ala) β-sheet (Protein Data Bank identification code 2slk) using UCSF Chimera and Modeller. The system was then equilibrated in a transferable intermolecular potential 3P (TIP3P) explicit water box. Simulations were run for 40 ns with a time-step of 2 fs at constant temperature (300 K) and pressure (1 bar) using GROMACS. The force field used was GROMOS53a6. Alpha,alpha-Trehalose structure was provided by the Automated Topology Builder (ATB) and Repository available from the Molecular Dynamics Group at the University of Queensland, QLD, Australia. The stability of the β-sheet assembly was verified from hydrogen-bond dynamics and root mean squared deviation (RMSD) data obtained from the molecular dynamics' trajectory.

Materials fabrication – To investigate biopolymer preservation mechanism, films were fabricated via drop casting and spray drying. Suspensions were made of gram negative PGPRs (*Rhizobium tropici* CIAT 899 Martinez-Romero et al. - ATCC 49672) mixed with the silk fibroin, trehalose or silk fibroin – trehalose (ST) mixtures. NaCl solution was used as a negative control. Alamar Blue metabolic assay and agar streaking were used to evaluate bacteria viability upon resuscitation. Biopolymer solutions at a concentration of 1 dry wt% solution were prepared for consistency throughout the manuscript. Silk fibroin aqueous suspensions were prepared as described in "Materials fabrication of Bombxy silk". Trehalose (TCI America, Portland, OR, USA), polyvinylpyrrolidone (PVP, 30kDa) (Sigma Aldrich, St Louis, MO, USA), methyl cellulose (Sigma Aldrich, St Louis, MO) and NaCl (Sigma Aldrich, St Louis, MO) were all

dissolved in H_2O. However, for methyl cellulose the H_2O had to be cooled at 4ºC for mixing to occur. For bacteria handling and culture, 50% tryptic Soy Broth (Becton Dickinson, Franklin Lakes, NJ, USA) was generated by mixing 500ml of H_2O with 7.5g BDTM Tryptic Soy broth (Soybean-Casein Digest Medium) (Becton Dickinson, Franklin Lakes, NJ, USA). The media was autoclaved for 60 min at 121ºC. CIAT 899 was sourced and cultured in a shaker incubator at 200rpm and 30ºC up to an OD600 measure of 0.7. Once bacteria reached an OD600 of 0.7, 11ml of bacteria broth solution was centrifuged at 4300 rpm for 20 min. The bacteria formed a pellet and the supernatant was discarded. Concentrated bacteria suspension was made, 1.1ml of biopolymer was pipetted into the pelleted bacteria strain and uniformly mixed by thoroughly pipetting up and down. This was carried out for all biopolymers used. These were labeled as *live* samples. For the *dead* samples, used as negative control, the bacteria pellet was mixed with 70% (w/v) isopropanol and incubated for 60 min. After the 60 min the bacteria solution was centrifuged as above and supernatant discarded. Concentrated bacteria suspension was made, 1.1ml of biopolymer was pipetted into the pelleted bacteria and uniformly mixed by thoroughly pipetting up and down. This was carried out for all biopolymers used. 5 repeats of 100 ml of *live* and *dead* samples were produced for each time point. Bacteria-biopolymer suspensions were drop cast onto 1" by 1" PDMS slab and left to air-dry. Films were then preserved at room temperature for the required period of time in petri dishes. Samples that required water annealing were placed in a vacuum chamber with H_2O, sealed for 6 hours to anneal, removed and placed in petri dishes.

Live dead assay – To develop calibration curve of alamar Blue assay (Resazurin), the following procedure was followed: (*i*) OD600 = 0.7 bacteria solution was collected and centrifuged. (*ii*) For

negative control (*dead* bacteria), centrifuged bacteria were resuspended into 1ml of 70% isopropanol (Sigma Aldrich, St Louis, MO, USA) for 60 min., then centrifuged and resuspended into 1ml of NaCl.(*iii*) The bacteria suspension obtained at point (*i*) was centrifuged and resuspended in NaCl (*live* bacteria). (*iv*) Dead and live bacteria were mixed in increasing relative concentration to form the following ratios: 100% live, 80:20 live:dead, 50:50 live:dead, 20:80 live:dead and 100% dead and the relative OD600 was measured (*v*) 100 ml of the above suspensions were added to 96 well plate and viability was measured. To measure viability an alamar Blue assay was performed following the manufacturer protocol. The excitation wavelength was 570nm and emission wavelength 585nm. Samples were prepared in the dark and kept under wrap of foil paper for 60 minutes before the analysis was performed. Alamar Blue assay microplate reader (Tecan Safire 2, Mannedorf, Switzerland) gain was kept constant for all experiments performed.

Film degradation – Biopolymer films encapsulating GFP-CIAT 899 were placed in contact with 1cm thin phytagel films (artificial soil) and time-dependent degradation was studied with the ChemiDoc MP Imager (Bio-rad, Hercules, CA) at GFP emission and excitation. Phytagel gel (Sigma Aldrich, St Louis, MO) was made by following protocol from Sigma Aldrich by mixing 2g/l phytagel and 1.5% $CaCl_2$ in water solution. Further, film degradation was studied under fluorescence microscopy and videoed (Movie S1). Phytagel films were placed above air-dried films.

DLS – Dynamic Light Scattering (DLS) measurements were performed on a DynaPro NanoStar Light Scatterer (Wyatt Technology). Samples at 0.1 mg/ml were measured in plastic cuvettes

(UVette, Eppendorf). The laser was set at 658 nm. The acquisition time for each data point was 5 seconds, and ten data points were acquired for each sample.

CD – Circular Dichroism (CD) spectra were recorded from 185 to 260nm using a JASCO J-1500 spectrometer, with each spectrum averaged from three consecutive scans. Samples of concentrations higher than 0.1mg/ml were diluted to 0.1mg/ml with the corresponding buffer and measured in a 1 mm path length quartz cuvette (Starna Cells, Inc.).

FTIR – Drop cast films were analyzed using Thermo Fisher FTIR6700 Fourier Transform Infrared Spectrometer through attenuated total reflection (ATR) germanium crystal. For each sample, 64 scans were coadded with a resolution of 4 cm^{-1}, at wave numbers between 4000 and 650 cm^{-1}. The background spectra were collected under the same conditions and subtracted from the scan for each sample.

SEM – Drop cast films were freeze cracked after being dipped in liquid nitrogen and analyzed with a Zeiss Merlin High-resolution scanning electron microscope. Samples prepared did not charge, therefore no gold plating or any preparation of samples was required. An EHT of 1.00kv was used with a 100pA probe.

Nanoindentation – Nanoindentaion measurements were performed on a Hysitron TriboIndenter with a nanoDMA transducer (Bruker). Samples were indented in load control mode with a peak force of 500 μN and a standard load-peak hold-unload function. Reduced modulus was calculated by fitting the unloading data (with upper and lower limits being 95% and 20%,

respectively) using the Oliver-Pharr method and converted to Young's modulus assuming a Poisson's ratio of 0.33 for all samples. Each type of sample was prepared and indented in triplets to ensure good fabrication repeatability. For each sample, indentation was performed at a total of 49 points (7×7 grid with an increment of 20 μm in both directions) to ensure statistical reliability of the modulus measurements.

Viscosity – Rheological measurements were performed on 6 dry wt% biopolymer suspensions using a TA Instruments (New Castle, DE, USA) stress-controlled AR-G2 rheometer with a 60mm, 2° cone-and-plate fixture at 25℃. Solutions were allowed to equilibrate on the rheometer before running stepped flow from 0.1000 1/s to 1000 1/s.

Contact angle measurement – Contact angle analysis was performed using an optical contact angle apparatus Rame-hart goniometer (Succasunna, NJ, USA) equipped with a video measuring system with a high-resolution CCD camera and a high-performance digitizing adapter. SCA 20 software (Data Physics Instruments GmbH, Filderstadt, Germany) was used for data acquisition. Soda lime glass slides were fixed and kept flat throughout the analysis. The contact angle of silk fibroin, trehalose and their mixtures was measured by the sessile drop method by gently placing a droplet of 5 ml of biopolymer suspension onto the seed surface, according to the so-called pick-up procedure. All droplets were released from a height of 1 cm above the surface to ensure consistency between each measurement. The contact angle (θ, the angle between the baseline of the drop and the tangent at the drop boundary) was monitored using a software-assisted image-processing procedure. Five droplets were examined for the different biopolymer formulations considered on both the left and right sides and the resulting mean θ values were used.

Seed coating – *Phaseolus vulgaris* seeds were surface sterilized with 50% bleach for 3 minutes, rinsed in H_2O three times and left to air dry. GFP-CIAT 899 was grown overnight to an OD600 measurement of 1. 80 ml of GFP-CIAT 899 was centrifuged at 4200 rpm in a Eppendorf (Hamburg, Germany) centrifuge 5910 R. The supernatant was discarded and 8ml of 6 dry wt% silk fibroin-trehalose (1:3) suspension was added to the spun down GFP-CIAT 899. Air-dried seeds were then dipped into this solution for 120 seconds, taken out and left to dry. After drying, the seeds were planted at the 24-hour mark. When water annealing post-processing was applied, seeds were placed in a vacuum chamber with H_2O, sealed for 6 hours to anneal and planted at the 24-hour point.

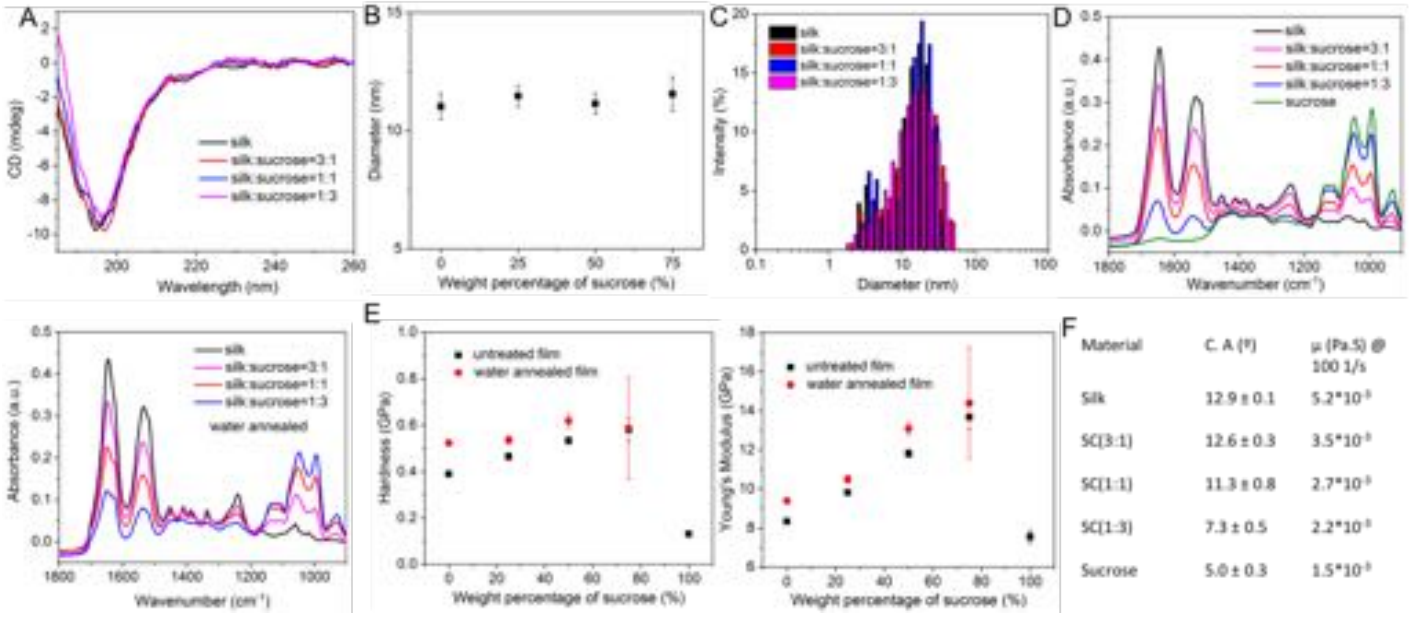
Encapsulation of bacteria on seed models – 50 seed models made by borosilicate glass beads (diameter= 5 mm, VWR, Radnor, PA, USA) were coated and air-dried. Once coatings dried, the glass beads were dropped into a measuring tube with 20ml of 1X phosphate buffer solution (Thermo Fisher scientific, Waltham, MA, USA). The solution was mixed, diluted and plated on agar plates for colony counting. For glass bead fluorescence imaging, beads were imaged under UV light and glass bead fluorescence imaged for the following conditions: uncoated, dip coated with silk fibroin, dip coated with silk fibroin and GFP-CIAT 899 and spray dried with silk fibroin and GFP-CIAT 899. The glass beads were left to air-dry.

Planting – Twelve square pots per experimental run were used and two seeds planted per pot. Four experimental runs were conducted with control (surface sterilized seeds) and GFP-CIAT 899 coated seeds. Two conditions were used, non-saline (4 ds/m) and saline (8 ds/m). Artificial

salinity was created by adding 12g of NaCl into 1.2 litres of water, which was then mixed with 650g of soil. Salinity was measured with a salinity meter. The plants were then watered every other 3rd day. Plant heights and root lengths were then recorded at week 1 and week 2 after germination.

Fluorescence microscopy – Root nodules were imaged under GFP fluorescence with a Nikon Eclipse TE2000-E to confirm GFP-CIAT 899 root nodulation.

Figure S3.1



Material	C. A (°)	μ (Pa.S) @ 100 1/s
Silk	12.9 ± 0.1	$5.2*10^{-3}$
SC[3:1]	12.6 ± 0.3	$3.5*10^{-3}$
SC[1:1]	11.3 ± 0.8	$2.7*10^{-3}$
SC[1:3]	7.3 ± 0.5	$2.2*10^{-3}$
Sucrose	5.0 ± 0.3	$1.5*10^{-3}$

Figure S3.1. Physical analysis of dried silk film, sucrose film and their mixture films. (A) CD of air-dried films (B) DLS intensity average diameter (C) DLS size distribution (D) FTIR spectra of films (E) Mechanical properties of films using nanoindentation (F) Contact angle and viscosity table of solutions. S=silk, C=sucrose, SC(3:1)=silk:sucrose=3:1.

Figure S3.2

A

Material	C. A (º)	μ (Pa.s) @ 1/100 1/s	Thickness (mm)
Silk	15.9 ± 0.1	$5.2*10^{-3}$	0.118 ± 0.004
ST(3:1)	12.9 ± 0.3	$3.6*10^{-3}$	0.125 ± 0.006
ST(1:1)	10.7 ± 0.2	$3.0*10^{-3}$	0.123 ± 0.005
ST(1:3)	7.9 ± 1.0	$2.2*10^{-3}$	0.116 ± 0.003
Trehalose	4.7 ± 1.0	$1.2*10^{-3}$	N/A

B

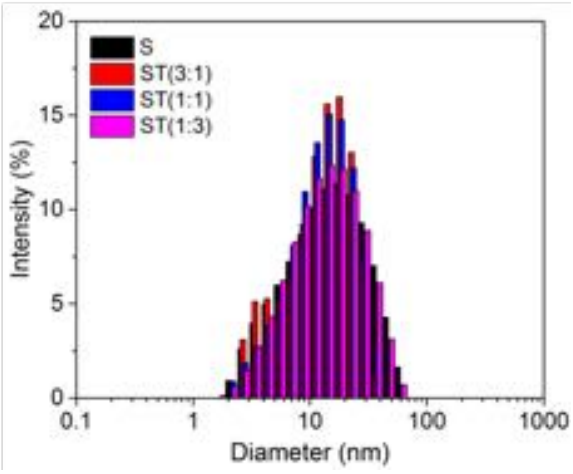


Figure S3.2. Physical analysis of dried silk film, trehalose film and their mixture films. (A) Viscosity and contact angle of solutions (B) DLS size distribution. S=silk, T=trehalose, ST(3:1)=silk:trehalose=3:1 relative dry weight concentration.

Figure S3.3

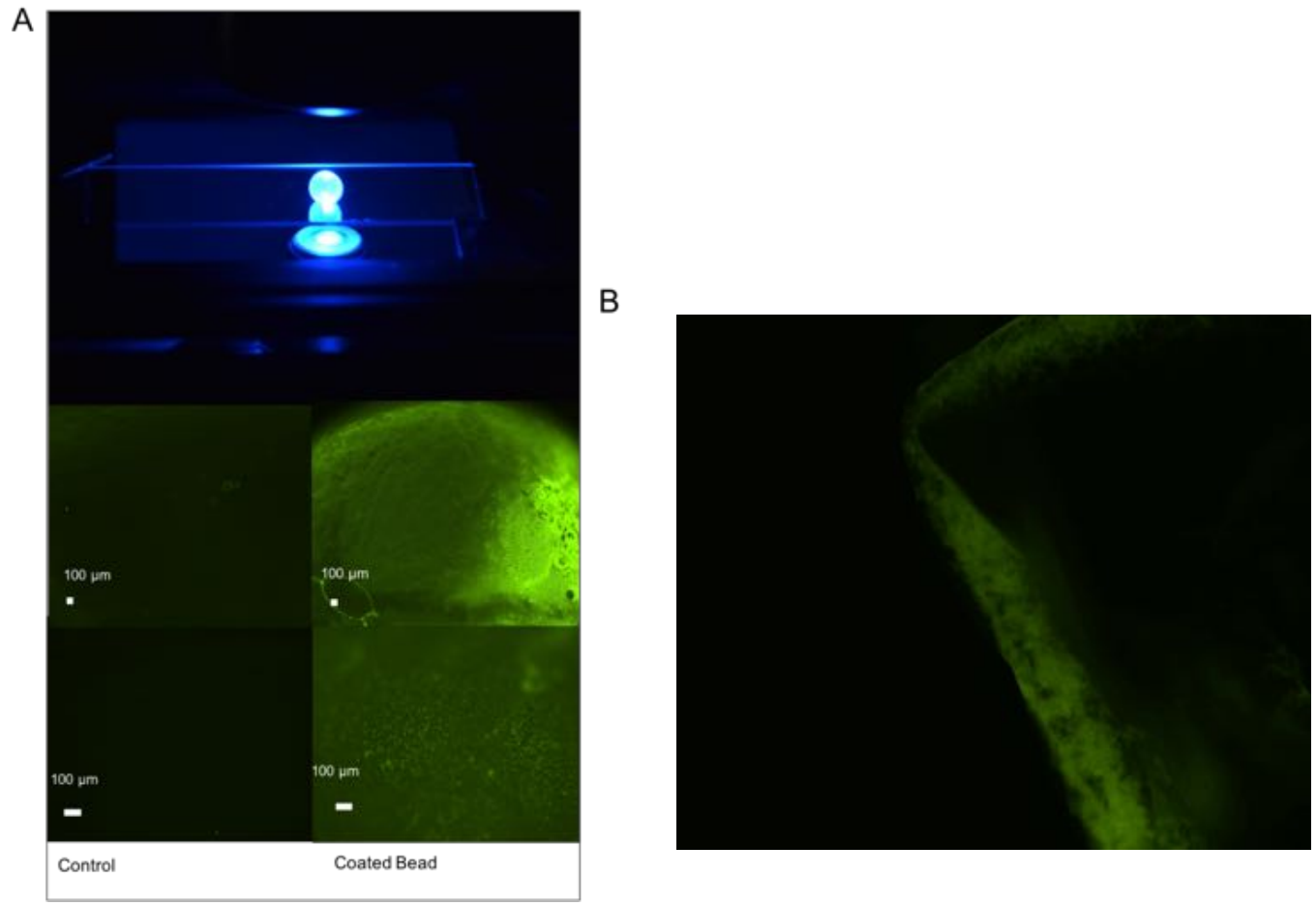


Figure S3.3. (A) GFP-CIAT 899 coated glass beads setup (B) Framework of the video depicting diffusion of CIAT 899 embedded in ST(1:3) films in artificial soil (phytagel).

Figure S3.4

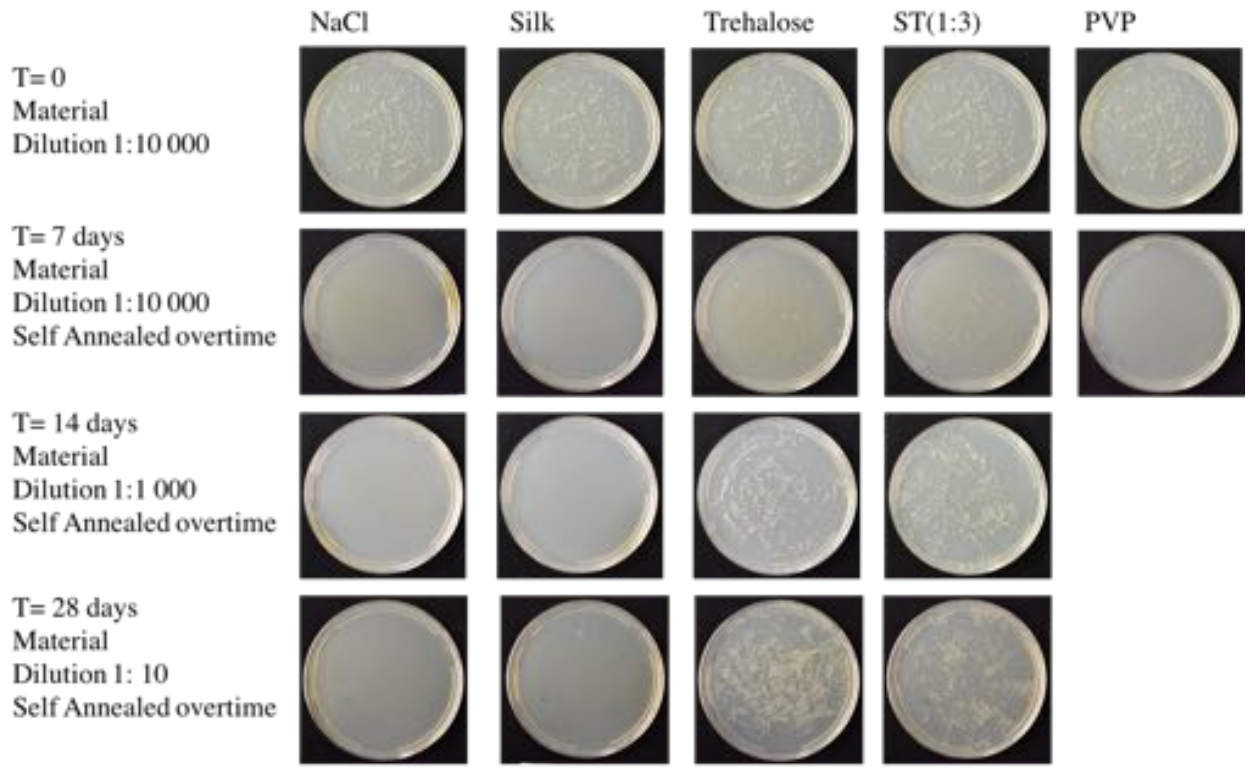


Figure S3.4. Selected agar colony counts for stability of CIAT 899 in silk film, trehalose film and their mixture films after storage at 23 ℃ for 4 weeks at 25% humidity.

Figure S3.5

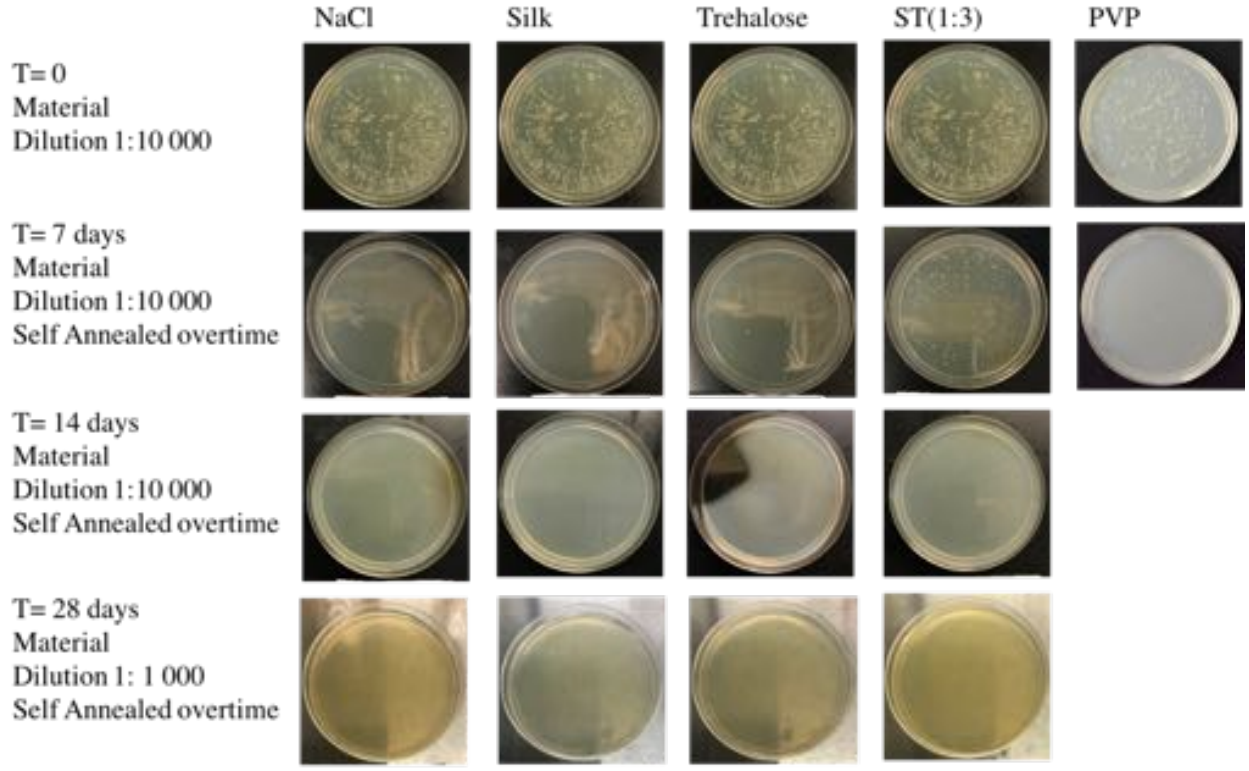


Figure S3.5. Selected agar colony counts for stability of CIAT 899 in silk film, trehalose film and their mixture films after storage at 23 ℃ for 4 weeks at 50% humidity.

Chapter 4
Programmable Design of Seed Coating Function Induces

Water-Stress Tolerance in Semi-Arid Regions

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4.1 Abstract

In semi-arid regions, water stress during seed germination and early seedling growth is the highest cause of crop loss. In nature, some seeds (e.g. chia, basil) produce a mucilage-based hydrogel that creates a germination-promoting microenvironment by retaining water, regulating nutrients entry, and facilitating interactions with beneficial microorganisms. Inspired by this strategy, a two-layered biopolymer-based seed coating has been developed to increase germination and water stress tolerance in semi-arid, sandy soils. Seeds are coated with a silk/trehalose inner layer containing rhizobacteria and with a pectin/carboxymethylcellulose (CMC) outer layer that upon sowing reswells and acts as a water jacket. Using Phaseolus vulgaris (common bean) cultured

under water stress conditions in an experimental farm in Ben Guerir, Morocco, the proposed seed coating can effectively deliver rhizobacteria to form roots nodules, results in plants with better

4.2 Introduction

In semi-arid regions, which constitute circa 15% of the world's land, water is the determining factor for crop production[222] and water stress during seed germination and early seedling growth is the highest cause of crop loss,[223] with dramatic impact on food security for 1 billion people that are threatened by desertification and already live in conditions of malnutrition.[176]

For semi-arid soils, water-holding compounds like hydrophilic and superabsorbent polymers can be applied to seeds, mixed into the soil or deposited on roots before planting to increase water retention and usage efficiency.[224] However, the application of these polymers is labor and energy intensive and often results in the release of synthetic plastics in the soil.[225] As a complementary approach, plant-growth promoting rhizobacteria (PGPRs) can be used to enhance plant health in conditions of water scarcity.[226,227] PGPRs are biofertilizers that interact with plant roots to increase availability of nutrients and phytohormones and enhance plant response to heat, saline soil and drought.[228] Some PGPRs, like rhizobia, can infect legume roots to form symbiotic nodules that fix nitrogen, limiting the use of fertilizers and enhancing plant health in semi-arid regions.[229] Nonetheless, the use of PGPRs is limited by their low resistance to desiccation stress[217,230] and competition upon resuscitation with a diversity of microorganisms present in the soil environment (i.e. spermosphere)[100]. Together, these limitations make difficult the integration of rhizobia in simple delivery systems – as seed coating technologies – that do not require the use of skills, agricultural practices and resources often not available in semi-arid regions of the world.[34]

In nature, polysaccharides present in the seed coat of myxospermous species occupying arid environments (e.g. *Salvia hispanica*, *Ocimum basilicum* and *Plantago ovata*) swells and extrudes, upon sowing, a halo of mucilage that completely envelops the seed.[231–233] The extruded mucilage generates a growth-promoting spermosphere that retains water, regulate nutrients entry, and facilitates interactions with PGPRs.[231] Seed mucilage is of increasing interest for the pharmaceutical, biomedical and food industry and it is usually a composite of pectic, non-cellulosic, and cellulosic polysaccharides.[234] In this study, we have been inspired by the multifunctional coat of myxospermous seed to develop a seed coating technology with programmable function that creates a spermosphere that positively affects the seed niche and promotes water stress-resistance in semi-arid soils.

4.3 Results

4.3.1 Design of the seed coating

The coating consists of a two-layered structure (Fig. 4.1). An SEM image of the dry two layered coating deposited on the surface of a *Phaseolus vulgaris* seed is depicted in Fig. 4.1a. The inner layer (Layer 1) is made of a 1:3 by weight mixture of silk fibroin and trehalose that contains *Rhizobium tropici* CIAT 899 – referred to as *R. tropici* CIAT 899 onwards. Silk fibroin is a 395kDa structural protein that is extracted from the *Bombyx mori* silk cocoon with a yield of 70-75% and is the main component of the silk textile fibers.[84,188] Through a water-based process applicable also to silk waste – cocoons unsuitable for reeling, yarn waste and garneted stock – silk fibroin can be regenerated in a water suspension and then easily applied to form coatings through dip-coating and spray-coating techniques.[235] The physical, mechanical, biological and biodegradation properties of silk fibroin can be modulated by controlling the protein secondary

and tertiary structures (random coil, alpha helix and beta-sheet) at the point of material assembly or with post-processing techniques (silk fibroin polymorphism).[236–240] Silk fibroin is also known to preserve biological entities and biomolecules encapsulated in silk-based materials by providing a barrier to oxygen stress and minimize contact with water.[195] Trehalose is a disaccharide ubiquitously used by natural organisms to impart osmoprotection and that can act as carbon source for rhizobium.[83,198,201] The mixture of silk fibroin and trehalose enables Layer 1 adhesion to the seed coat, preservation of *R. tropici* CIAT 899 by mitigating oxidative and osmotic stresses, and release of the biofertilizer upon sowing,[83,195,241] Given Layer 1 coating thickness (t), an ellipsoidal seed shape (a, b, c), the known concentration of *R. tropici* CIAT 899 (C_a) and assuming a homogeneous dispersion of *R. tropici* CIAT 899 and the formation of a homogeneous coating, it is possible to estimate the number of *R. tropici* CIAT 899 in the inoculum (N) by multiplying C_a with the volume (V) as shown in equation (1).

$$N = C_a \times V = C_a \times \left(\frac{4}{3}\pi ABC - \frac{4}{3}\pi abc\right) \qquad (1),$$

where A=a+t, B=b+t, and C= c+t. Using a = 0.25 cm, b = 0.25 cm, c = 0.5 cm, t = 0.000 5cm, and $C_a = 10^{10}/cm^3$, then N = $6.56*10^6$ *R. tropici* CIAT 899 were encapsulated per seed. The external layer (Layer 2) is a mucilage-like mixture of pectin-carboxymethyl cellulose (P:C 1:1) that contains nutrients and upon sowing forms a hydrogel that acts as a water jacket and provides a suitable environment for rhizobia resuscitation and growth (Fig. 4.1a-b). Layer 2 was designed as food gel[242] and contains Ca^{2+} ions that act as crosslinker for pectins' galacturonic acid residues, providing stability to the gel and yielding a gel content of circa 65% (Fig. 4.1c). CMC molecules present in Layer 2 fill the gaps in the pectin network and confer water superabsorption properties and enhance water retention. In Supplementary Fig.4.1, we report the investigation of P:C hydrogels volume variation in 154 mM NaCl solution due to water absorption and gel

content (GC) as a function of relative pectin and carboxymethyl cellulose (CMC) content and of increasing Ca^{2+} concentrations. Volume variation upon re-hydration and GC of pectin, CMC and their mixtures indicated that P:C 1:1 provides the best performance as a trade-off between volume variation (indication of water uptake) and GC properties (indication of gel stability over time). The effects of biologically-relevant low, medium and high Ca^{2+} concentrations in P:C gels (5 mM or LCC, 10 mM or MCC and 15 mM or HCC) were then investigated, given the strong beneficial effect of the dication on rhizobia symbiosis with leguminous plants[243]. P:C 1:1 volume increased in the first three hours upon rehydration, decreased at the 6 h time point, recovered and plateaued at 12 h (Fig. 4.1e). The decrease in volume at 6 h was similar for all the materials considered and could be explained with the wash-off of non-crosslinked CMC molecules. Previously reported maximum swelling of pectin hydrogels crosslinked with glutaraldehyde matched the results obtained in this study,[244] though the use of toxic crosslinkers should be avoided for agricultural applications. Further, studies of biodegradable hydrogels used to modify soil water holding capacity have reported water absorption in the same order of Layer 2.[245] Water retention is another important parameter to consider in semi-arid soils as the hydrogels can stabilize the humidity around seeds for extended periods of time, acting as a water buffer in-between watering periods[246]. The measurement of volume variation during air-drying indicated that all the P:C gels considered had similar water loss trends, but HCC and MCC gels could retain water longer than LCC, given the higher initial volumes (Fig. 4.1f). The use of different pectin to CMC ratio in P:C and lower concentrations of Ca^{2+} ions may further be used to regulate water retention (Supplementary Fig. 4.2). Water absorption studies for gels were also conducted in soil, to simulate how Layer 2 would swell upon sowing. Volume variations of gels were measured after 24h in three soil water content (SWC) conditions (Fig. 4.1g). Gels' volume

significantly increased with SWC ($p<0.05$), but Ca^{2+} ions did not have statistically significant effect on the swelling for the concentrations considered ($p>0.05$). Interestingly, an order of magnitude was lost in P:C water uptake, when compared to swelling in saline solution, probably due to the lower water potential of soil and the compressive forces that soil applies on the gel and that limit swelling. However, even in semi-arid soils (SWC=10%), volume variation around 250% indicated the capability of the coating to extract water from the environment and make it available to the rhizobacteria and the seed. A pressure of circa 412 Pa is applied on the surface of a *P. vulgaris* seed (1.4x0.8x0.7 cm) sowed 3 cm below the surface of a semi-arid soil ($\rho_{avg}\cong 1.4$ g/cm^3). Additionally, soil has a compression modulus of circa 4-7 MPa[247], indicating that the hydrogels may have complex interactions with the surrounding soil while reswelling occurs as a combination of soil deformation and expansion in air pockets present in the soil.[248]

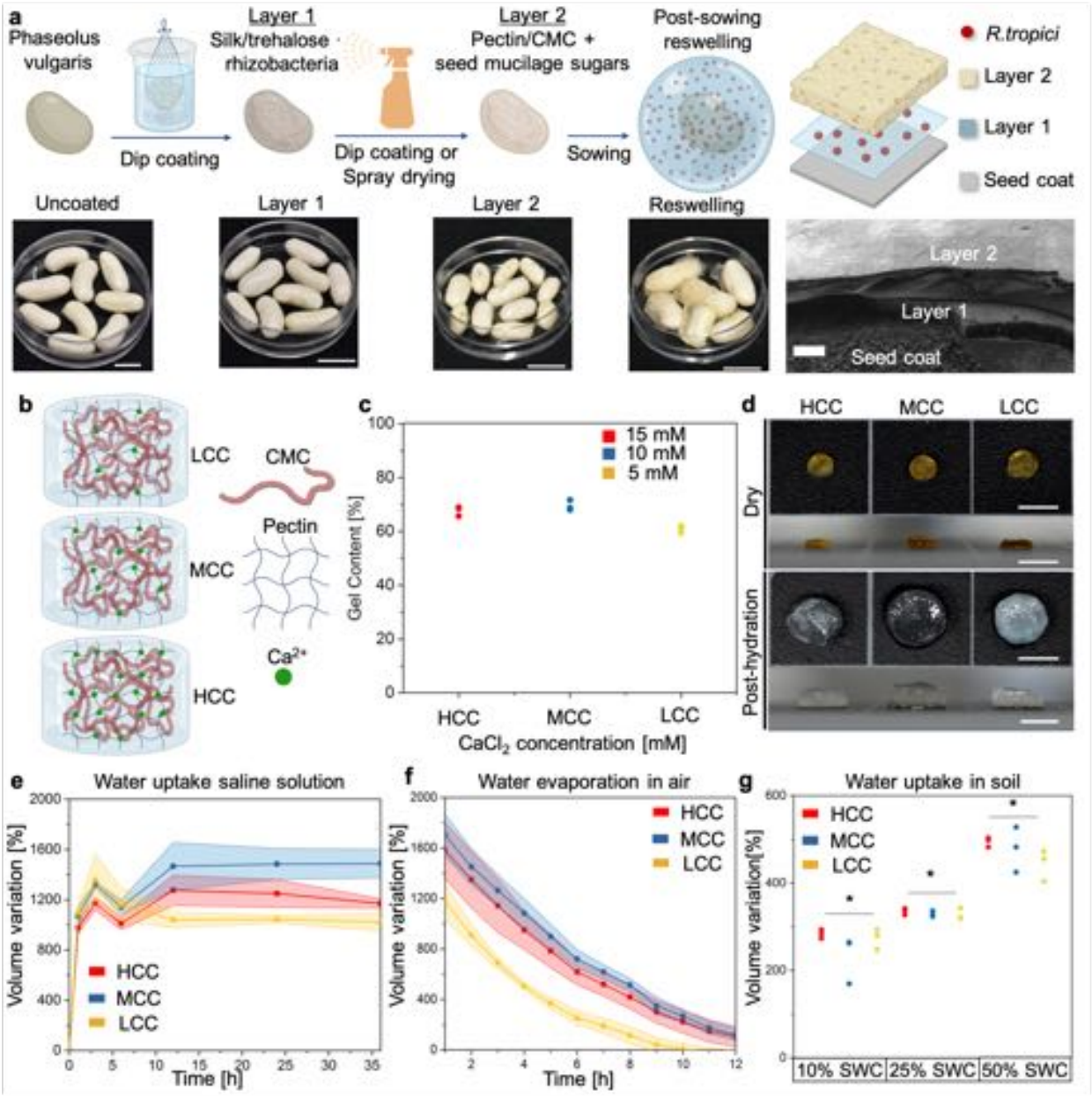


Fig. 4.1. Material design, fabrication and selection. a) Schematic diagram of the two-layered seed coating fabrication process, relative pictures of *P. vulgaris* coated seeds and crossection of coated seeds. Layer 1 contains a 1:3 mixture of silk-trehalose that adheres on the silk coat, encapsulates, preserves and releases *R. tropici* CIAT 899. Layer 2 is made of a 1:1 mixture of pectin-CMC (P:C). When the seed is watered, Layer 2 swells into a hydrogel and hydrates Layer 1, which dissolves and releases *R. tropici* CIAT 899. The hydrogel provides an appropriate environment for *R. tropici* CIAT 899 resuscitation and growth. Scale bars fo pictures correspond to 10 mm. Scale bar for SEM image of the coating is 10 □m b) Schematic of the pectin-CMC hydrogel structure, where Ca^{2+} are used to crosslink pectin chains while CMC acts as a filler to enhance water uptake. c) Effects of high, medium and low Ca^{2+}concentrations (HCC, MCC and LCC) on gel content. Stars above bars indicate a statistically significant difference in the mean of

a material ratio group compared to all other groups (* $p<0.05$). The black error bars represent the standard error of the mean. d) Representative images of dry and swollen (12h in 154 mm NaCl solution) hydrogels. Scale bars=10 mm. e) Water uptake over time of dried P:C in 154 mm NaCl solution. f) Water evaporation over time of hydrogels after a 12h immersion in 154 mm NaCl solution. Highlighted areas around curves correspond to the standard error of the mean. g) Water uptake of P:C hydrogels in soils of increasing moisture content after 24h. Stars above bars indicate a statistically significant difference in the mean swelling ratio of a soil humidity group compared to all other groups (* $p<0.05$). No statistical significant difference was found in the mean swelling ratios between $CaCl_2$ concentrations at similar soil humidity. The black error bars represent the standard error of the mean.

4.3.2 Mechanical Properties and Gel Microstructure

Rheological measurements help to choose the required settings for material application onto seed surface. Rheological investigation of LCC, MCC and HCC solutions (i.e. before gelling is triggered) showed a shear thinning behavior (Supplementary Fig. 4.3) and the stability of the solutions' storage and loss modulus (G' and G'', respectively) (Fig. 4.2a) over time upon addition of Ca^{2+} ions into the P:C suspension. Exposure of P:C suspension to NaOH, causes a rapid increase in pH that results in immediate gelation of LCC, MCC and HCC. The rapid gelation hinders the application of the Winter-Chambon rule to calculate gelation time. Nonetheless, the evolution of G' and G" post gelling was monitored over time (Fig. 4.2b) and showed an increase of G" that was positively correlated with Ca^{2+} concentration. To further evaluate the suitability of P:C gels to work as seed coating, we measured the mechanical properties of P:C 1:1 hydrogels through unconfined compression tests (Fig. 4.2c). MCC hydrogels displayed both the highest compressive strength (5.43 ± 0.31 kPa) (Fig. 4.2d) and Young's modulus (33.86 ± 4.60 kPa) (Fig. 4.2e). Nanoindentation tests were also conducted on dry P:C to determine their capability to sustain transportation and storage periods without being damaged. The measured Young's modulus (~ 15-20 GPa, Fig. 4.2e) and hardness (~1-1.5 GPa, Fig. 4.2f) were of the same order of currently available seed coatings.[182] Cross-sectional cryo-

SEM was used to evaluate the microsctructure of the hydrogels. Micrographs showed an interconnected microstructure with an assumed pore size of ~ 5 µm (Supplementary Fig. S4.4). However, the pore shape and dimension may have been affected by the formation of ice crystals during the sample preparation, which is known to artificially increase the pore size, particularly in hydrogels with a weak structural integrity such as LCC.[249]

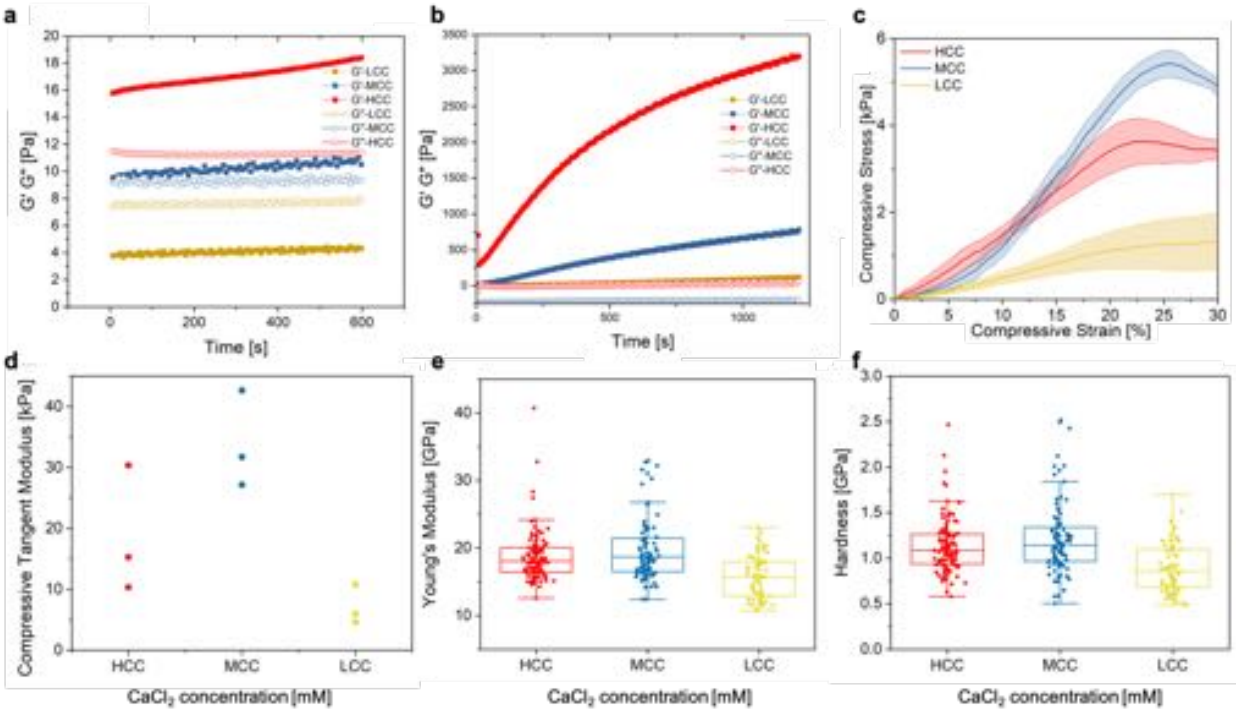


Fig. 4.2. Mechanical characterization of P:C hydrogels. a) Storage (G') and loss modulus (G") prior to NaOH addition. b) Storage (B') and loss modulus (G") after NaOH addition (last step in hydrogel fabrication process). c) Unconfined compression of P:C post hydration for 12h in 154 mm NaCl solution. Highlights around the curves correspond to the standard error of the mean. d) Calculated compressive tangent Young's moduli post hydration for 12h in 154 mm NaCl solution. Statistical significant difference in the mean Young's moduli of MCC compared with LCC (* $p<0.05$). e) Young's moduli on dry P:C hydrogels measured from nanoindentation studies. No significant statistical difference was measured in the young's moduli of dried materials. For HCC, MCC, LCC Upper quartile = 20.0, 21.4, 17.9; Lower quartile = 16.4, 16.6, 12.9; Median = 18.1, 18.7, 15.7; Mean = 18.8, 19.8, 15.7; Min = 12.6, 12.4, 10.8; Max = 40.7, 32.9, 23.0 f) Hardness of dry P:C 1:1 measured with nanoindentation. No significant statistical difference was measured in the hardness of dried materials. For HCC, MCC, LCC Upper quartile = 1.26, 1.34, 1.10; Lower quartile = 0.94, 0.97, 0.68; Median = 1.09, 1.14, 0.86; Mean = 1.13, 1.22, 0.90; Min = 0.58, 0.50, 0.49; Max = 2.47, 2.52, 1.70. Boxplots show the median (horizontal line), 25th and 75th percentiles (lower and upper boundaries, respectively). Whiskers extend to data points that lie within 1.5 interquartile ranges of the 25th and 75th quartiles; and observations that fall outside this range are displayed independently.

4.3.3 The effectiveness to support rhizobacteria growth

To investigate the effectiveness of P:C hydrogels as niche to support *R. tropici* CIAT 899 resuscitation and growth, we designed two experimental set-ups. In the first study, *R. tropici* CIAT 899 were released from dissolving silk films into swelling P:C 1:1, as shown in Fig. 4.3a. In particular, we used *R. tropici* CIAT 899 harboring a green fluorescent protein reporter (*R. tropici* CIAT 899-GFP) to use the fluorescent intensity signal over time as an indication of bacterial colonization and growth. After verifying that *R. tropici* CIAT 899-GFP could use seed mucilage as carbon source (using simulated basil mucilage as an example, Supplementary Fig. 4.5), we investigated the migration of *R. tropici* CIAT 899-GFP from silk film into P:C 1:1 formed at increasing concentrations of Ca^{2+} (Fig. 4.3a) and containing nutrients found in seed mucilage (*i.e.* 20.9 mM (D-(+)-xylose, 6.28 mM L-(+)-arabinose, 6.28 mM DL-arabinose, L-8.94 mM rhamnose with a ratio of 30:9:9:14)).[250]

The release of *R. tropici* CIAT 899-GFP from silk films and the subsequent P:C colonization was investigated using fluorescent microscopy (Fig. 4.3b)[251]. Silk films were dissolving gradually over time while green spots, corresponding to *R. tropici* CIAT 899-GFP microcolonies, were growing in size and numbers within the depth of the hydrogels. *R. tropici* CIAT 899-GFP microcolonies were more concentrated at the surface of the LCC and HCC gels at day 7, while *R. tropici* CIAT 899 -GFP colonization was more homogenously distributed in MCC samples. Interestingly, a size gradient could be observed in microcolonies present in MCC hydrogels, with larger colonies visible closer to the source of *R. tropici* CIAT 899-GFP. To further investigate hydrogel colonization from the environment, we designed a second study where dry P:C 1:1 were immersed in a solution containing *R. tropici* CIAT 899-GFP. GFP intensity in the hydrogels increased over time and plateaued at 32h (Fig. 4.3c). GFP intensity was

inversely proportional to the crosslinker content, i.e. the lower the content of Ca^{2+} ions the higher the GFP intensity. To determine the interplay between Layer 2 swelling and bacteria colonization, we immersed dry P:C 1:1 in a 154 mM solution containing *R. tropici* CIAT 899-GFP (OD_{600} ~0.1). Histological sections at 6h and 54h post re-hydration showed the presence of gram-negative *R. tropici* CIAT 899-GFP within the gels (Fig. 4.3d and Supplementary Fig. 4.6), suggesting that Layer 2 swelling may be able to recruit endogenous microorganism present in the soil and further attract them post-swelling due to the presence of nutrients in the hydrogel. To further support this assumption, diffusion of saccharides in the hydrogel was studied using fluorescein as a working model. Calculated diffusion coefficients were ~10^{-5} cm^2/s and not influenced by the amount of crosslinks present in the hydrogels (i.e. Ca^{2+} concentration during material fabrication) (Fig. 4.3e, Table 4.1).[252] This result suggests that the rate of diffusion of nutrients was not the determining factor of *R. tropici* CIAT 899-GFP growth, but other features such as different pore sizes and geometries may have caused an increased colonization in MCC gels.[253,254]

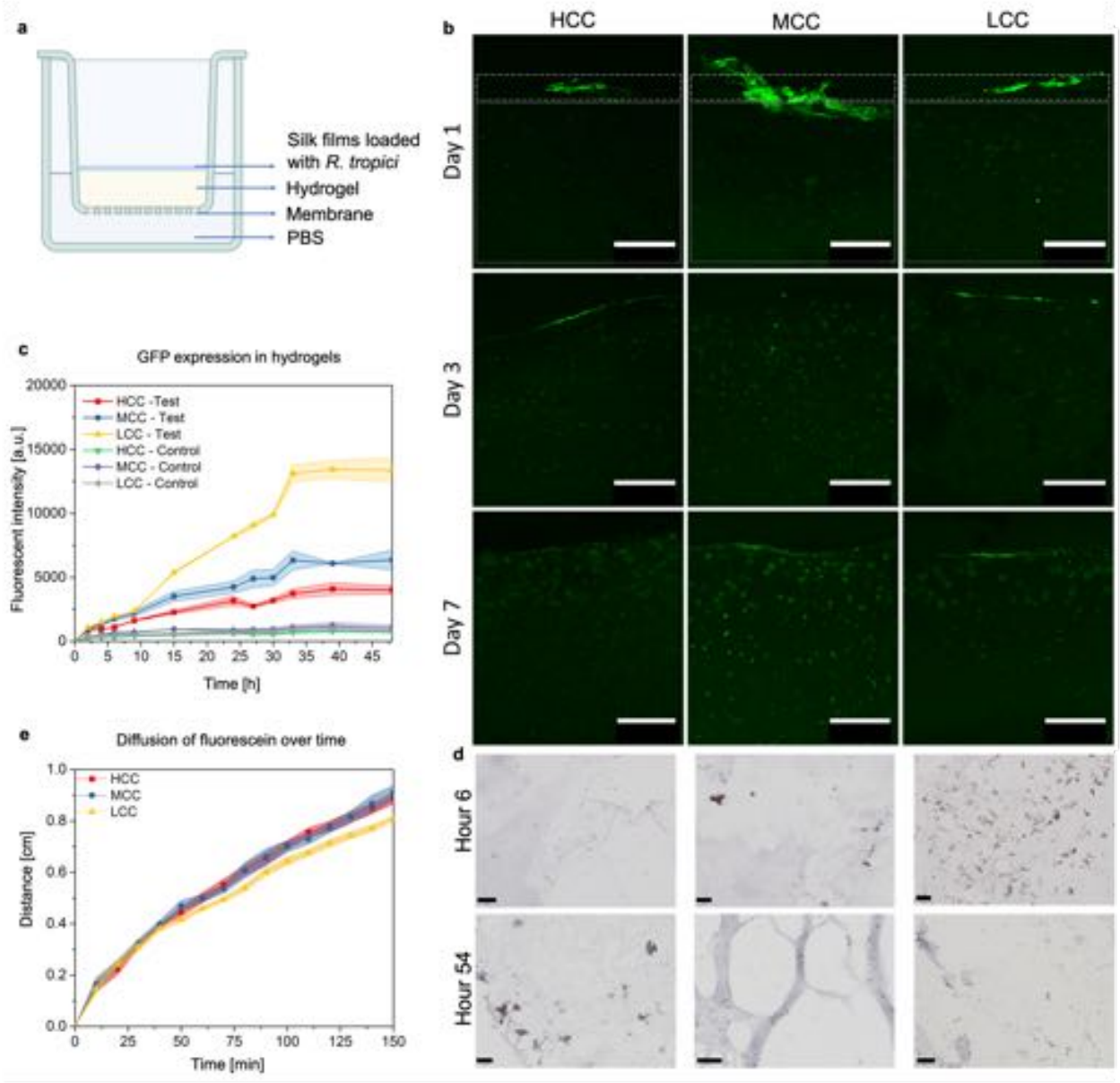


Fig. 4.3. Use of P:C hydrogels as niche to grow *R. tropici* post rehydration. a) Experimental setup showing *R. tropici* CIAT 899 released from silk films, migrating and growing in P:C hydrogels simulating dry to swollen states. b) Representative confocal cross-sections images showing microbe migration and growth in hydrogels, simulating post-sowing phenomena in soil. Dotted box highlights applied silk film location. Setup of experiment is shown in (A). Scale bars represent 500 µm. c) *R. tropici* CIAT 899-GFP expression in hydrated hydrogels, using the polysaccharides as only energy source. Highlighted areas around curves correspond to the standard error of the mean. d) Representative histology sections of the hydrogels used as only energy source, showing attraction of microbes in the hydrogels. Scale bars represent 20 µm. e) Diffusion of fluorescein in hydrogels to model movement of nutrients/sugars.

Medium	Diffusion Coefficient (Fluorescein) [cm^2/s]
HCC	$3.32 \cdot 10^{-5} \pm 8.83 \cdot 10^{-7}$
MCC	$4.00 \cdot 10^{-5} \pm 1.31 \cdot 10^{-6}$
LCC	$3.96 \cdot 10^{-5} \pm 1.38 \cdot 10^{-6}$
Water	$4.20 \cdot 10^{-6}$

Table 4.1. Calculated diffusion coefficients.

4.3.4 Coating biodegradation

The biodegradation of the seed coating was evaluated by exposing MCC hydrogels to soil. Biopolymers degradation in the absence of light is mostly catalyzed by microbial activity through enzymatic degradation that accelerate proteolytic and carbohydrolytic processes. Important parameters such as type of indigenous microorganisms, temperature, soil type, composition, pH, salinity, water and carbon contents play prominent roles in determine how quickly biopolymers may be degraded. Fig. 4.4a depicts the mass loss of MCC hydrogels over time when investigated at 16°C in a soil containing microorganisms. At day 14 and 28, almost 50% and 70% of the dry weight of the gel was lost. When exposed to soil containing sodium azide – a bacteriostat – the mass loss rate of MCC decreased for the first 14 days, when compared to untreated soil, indicating the critical role of microorganism in biopolymer degradation in soil. For longer time points (up to day 28), the mass loss increased and reached values similar to the one measured for untreated soil, probably indicating the inefficacy of the sodium azide treatment in the long term. Biodegradation studies were also conducted in untreated soil at 25°C and resulted in the complete biodegradation of LCC, MCC and HCC materials in less than 30 days (Supplementary Fig. 4.7). Biodegradation in sterilized soil only resulted in less than 50% of mass loss over a period of 30 days. The complete biodegradation of

the coating within the life cycle of the plant is an important feature to minimize the environmental impact of agriculture and the release of pollutant in the soil. Recently, policymakers have promoted new laws that strictly regulate the biodegradation of polymers released in the environment for agricultural application. For example, in January 2018 the European Chemicals Agency (ECHA) examined the need for an EU-wide restriction on the use of intentionally added microplastic particles (IAMPs) in products placed on the EU market, including food and agriculture, with non-biodegradable microplastics forecasted to be banned in 2025[255].

4.3.5 Seedling germination and growth in semi-arid conditions

All together, these results suggest that the seed coating properties may be programmed by varying several parameters including relative P:C concentration, amount of Ca^{2+} during materials fabrication and presence of nutrients to design an environment that can support resuscitation and growth of plant growth promoting rhizobacteria. In particular, for the purpose of our study, we selected MCC as the hydrogel of choice to conduct germination studies, given the combination of homogenous *R. tropici* CIAT 899 colonization, water absorption and mechanical properties. In a preliminary study, germination of *P. vulgaris* seeds was tested in a greenhouse setting by applying the following treatments: i) no treatment (negative control), ii) inoculation with 3% poly(vinylpyrrolidone) (PVP) solution with *R. tropici* CIAT 899-GFP (positive control), iii) bilayer seed coating with Layer 2 applied via spray-coating, and iv) bilayer coating with Layer 2 applied via dip-coating. Seedlings were checked for nodulations at day 14 after germination, to ensure successful delivery of rhizobacteria and root colonization (Fig. 4.4b). A 100% nodulation rate was observed for all three procedures where *R. tropici* CIAT 899 were added to the soil.

Application of Layer 2 both via spray-coating and dip-coating resulted in roots that were more developed when compared to the positive control, suggesting that the seed enhancement technology outperforms current standard materials and can be applied with tools largely available by growers.

To further test the efficacy of the two-layer coatings (L2) in mitigating environmental stressors typical of semi-arid regions, we conducted germination studies at the UM6P experimental farm in Ben Guerir, Morocco, using native soil (Fig. 4.4c). Soil analysis revealed that a composition typical of sandy soils, slightly alkaline and prone to induce drought stress due to limited water retention capacity. The soil was rich in nitrogen and phosphate and concentrations of oligoelements such as manganese and zinc were sufficient, while there was a slight deficiency in copper content (Supplementary Table 4.1). To investigated the effectiveness of L2 to induce water-stress tolerance, we exposed L2-coated *P. vulgaris* seeds to soils with a decreasing water potential Ψs), by altering the watering conditions to induce water stress regimes (Fig. 4.4c). Seeds with no coating (C) and seeds coated only with Layer 1 (L1) were used as control and one-way ANOVA test with Bonferroni's correction was used to analyze the data collected. When comparing the different watering conditions in 150 g of soil per plant, it was observed that for mild (Ψs = -12 kPa) and severe (Ψs = -20 kPa) water-stressed conditions, L2 coating statistically significantly influenced germination and plant health, when compared to the two controls. L2 seeds resulted in shoot dry mass that were higher, respectively, when compared to C and L1 seeds for Ψs at -1kPa, -12 kPa and -20 kPa. Shoot length was not a factor that seemed to be determined by coating maybe because this was early on in the growth process. No statistical significant difference was found for shoot length between L2, L1 and C seeds for Ψs = -20 kPa. It was also observed that root architecture was significantly affected by seed and water

treatments. Seeds germinated in soil with Ψs = -1 kPa and Ψs = -5 kPa had the longest roots in comparison with seeds germinated in water stress conditions, i.e. Ψs = -12 kPa and Ψs = -20 kPa. Under water stress regimes, roots in L2 and L1 seeds were statistically significantly longer when compared to C seeds, indicating that the coating treatments provided a better environment for early root establishment. Measurement of chlorophyll content in leaves showed that watering regime but not seed coating affected the production of the green pigment. Measurement of total phenolic compounds (TPC) is correlated to drought stress as plants release phenolic compounds to mitigate water deficit.[256] TPC was statistically significantly lower for L2 seeds when compared to L1 and C seeds, indicating the contribution of the designed hydrogel to induce water-stress tolerance. Measurement of stomatal conductance corroborated this finding. *P. vulgaris* cultured in water stress regimes lower stomatal conductance to limit water loss.[257] In our experiments, L2 seeds showed statistically significant higher stomatal conductance for Ψs = -1 kPa, -5 kPa and -12 kPa, when compared to the L1 and C seeds and a higher stomatal conductance for Ψs = -20 kPa to C seeds, indicating a better tolerance to water deficiency. Altogether, these results indicate that the L2 coating positively influence plant establishment of *P. vulgaris* in a semi-arid soil and under water stress.

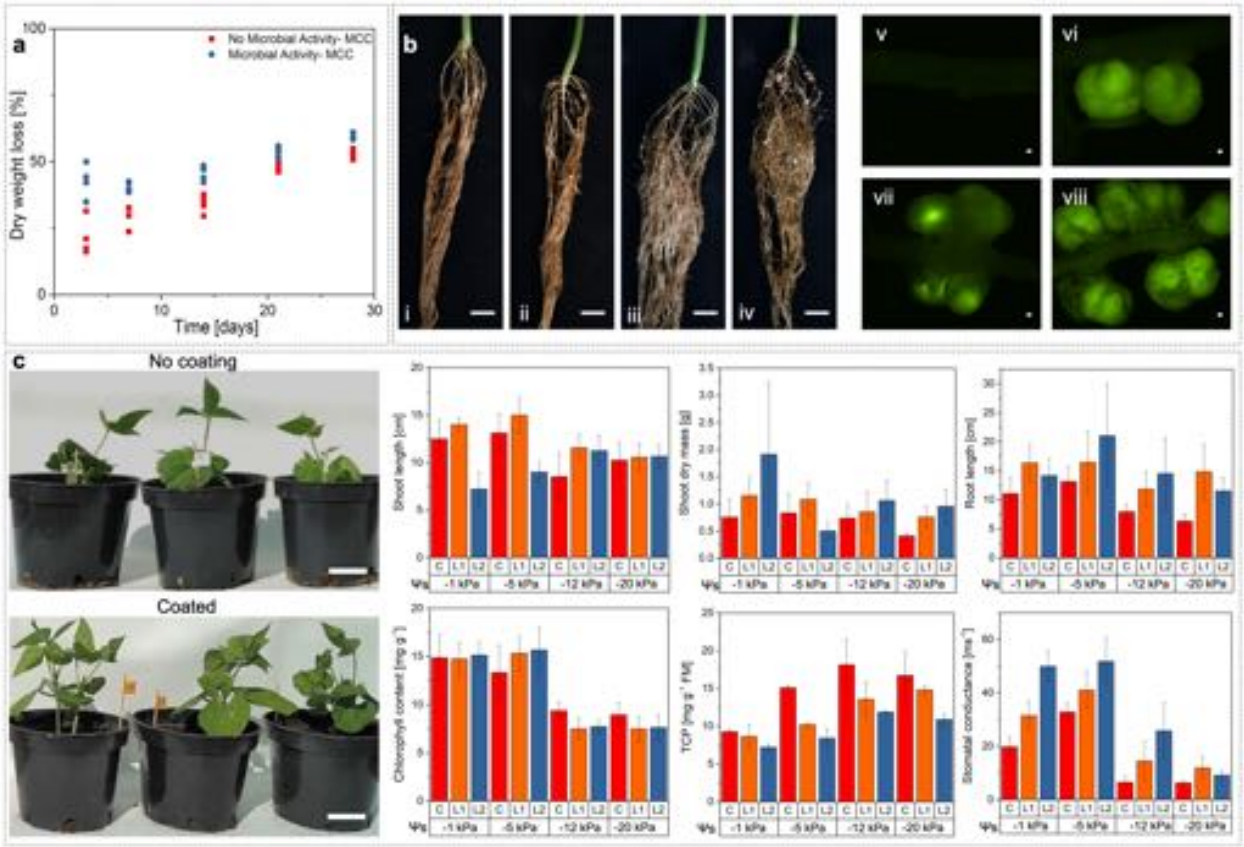


Fig. 4.4. Degradation in soil of seed coating material and application to *P. vulgaris* to mitigate water stress. a) Degradation of P:C hydrogel with soil microbe activity and without soil microbe activity in 25% humidity soil over a month at 16ºC. Dots correspond to collected data. b) Representative root images and corresponding fluorescent microscopy images of nodule formation for plants established from the following treatments: *i*) and *iv*) no coating and no inoculation; *ii*) and *vi*) inoculation of *R. tropici* CIAT 899-GFP using a 3% PVP solution; *iii*) and *vii*) bilayer coating with Layer 2 applied via spray-coating; and *iv*) and *viii*) bilayer coating with Layer 2 applied via dip-coating. Scale bars represent 10 mm for roots pictures and 100 µm for fluorescent microscope images. N=18 plants were tested per treatment type with a 100% nodulation rate for seeds treated with *R. tropici* CIAT 899. No nodulation was visible at day 14 for the negative control. c) Growth of *P. vulgaris* at week 6 in water stress regime for seeds that had no coating and that were coated with L1 and L2 coatings. Scale bars represent 10 cm. *P. vulgaris* establishment investigation is shown as a function of coating and water potential (Ψs) levels. Ψs = -1kPa and -5 kPa correspond to healthy soil moisture content. Ψs = -12kPa and -20 kPa represent mild and severe water stress conditions, respectively. *P. vulgaris* plant establishment has been investigating by measuring shoot length, shoot dry mass, root length, chlorophyll content, total phenolic content (TPC) and stomatal conductance. Error bars represent standard deviation; five repeats were used per analysis and condition.

4.4 Discussion

In this study we investigated the use of a biomaterials and drug delivery approach to engineer a programmable seed coating technology for effective delivery and growth of rhizobacteria in the spermosphere. Activated upon sowing, the two-layered coating technology enabled the resuscitation and self-replication of rhizobia within a biopolymer-based hydrogel that resembles seed mucilage and resulted in the formation of microbial colonies that formed symbiotic nodules with plant roots and induced water stress tolerance in semi-arid conditions. Furthermore, the use of biopolymers generally used in food gels provide a largely available, cost-effective and non-toxic solution to mitigate abiotic stress. Together, these findings open the door to the use of enhanced seed coating technologies to address specific weather and soil conditions, to adapt agriculture to changes in climate patterns while also minimizing the use of scarce and energy-intensive inputs.

4.5 Materials and Methods

Materials

Materials fabrication, hydration and dehydration studies are reported below. Extensive details of the experimental procedures are reported in Supporting Information

Hydrogel fabrication: Four types of gels were prepared with different ratios of low-methoxylated pectin (P) from citrus peel (> 74% galacturonic acid, > 6.7% methoxy groups, Sigma-Aldrich, St. Louis, MO) to NaCMC (C) (molecular weight 90,000 g/mol, degree of substitution 0.7, Sigma-Aldrich, St. Louis, MO). The tested P:C ratios were 1:0, 3:1, 1:1, and 1:3, but the total amount of polysaccharides was 5 wt% for all solutions. For the gel preparation, monomeric sugars (D-(+)-xylose 20.9 mM , L-(+)-arabinose 6.28 mM, DL-arabinose 6.28 mM, L-rhamnose 8.94 mM (Sigma-Aldrich, St. Louis, MO) with the ratio 30:9:9:14) were first added to

deionized (DI) water to mimic the ratio found in Basil seeds[250]. After complete mixing of the polysaccharides in the sugar solution, three calcium chloride ($CaCl_2$, Sigma-Aldrich, St. Louis, MO) concentrations were added to each mix from a 1 mM $CaCl_2$ stock solution in DI water: HCC (15 mM), MCC (10 mM), and LCC (5 mM). After leaving the solution for 24 hours, 70 mol% (mol percentage of polysaccharides) of OH- (from 2 M NaOH stock solution) was added to each gel, to increase the pH and lead to gelation, according to the egg-box model. 48 hours later, the samples were prepared and air-dried for 24 hours at RT. From those gels, the best P:C ratio was selected by analyzing both the water content over time and the GC. Only materials with selected P:C ratio were then optimized (pH and $CaCl_2$ concentrations). Indeed, because the pH is only important for pectin gelation and not NaCMC, the added NaOH amount had to be adjusted: 70 mol% of galacturonic acid (pectin's principal monomer) rather than 70 mol% of all polysaccharides. $CaCl_2$ concentrations subsequently needed to be increased for the mechanical integrity of the gel. Finally, full characterization was only done for the materials with the selected P:C ratio.

Mechanical properties

To evaluate the strength of wet samples, non-confined compression tests were conducted (5943 Instron, Norwood, MA) on round gel samples (n = 3, diameter 38 mm, thickness 16 mm). The strain speed rate was set to 100% min^{-1}, and the whole experiment was filmed, until a strain compression of 30%. Tangents to all compressive stress-strain curves were calculated at 5% strain, 10% strain, and 15% strain, to evaluate which tangents were fitting the best the linear (elastic) regions of each gel type.

Nanoindentation: Nanoindentaion measurements were performed on a Hysitron TriboIndenter with a nanoDMA transducer (Bruker, Billerica, MA). Samples were indented in load control

mode with a peak force of 500 μN and a standard load-peak hold-unload function. Reduced modulus was calculated by fitting the unloading data (with upper and lower limits being 95% and 20%, respectively) using the Oliver-Pharr method and converted to Young's modulus assuming a Poisson's ratio of 0.33 for all samples. Each type of sample was prepared and indented in triplets to ensure good fabrication repeatability. For each sample, indentation was performed at 3 locations a total of 36 points (6×6 grid with an increment of 20 μm in both directions) at each location to ensure statistical reliability of the modulus measurements.

Bacterial growth

To assess bacterial growth in the presence of the selected hydrogels, two experiments were conducted. For both, a growth media was prepared by mixing 5 g of BactoTM Peptone (BD Biosciences, Franklin Lakes, NJ), 3 g of yeast extract (Sigma-Aldrich, St. Louis, MO), and 10 mL of 0.7 M $CaCl_2$ solution to 1L of DI water. The following antibiotics (Sigma-Aldrich, St. Louis, MO) were then added: Rifampicin (25 mg/mL), Nalidixic acid (20 mg/mL), and Tetracycline (10 mg/mL). The media was sterilized by autoclaving for 50 minutes at 120°C. *R. tropici* CIAT 899 was grown in media overnight (28˚C, 250 rpm) in 14 mL falcon round-bottom tubes (Corning Inc., Corning, NY). The tubes were then centrifuged at 3850 g for 10 minutes (5910 R, Eppendorf, Hauppauge, NY) and the bacterial pellets were resuspended in 5 mL PBS (BupHTM phosphate-buffered saline packs, ThermoFisher Scientific, Waltham, MA). The OD600 was finally adjusted to 0.1 before the following two experiments were performed.

Experiment 1: Two MCC gels were fabricated, one with the usual basil-inspired monosaccharides and one with the same amount of sucrose instead. Because it is known that *R. tropici* CIAT 899 can digest sucrose, this experiment would thus give information about the bacteria digestion of basil sugars. 48 hours after the NaOH addition, twelve square hydrogel

samples (1 cm^3) were cut out and air-dried for 24 hours at RT. The gels were then rehydrated in PBS for 12 hours in 15 mL tubes. For controls (n = 3), the PBS was renewed, and for positive samples (n = 3), the initial PBS was replaced by PBS with *R. tropici* CIAT 899 (OD600 = 0.1). *R. tropici* CIAT 899 synthesizing green fluorescent protein (*R. tropici* CIAT 899-GFP)[251] were used to conduct hydrogel colonization studies. GFP fluorescence intensity of the solution was repeatedly measured at the following time points: 5 h, 8 h, 24 h, 30 h, 36 h, and 48 h (Safire2, Tecan, Switzerland). The excitation light was 491 nm, the emission light was 530 nm, and the gain was set to 70. GFP fluorescent intensity gave information on the amount of the fluorescent reporter expressed by *R. tropici* CIAT 899-GFP and it was used as an indication of living bacteria and their growth.

Experiment 2: The fluorescence experiment was repeated with the three P:C 1:1 gels (LCC, MCC, and HCC). The time points at which fluorescence was repeatedly measured were 2 h, 4 h, 6 h, 9 h, 15 h, 24 h, 27 h, 30 h, 33 h, 39 h, and 48 h.

Histological sections

To determine if bacteria were attracted by the hydrogel and migrated inside them, pieces of hydrogels were retrieved after 6 and 54 hours of incubation with *R. tropici* CIAT 899-GFP, and fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO) for 18 hours, washed twice with PBS, and casted into HistoGelTM (ThermoFisher Scientific, Waltham, MA). Distinct samples were used per each time point considered. After thirty minutes, two slices were cut out from the sample and placed in a cassette in formalin for an additional hour. Finally, all gels were conserved in 70% ethanol before being brought for histology (Gram-negative staining, Hope Babette Tang Histology Facility, Koch Institute, MIT, MA).

Migration studies: 1% (w/v) 45 minute-boiled silk fibroin aqueous solution was prepared from silkworm cocoons (Tajima Shoji Co., LTD., Yokohama, Japan) as described in Rockwood et al.[235]and mixed with a 1% (w/v) aqueous solution of trehalose (D-(+)-trehalose anhydrous, TCI Chemicals, Tokyo, Japan). The two solutions were added together to a final silk:trehalose ratio of 1:3. *R. tropici* CIAT 899-GFP were centrifuged at 3850 g for 10 minutes (Eppendorf 5910 R, Hamburg, Germany) and the pellet was resuspended in the silk:trehalose solution until an OD600 of 0.1 was reached. 50 mL of this solution was drop-casted on a PDMS sheet and air-dried for 48 hours at RT to form a film. In the meantime, the three P:C 1:1 gels were prepared as explained earlier. Right after the addition of NaOH, each viscous solution was transferred to the inserts of a 12-transwell plate (Corning Inc., Corning, NY) (n = 4) to reach a height of 2 mm. The gels were finally air-dried for 48 hours at RT. On top of the dried gel, a dry film was deposited on Day 0. At the bottom of the transwell plate, PBS was added so that only the bottom of the gel touched the solution through the permeable membrane. One sample of each gel was taken out on day 1, day 3, and day 7, i.e. distinct samples were used per each time point considered. The samples were fixed with 10% formalin solution for 30 minutes (Sigma-Aldrich, St. Louis, MO) and incubated with the TrueVIEWTM kit (Vector Laboratories, CA) for 5 minutes to quench the autofluorescence of the hydrogels. A cross-section of each gel was then imaged with a confocal microscope (inverted Ti Nikon1AR ultra-fast spectral scanning confocal microscope, Nikon Tokyo, Japan) at each time point to observe the migration of the microbes across the hydrogel. All images were taken with the exact same parameters. The fluorescent images were then processed with the ImageJ software to obtain a maximum intensity Z-projection.

Diffusion studies

To compare the diffusion of nutrients through hydrogels, 4-mL solutions of LCC, MCC and HCC hydrogel were prepared and added to 5-mL tubes and solidified for 48 hours. Gels were then hydrated in 154 mM NaCl solution for 24 hours before the diffusion study. 1 mL of a solution of fluorescein (100 mM) in water was pipetted on top of each hydrogel and the gels were photographed every 10 minutes for 150 minutes. The distance the fluorophore had traveled was measured at each time point and these data were used to calculate the diffusion rate of small molecules through the hydrogel[252]. Samples were measured repeatedly for time points considered.

Material biodegradation

Degradation of hydrogels in soil was evaluated over a one-month period. Dry samples of each gel (five 8 mm punches, n = 3) were weighed beforehand (Wi). The samples in 100 mm mesh tea bags were buried in 25% hydrated soil. 50 mL silk films containing *R. tropici* CIAT 899 (OD600 = 0.1) were prepared as explained above and one film was added to each tea bag. The moisture condition of the soil was monitored with a hydrometer and DI water was added when needed to maintain the 25% humidity level (5 mL every three days). A sample of each gel was taken out on days 1, 3, 7, 14, and 28 (measurements were then taken from distinct samples). They were quickly washed in 154 mM NaCl solution and then air-dried for 24 hours at RT before their weight was measured (W_d). Degraded gel (DG) amount was calculated as described in equation (4):

$$DG = \frac{W_i - W_d}{W_i} \cdot 100 \qquad (4)$$

where, W_i is the initial dry weight of the sample and W_d the dry weight of the samples after some time in PBS and bacteria or in soil, depending on the experiment. To investigate the effects of a

reduced microbial activity on the material biodegradation, 2% sodium azide solution was mixed with the soil (1 ml per 1 g of soil)[258]. Biodegradation studies were conducted at 16°C and 24°C.

Rheology: Isothermal gelation studies were conducted with a TA Instruments (New Castle, DE) stress-controlled AR-G2 rheometer with a 40 mm, 2∞ cone-and-plate fixture at 25°C. NaOH was added to the polymer mixture to induce bond formation (time t = 0) then immediately transferred onto just rheometer plate, and measurement started at t = 60-90 s. For time sweeping tests, storage moduli G′ and loss moduli G″ were monitored as a function of time at a 1 Hz frequency and a 2% stress strain under constant temperature (25°C).

Fluorescence Calibration

To convert all fluorescence intensity numbers to OD600 values, a calibration curve was made. *R. tropici* CIAT 899 were diluted in PBS at seven different OD600: 0, 0.1, 0.3, 0.5, 0.7, 1, and 1.1. The corresponding fluorescence intensities were measured and plotted against the OD600. The excitation light was 491 nm, the emission light was 530 nm, and the gain was set to 70. A linear model was fitted with MATLAB (R2018a, MathWorks, Natick, MA).

Statistical Analysis

Statistical analysis was performed on SRs, GCs, Es, and DGs with MATLAB (R2018a, Math-Works, Natick, MA). Normality of the data was verified using the Jarque-Bera test. A One-Way ANOVA test was applied, followed by pairwise comparison testing if the results showed a statistically significant difference between the groups ($p<0.05$). Bonferroni's correction was applied to counter the effects of multiple comparisons.

Seed coating: *P. vulgaris* seeds were surface sterilized with 10% bleach for 3 minutes, rinsed in H_2O three times, and left to air dry. 80 mL of *R. tropici* CIAT 899-GFP (OD600 = 1) was centrifuged at 3850 g in an Eppendorf centrifuge 5910 R (Hamburg, Germany). The supernatant

was discarded and 8 mL of dry 6 wt% silk fibroin-trehalose (1:3) solution was added to the spun down *R. tropici* CIAT 899-GFP. Air-dried seeds were then dipped into this solution for 120 seconds, taken out and left to dry (*Layer 1*). After slightly drying, the seeds were either dip-coated into a hydrogel gelation solution just after adding NaOH and left to dry (*Layer 2 dip*) or slightly sprayed with the hydrogel solution (*Layer 2 spray*). After drying, the seeds were planted at the 24-hour mark.

Growth conditions

Initial assessment of seed growth and nodulation was carried out in African Violet soil. Germination of *P. vulgaris* seeds was tested in a greenhouse setting by applying the following treatments: i) no treatment (negative control), ii) inoculation with 3% poly(vinylpyrrolidone) (PVP) solution with *R. tropici* CIAT 899-GFP (positive control), iii) bilayer seed coating with Layer 2 applied via spray-coating, and iv) bilayer coating with Layer 2 applied via dip-coating[82,259]. Seedlings were checked for nodulations at day 14 after germination, to ensure successful delivery of rhizobacteria and root colonization. N=18 seeds per treatment were used. Additionally, no statistically significant changes in soil electrical conductivity (EC) were measured in soil where coated and uncoated seeds have germinated (2.13±0.03 and 2.12±0.02 mS/cm, respectively), indicating that the ions present in the coating do not significantly affect EC.

Following this preliminary assessment, seed growth was carried out in semi-arid soil at UM6P experimental farm in Ben Guerir, Morocco. Watering conditions were adjusted to obtain soils with an average water potential (Ψs) of -1kPa, -5 kPa, -12 kPa and -20kPa over a 48 hour time interval. Mild and severe water stress growth condition corresponded to Ψs = -12 kPa and -20kPa, respectively. Soil tensiometers were made from a ceramic cup connected to an acrylic

glass tube and has a 136PC15G1 bridge pressure sensor (Micro Switch, Freeport, Il, USA) on the top to measure the pressure inside the tubing caused by water dynamics between the soil and water filled tube.) Tensiometers were used for each treatment in three replicates to monitor the water potential. Lost water through evapotranspiration was replenished to maintain the desired water potential. Healthy seeds from each treatment were evenly germinated on plastic trays of 10 cm containing 150 g of a substrate mixture composed by 30% of sieved sand (2 mm) and topsoil, manually shifted from stone. After adding water treatment solution to each replicate, trays were placed in the growth chamber, where relative humidity was 65 to 70%, night temperature 16˚C and day temperature 24˚C and photoperiod was 16/8 h. All the treatments were laid out in a completely randomized design, replicated five times, and kept for recording physiological attributes. To find out the role of seed coating in alleviating drought stress and to evaluate the effect on plant establishment and behavior along the growth cycle, three random seedlings were transplanted to large pots (30/20 cm), 20 days after sowing. Pots, containing 4 kg of the substrate mixture, were kept under the greenhouse to allow roots and appropriate leaves development. Irrigation was applied following the same water treatments used before. Different measurements from distinct samples were taken to investigate the treatment general effects: (*1*) Shoot length over time; (*2*) Shoot dry weight; (3) Root length measured with WinRhizo (Regent Instruments Inc., Quebec City, Canada) root scanner; (4) Stomatal conductance was measured using a SC-1 Leaf Porometer (Decagon Devices, Inc. USA); (*5*) Chlorophyll content was measured with a the CL-01 Chlorophyll Content System (Hansatech Instruments Ltd, Norfolk, England); (*6*) Total Phenolic Compounds (TPC) was measured by grounding in a mortar containing 5 ml of 50% ethanol solution fragments of leaves and roots (0.5 g FM). The extracts were collected in tubes with lids and labelled, then left in the refrigerator overnight. Upon adding 0.5 ml of chloroform

in 3 ml of extract, tubes were vortexed and centrifuged for 5 min at 7100 g. TCP assay was performed using the Folin-Ciocalteu reagent. Briefly, 0.5 ml of extract, 3 ml of distilled water and 0.5 ml of Na_2CO_3 (20%) were mixed in a test tube. After 3 minutes, 0.5 ml of Folin-Ciocalteu reagent was added. The test tubes were left for 30 min at 40°C before measuring absorbance at 760 nm. The content of phenolic compounds was calculated using gallic acid for the standard curve and expressed in milligrams per gram of fresh leaf matter.

4.6 Acknowledgements

We acknowledge Miguel Lara for *R. tropici* CIAT 899-GFP from Universidad Nacional Autonoma de Mexico. This work was partially supported by Office of Naval Research (Award No. N000141812258), the National Science Foundation (Award No. CMMI-1752172), the MIT Paul M. Cook Career Development Professorship. The authors acknowledge OCP S.A. and Université Mohammed VI Polytechnique-MIT Research Program.

4.7 Data availability

All relevant data are included in the paper and/or its Supplementary Information. All raw data are available from the authors on request.

4.8 Contributions

A.T.Z., J.L., M.M., B.M. and L.K. designed the study. A.T.Z., J.L., M.M., H.S., S.M., D.K. and H.M.E.F. collected and analyzed the data. All authors contributed to the discussion and interpretation of the results. The manuscript was drafted by A.T.Z., J.L., H.S., M.M, L.K and B.M. and reviewed and approved by the other authors.

4.9 Competing interests

B.M and A.T.Z. are co-inventors in a patent application that describes the coating technology reported in this study. BM is co-founder of Mori, Inc, a company that develops silk-based edible coatings to extend the shelf-life of food.

4.10. Supplementary Methods

Hydration studies

To understand the swelling kinetics of the selected hydrogels, a hydration study was conducted in solution. Wet 100 mm mesh nylon tea bags (DulytekTM, Seattle, WA) after five flicks and dry hydrogel samples (ten 3.5 mm punches, n = 3) were weighed (Mettler Toledo, Greifensee, Switzerland). To measure the swelling, the gel punches were placed in the tea bags in 154 mM NaCl solution (Sigma-Aldrich, St. Louis, MO) and their weights (punch + bag) were repeatedly measured after 1 h, 3 h, 6 h, 12 h, 24 h, and 36 h. After each time point, the bags with the punches were put back in solution. The swelling ratio (SR) was calculated as follows: SR = (Ws - Wi) / Wi * 100, with Ws being the weight of the swollen samples at the different time points and Wi being the initial weight of the dried sample before the experiment. Ws was calculated as the mass of the wet tea bag with the wet sample minus the weight of the wet tea bag. Another hydration study was then conducted in soil (African Violet Potting Mix, Scotts Miracle-Gro, OH) to highlight the effect and interaction of compressional soil forces vs. hydrogel swelling forces. Dried punches of each gel (8 mm diameter, n = 3) were put in 100 mm mesh nylon tea bags. Dried punches were weighed beforehand. Samples in bags were buried in the soil at three different moisture conditions (hydrated with deionized water): 10%, 25%, and 50% over a 24-hour period. Hydration was monitored with a hydrometer (Soil Moisture Sensor and LabQuest mini, Vernier, Beaverton, OR). Then, tea bags were taken out and cut to retrieve the wet

punches, which were weighed. Their SR was finally calculated according to the SR equation previously described.

Dehydration

Dry hydrogel samples (ten 3.5 mm punches per sample, n = 5), as well as wet and dry 100 mm mesh nylon tea bags, were weighed. Dried samples (Wi) were put in the tea bags and immersed in 154 mM NaCl solution for 12 hours. Teabags containing the samples were then retrieved from the water and flicked five times before being weighed to determine the SR as described above. Samples were then let to air-dry at RT. From there on, weight was measured every hour (Ws) until the samples were dry to obtain a water content curve (changes in SR over time). Ws was calculated as the mass of the teabag with the sample minus the weight of the teabag (wet weight for the first hour and then dry weight). The pH of the 154 mM NaCl solution in which the bagged samples were swollen was measured after 12 hours with a pH meter (Orion Dual Star, ThermoFisher Scientific, Waltham, MA).

Gel content

The dry weight of 8 mm hydrogel punches (n = 3) was measured before immersing them in 154 mM NaCl solution for 12 hours (W_i). The samples were then air-dried for 24 hours at RT and their weight measurement was taken again (W_d). The gel content (GC) was calculated as follows[260,261] :

$$GC = \frac{W_d}{W_i} \cdot 100 \qquad (2)$$

with W_i being the initial dry weight of the gel and W_d corresponding to the weight of the dried sample after swelling and deswelling the initial dry sample.

Macro and micromorphology

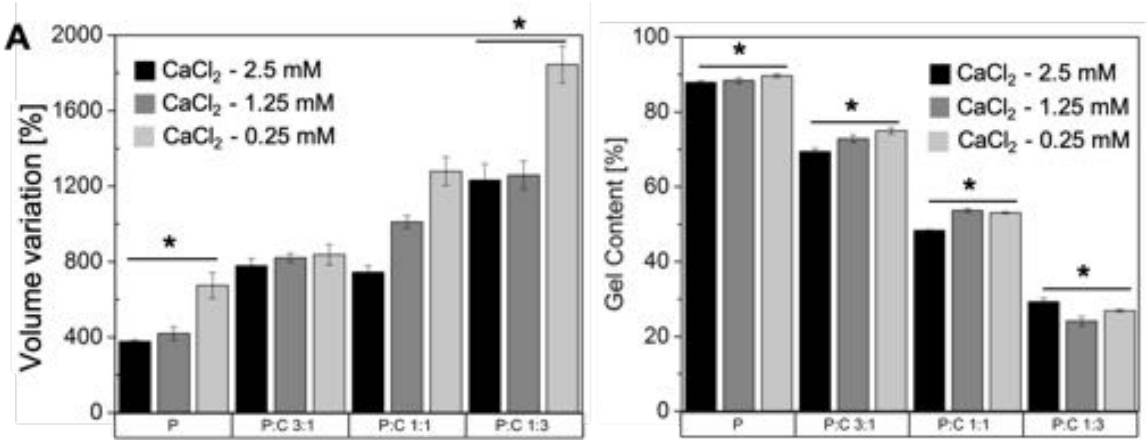
To have a visual sense of the swelling volume and integrity of the selected hydrogels, pictures of 8 mm punches were taken in the dry state and after being swelled in 154 mM NaCl solution for 12 hours (camera D3400, Nikon, Tokyo, Japan). Pictures were analyzed with ImageJ software available from https://imagej.nih.gov/ij/ (National Institutes of Health, MD). The volumes of the gels were calculated by measuring (*1*) the diameter of each gel in the dry and wet states at four different angles (0°, 45°, 90°, and 125°) and (*2*) the height of each gel in the dry and wet states at three spots (on each end and in the middle). The volume variation (VV) was calculated with the following equation:

$$VV = \frac{V_s - V_i}{V_i} \cdot 100 \qquad (3),$$

with V_i being the initial dry volume of the hydrogel and V_s being the swollen volume of the gel after 12 hours of immersion in 154 mM NaCl solution.

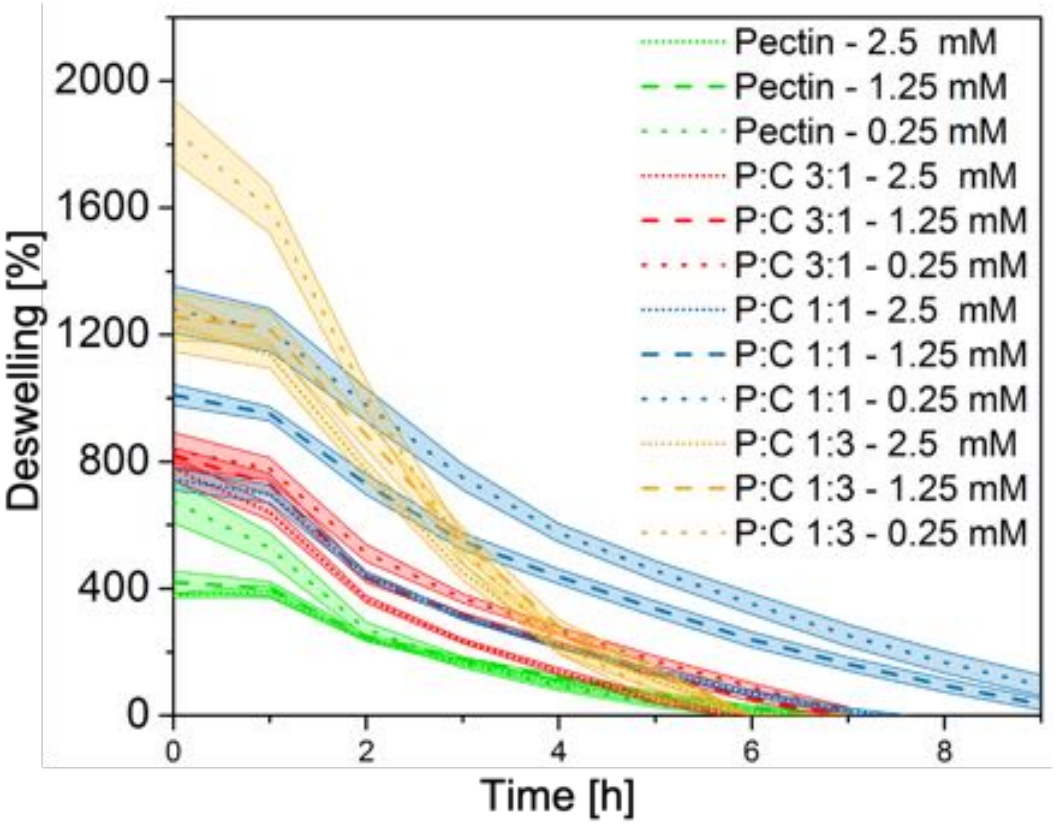
To visualize the microstructure of the hydrogels, 8 mm punches were swollen in 154 mM NaCl solution for 12 hours. Small pieces 2-3 mm were places in planchets at room temperature and plunged into liquid nitrogen. The frozen planchets were kept cold and transferred into a ACE900 freeze fracture machine. Samples were fractured, revealing the interior, then shadowed with 2 nm of platinum and coated with 10 nm of carbon. Coated samples were imaged directly in the Zeiss Crossbeam 540 FIB/SEM (Thornwood, NY) fitted with a Leica cryostage while held at below -150˚C. The LCC and MCC samples were soaked in 20% glycerol, a cryoprotectant for 30 minutes to prevent ice formation prior to freezing.

Supplementary Fig. 4.1



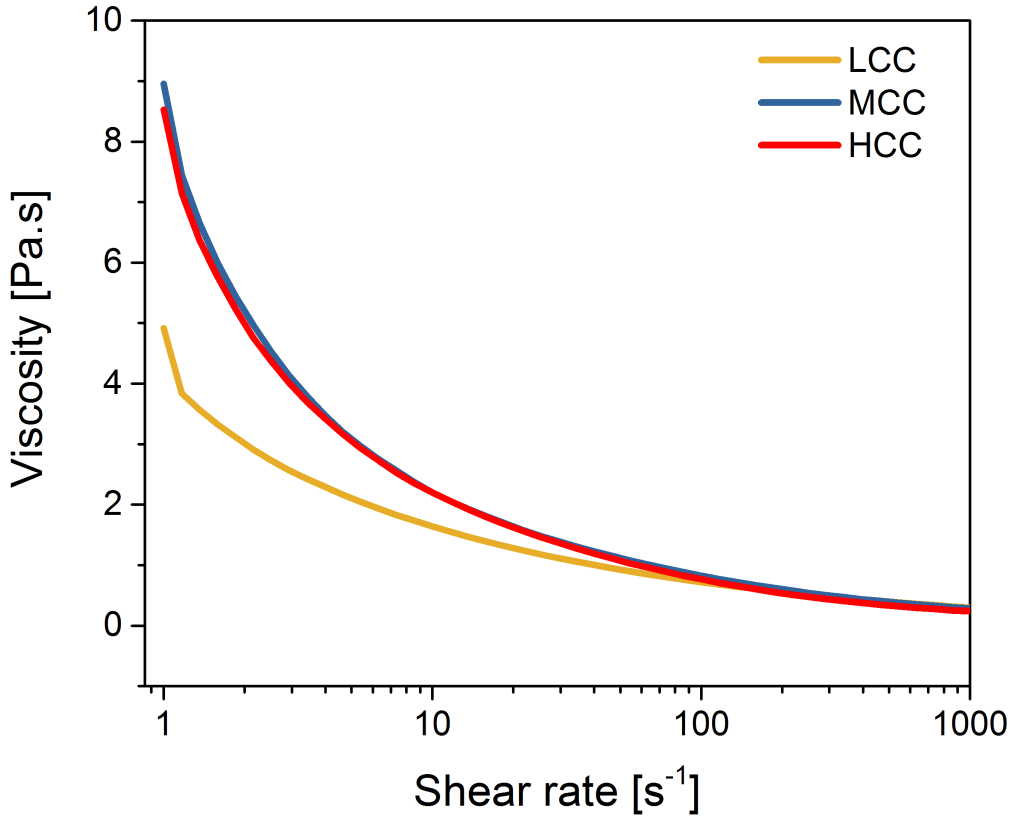
Supplementary Fig. 4.1. Investigation of P:C hydrogel properties as a function of relative mass of pectin and CMC in the dry gel and of increasing Ca^{2+} concentrations. Maximum swelling ratio (SR) and gel content (GC) of A) pectin (*P*) and CMC (*C*), and their mixtures. P:C 1:1 was selected to conduct further experiments based on the trade-off between swelling and gel content properties. Stars above bars indicate a statistically significant difference in the mean gel swelling ratio or gel content of a material ratio group compared to other groups (* $p<0.05$). The black error bars represent the standard error of the mean.

Supplementary Fig. 4.2



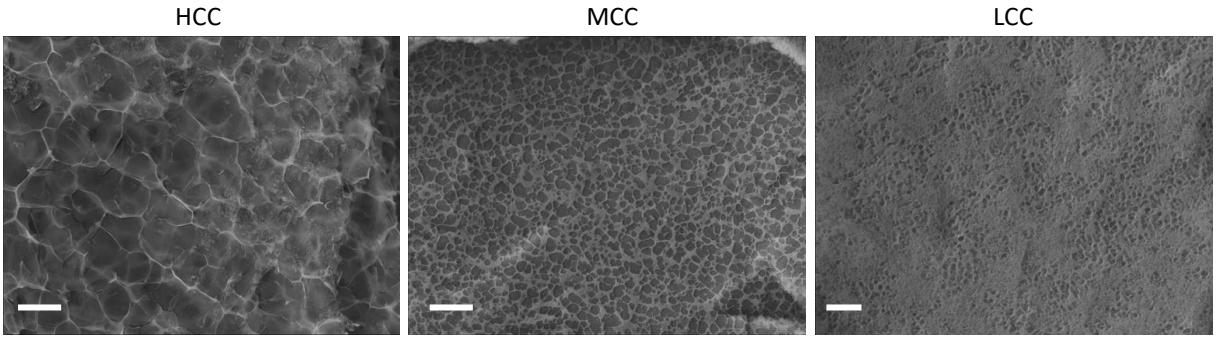
Supplementary Fig. 4.2. Deswelling over time for the tested hydrogels, upon 12h immersion in 154 mM NaCl solution. Concentrations refer to the amount of Ca^{2+} in each gel. Areas around curves indicate the standard error of the mean.

Supplementary Fig. 4.3



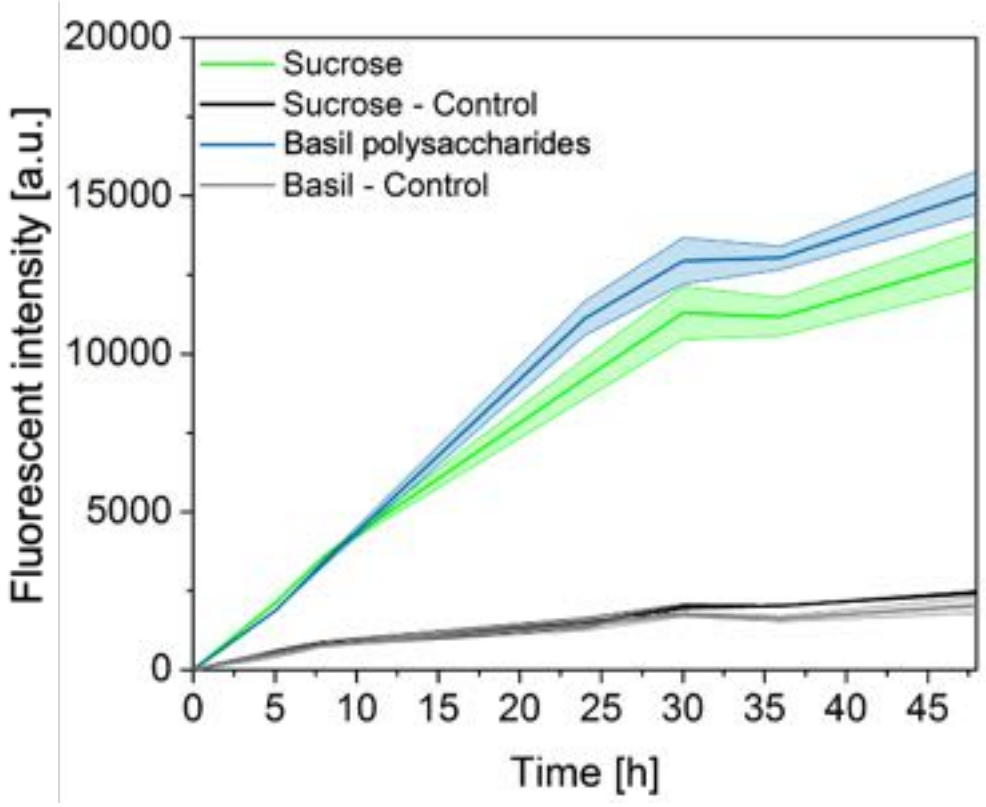
Supplementary Fig. 4.3. Viscosity of LCC, MCC and HCC solution prior to addition of NaOH to initiate gelation.

Supplementary Figure 4.4



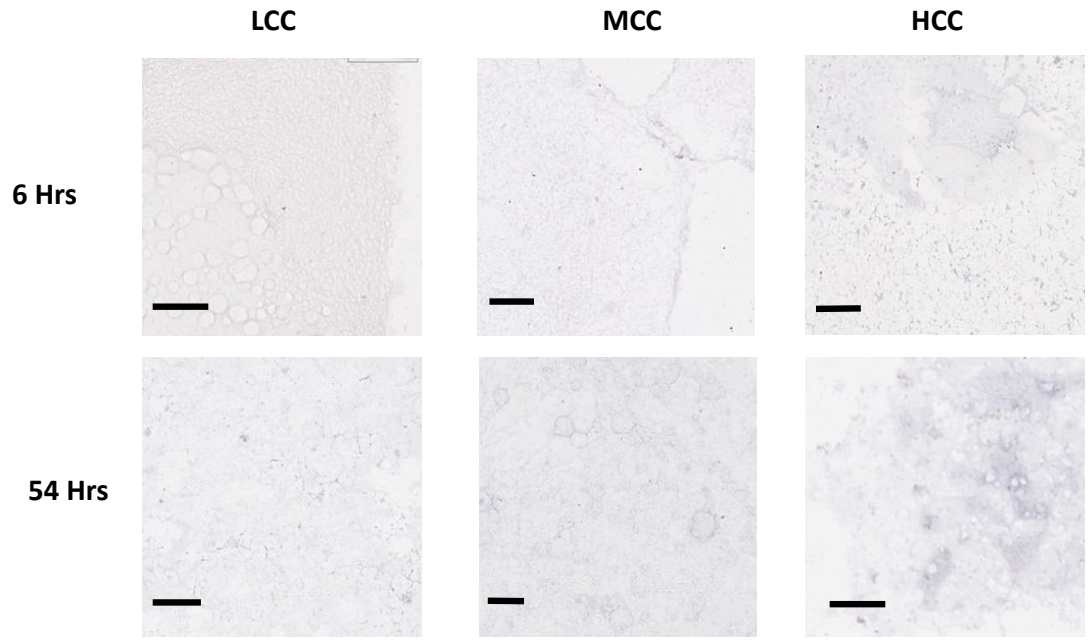
Supplementary Fig. 4.4. Material Characterization. Cross-section cryo-SEM images of swollen hydrogels, after 24h immersion in 154mM NaCl. A. HCC. Scale bar 5µm B. MCC. Scale bar 5µm C. LCC. Scale bar 1µm

Supplementary Fig. 4.5



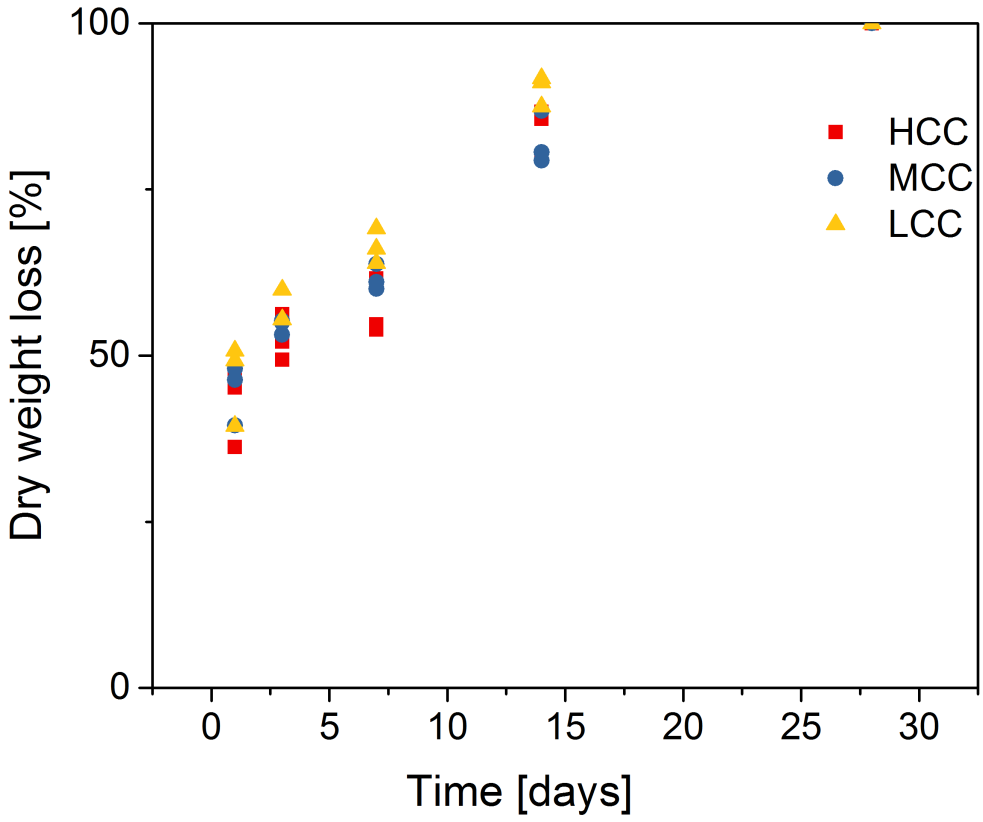
Supplementary Fig. 4.5. GFP expression in *R. tropici* CIAT 899 cultured in P:C 1:1 MCC hydrogels and exposed to different carbon sources. The fluorescent intensity signal over time may be used as an indication of bacterial growth. Sucrose and a mixture of polysaccharides that compose the basil seed mucilage were used as carbon source. Areas around curves correspond to the standard error of the mean.

Supplementary Fig. 4.6



Supplementary Fig. 4.6. Bacterial growth and migration. Representative histological sections of the LCC, MCC and HCC before being colonized by *R. tropici* CIAT 899-GFP. Scale bar 20µm.

Supplementary Fig. 4.7



Supplementary Fig. 4.7. Biodegradation of LCC, MCC and HCC in soil at 24°C.

Supplementary Table 4.1. Soil analysis for soil extracted from the UM6P experimental farm in Ben Guerir, Morocco.

Parameters		Results	Units	Method
Granulometry	Clay	12	%	NF X 31-107
	Silt	24	%	
	Sand	64	%	
pH		7.90		NFISO 10390
EC at 25°C		0.73	mS/cm	NF ISO 11265
$CaCO_3$		1.10	%	NF EN ISO 10693
Organic Matter		2.36	%	NF ISO 14235
P_2O_5		153	mg/kg	NF ISO 11263
Exchangeable elements	K_2O	544		NFX 31-108
	Na_2O	501		
	MgO	450		
	CaO	5243		
Oligo-elements	Cu	0.43		NFX 31-121
	Mn	3.06		
	Fe	5.21		
	Zn	2.05		
$N\text{-}NH_4$		2.87		Skalar
$N\text{-}NO_3$		181.87		

Chapter 5

Environmental Degradation of Silk Fibroin Based Films

The contents of this chapter is in the process of submission in Biomacromolecules as: Augustine T. Zvinavashe[1], Yunteng Cao[1], Hui Sun[1], Doyoon Kim[1], Benedetto Marelli[1,*]

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5.1 Abstract

There is a compelling need to find biodegradable materials that can be sustainably sourced for environmental applications. Regenerated silk fibroin has been shown to be biocompatible and environmentally stable and most critically has been successfully applied as microbial seed coatings, food coatings, plant phytoinjectors and microneedles for improving food security. In the present study, silk fibroin films with different secondary conformations in soil and seawater were used to study the degradation behavior of silk fibroin in environmental conditions. Silk fibroin with highest β-sheet content achieved lowest degradation rate, similar behavior with past studies. Further, it was shown microbial activity was a key driver for degradation in soil conditions. Salinity concentration did not affect degradation of films in soil as soil has numerous microbes with varying optimal conditions. In seawater silk fibroin film degradation after 8 weeks was minimal but different from a previous study that highlighted high biodegradability in seawater. Our study shows silk fibroin films biodegrade in the environment and controlling the degradation of silk can be used to optimize silk fibroin in the different environmental applications.

Keywords : silk fibroin; degradation; microbial; environment

5.2 Introduction

The degradability of silk based biomaterials used in tissue engineering and drug delivery is well studied[262–264] . However, the degradation of regenerated silk fibroin based biomaterials in environmental conditions is not well studied. There is a compelling need to find sustainable substitutes to synthetic polymers because of the global environmental threats plastics create such as not degrading in the natural environment and usage of fossil resources [265,266]. While the performance of natural protein is high, the low productivity by some of the aforementioned mammals and insects limits commercial use. However, commercial scale production of protein materials using microbial fermentation is just starting globally[266]. Recombinant structural protein is a promising alternative to conventional engineering plastics due to its good thermal and mechanical properties, potential biodegradability and production from biomass[266].

In January 2019 European Chemicals Agency (ECHA) proposed a wide-ranging restriction on intentionally added microplastics (IAMPs) in products placed on the European Union (EU) market with microplastics foreseen to be completely restricted by 2025[265,267]. These external pressures have created interest in biodegradable materials. The materials need to meet stringent biological, chemical and physical conditions involved in their daily uses but also be affordable and abundant[265]. Natural based materials have shown great promise in this area such as silk based materials. Silk based materials are interesting because of silk fibroin being suited for the generation of a number of biopolymer-based advanced material formats leveraging control of form and function[237]. Further, the numerous shown environmental applications silk possesses such as use in

microbial based seed coatings, food coatings and micro needles for food quality assessment and phytoinjectors [83,86,87,188,239,268,269].

The general thought is that silk textiles degrade in the environment due to humidity and ultraviolet (UV) exposure [270]. Research on environmental factors influencing silk objects in historic houses is generally performed by accelerated ageing test methods such as UV irradiation[270]. It is well known that silkworm silk is very weak with regards to UV irradiation[271]. UV irradiation leads to the cleavage of chemical bonds in the protein chains, which creates radicals that induce the cleavage of other chemical bonds. Thus, UV irradiation amplifies the number of radicals which accelerate the cleavage of chemical bonds in the protein chains, which decomposes silk chains into fragments[271]. However, previous studies suggest the role of UV irradiation is exaggerated as accelerated UV irradiation affects the structure and the properties of silk textiles in a different way than natural daylight exposure, resulting in crosslinking of the material and altered mechanical properties[270]. Mazibuko et al., compare the degradation of silk, cotton and denim textiles, the results show the effect of the soil on the degradability of textiles[272]. Silk has the highest degradation rate in controlled and uncontrolled environments. It was assumed the textile degradation might be as a result of the presence of microbes[272]. These experiments were not conducted on regenerated silk fibroin, however they give a general idea on the biodegradability of silk fibroin based materials. Further, humidity conditions, both at high and low levels, are known to have negative effects on silk by aiding deterioration. Also, temperature affects and accelerates deterioration, reduces molecular weight and has a negative effect on tensile strength[270]. Vilaplana et al., show oxidation, hydrolysis, chain scission and chain rearrangements (physical ageing) as the main degradation mechanisms affecting the structure and properties of silk textiles[270].

Silk fibroin (SF) produced by Bombyx mori L. insect makes an amazing environmental material because it displays remarkable properties like environmental stability, controlled biodegradability, flexibility, mechanical resistance and optical transparency, solution processability[189,267,273]. These properties combined with the water-based extraction and purification process make SF a promising material to replace synthetic, plastic-based and non-biodegradable material use [267]. Historically, silk based materials have fascinated scientists. In 1840 Parsley recovered silk garments of satin weave from the HMS Royal George which sunk in 1782[274]. The silk was intact and perfect, while pieces of leather, but no woollen clothing in the wreckage were found[274] .

These properties position silk fibroin as a next generation polymer to replace non renewable sources. We are living in a polymers age with a massive plastic production of more than 8.3 billion tons ever produced, which has led to worldwide plastic pollution[275] Modern agriculture heavily relies on the use of plastic materials while 4.8-12.7 million tons of plastic waste are added into oceans every year[276,277]. It is cited that 80% of marine debris originates from land, however this number is not well substantiated however it shows the connection of land and ocean and the importance of applying soil biodegradable materials[276]. Plastic films are commonly composed of non-biodegradable polyethylene and thus accumulate in environmental spaces leading to negative ecological and economic impacts [278,279].

Silk fibroin degradation is very important in environmental application. Even though the degradation of SF can be changed through the addition of enzymes or increase in beta-sheet content, the degradation of silk is still perplexing[262]. In order to control degradation in the

environment (soil or ocean) it is important to explore the factors that may control the degradation of SF materials. However, the environment includes microorganisms, such as bacteria and fungi, that can start the biodegradation process[280] . Biodegradation is the breakdown of polymer materials into smaller compounds by physical, chemical and biological factors[281]. Biodegradable polymers can be decomposed into CO_2, methane, water, inorganic compounds or biomass through microbial enzyme activity[282]. However, the process proceeds under different conditions because the microorganisms involved are different from each other and are particularly active in the environment[280,282]. They can also be broken down by non-enzymatic processes, such as chemical hydrolysis[282]. However, enzymes play a significant role in the degradation of silk fibroins[281]. Silk is a protein based biopolymer where amino acids are the fundamental building blocks, as a protein, silk degrades in vitro and in vivo in response to proteolytic enzymes which makes it a good biomaterial option for medical devices and environments with proteolytic enzyme activity[283].

In this study, we observe how silk fibroin film degrades in soil with microbial activity and without, how salinity affects the degradation and beta-sheet content and finally how silk fibroin films degrade in seawater. It will help in controlling or tuning properties (e.g., mechanics, degradation rates) in soils of different salinities and finally meeting environmental guidelines for silk fibroin based application.

5.3 Materials and methods

Preparation of silk solutions

Bombyx mori silk fibroin solutions were prepared according to previously published procedures[235]. Cocoons were boiled for 45 min in an aqueous solution of 0.02 M Na2CO3, and then rinsed thoroughly with water to extract sericin proteins. The extracted was dissolved in a 9.3M LiBr solution at 60℃. This solution was dialyzed in Milli-Q water using Slide-a-Lyzer dialysis cassettes (3.5 kDa MWCO) for 72h. The resulting SF suspension was then purified by centrifugation at 9000 rpm (~12,700g) over two 25 minute-long periods, at a constant of 4℃[189].

Preparation of silk and CIAT 899 solution

Suspensions were made of gram negative PGPRs (*Rhizobium tropici* CIAT 899 Martinez-Romero et al. - ATCC 49672) mixed with the silk fibroin. 50% tryptic Soy Broth (Becton Dickinson, Franklin Lakes, NJ, USA) was generated by mixing 500ml of H2O with 2.5g Bacto-peptone (Soybean-Casein Digest Medium) (Becton Dickinson, Franklin Lakes, NJ, USA), 1.5g Yeast extract and add 0.7M $CaCl_2$ (autoclaved), working concentration dilute 100X . The media was autoclaved for 60 min at 121oC. CIAT 899 was sourced and cultured in a shaker incubator at 200rpm and 30oC up to an OD600 measure of 0.7. Once bacteria reached an OD600 of 1, 10ml of bacteria broth solution was centrifuged at 4300 rpm for 20 min. The bacteria formed a pellet and the supernatant was discarded. Concentrated bacteria suspension was made, 10ml of silk fibroin was pipetted into the pelleted bacteria strain and uniformly mixed by thoroughly pipetting up and down.

Film formation

1 ml of ~7wt% silk solution was cast on polydimethylsiloxane (PDMS) sheet for regular silk fibroin films. For silk fibroin films embedded with rhizobium tropici CIAT 889, CIAT 889 was grown and centrifuged as reported above. The CIAT 889 pellet was mixed with silk fibroin solution and was cast on PDMS. The films were air-dried in a biological hood to control the drying rate. Once dried, the insoluble silk fibroin films were also prepared by the water annealing treatment. Water annealing (i.e enhancement of beta-sheet content) was done through exposure of silk fibroin films to water vapours under vacuum at 22℃, according to previously developed protocols (15) for 3 hours, 6 hours and 9 hours as required.

Degradation in soils

The degradation experiment proceeded in a) soils of varying salinity and b) with no microbial activity: soil was obtained from … Degradation was carried out for one type of soil at a time. .. Bags were loaded with silk fibroin films and sealed. The bags were then placed in soil with 25% water content at standard room conditions. The water moisture was then maintained by watering the soil every third day. The sealed bags would then be taken out at the relevant time point carefully removed with tweezers. The samples were rinsed with distilled water and air dried for 48 hours minimum.

Degree of degradation

The degree of degradation was determined gravimetrically. Sample mass initial was determined before degradation and sample mass final was determined after degradation. The samples were left to air dry for 48hours before final mass was measured.

Degradation in solution

Silk fibroin mass to be added in NaCl was noted. This mass and volume for degradation were kept constant for all experiments and repeats. Dissolved silk (protein) in solution was used as a measure for silk fibroin film degradation. The total silk fibroin dissolved in the solution would give us total protein content (100%). Using Sigma-Aldrich BCA Protein Assay kit the protein content was measured in each 50 ml conical tube. For every experiment, n=3.

Fourier Transform Infrared Spectroscopy (FTIR)

Drop cast films were analyzed using Thermo Fisher FTIR6700 Fourier Transform Infrared Spectrometer through attenuated total reflection (ATR) germanium crystal. For each sample, 64 scans were coadded with a resolution of 4 cm-1 , at wave numbers between 4000 and 650 cm . The background spectra were collected under the same conditions and subtracted from the scan for each sample.

Scanning Electron Microscopy (SEM)

Drop cast films were freeze cracked after being dipped in liquid nitrogen and analyzed with a Zeiss Merlin High-resolution scanning electron microscope. Samples prepared did not charge, therefore no gold plating or any preparation of samples was required. An EHT of 1.00kv was used with a 100pA probe.

Nanoindentation

Nanoindentaion measurements were performed on a Hysitron TriboIndenter with a nanoDMA transducer (Bruker). Samples were indented in load control mode with a peak force of 500 μN and a standard load-peak hold-unload function. Reduced modulus was calculated by fitting the unloading data (with upper and lower limits being 95% and 20%, respectively) using the Oliver-Pharr method and converted to Young's modulus assuming a Poisson's ratio of 0.33 for all samples. Each type of sample was prepared and indented in triplets to ensure good fabrication repeatability. For each sample, indentation was performed at a total of 49 points (7×7 grid with an increment of 20 μm in both directions) to ensure statistical reliability of the modulus measurements.

5.4 Results and discussion

Factors affecting enzymatic degradation of regenerated silk materials beside the environment can be categorized into two groups: a) structure related which include molecular weight, crystallinity, secondary structure and hierarchical structure b) morphology related which include material format, porosity, and surface morphology[283]. In this study silk fibroin (SF) based films were prepared as mentioned in the materials and methods section as a possible replacement for materials

used for environmental applications [83,86,239]. Figure 5.1 details the following four experiments that were conducted. Silk fibroin films degradation behavior was studied in soil and in seawater to understand how to design biomaterial for environmental application.

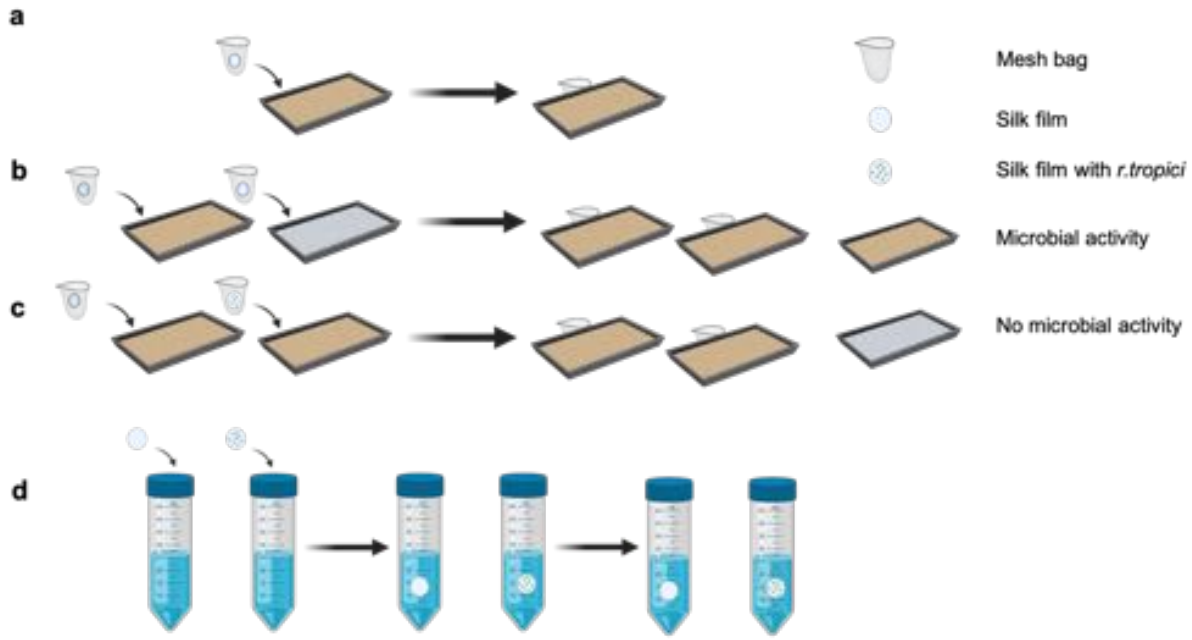

Figure 5.1. Schematic of degradation experiments. A) General degradation profile B) Microbial vs non-microbial degradation C) Effect of soil salinity D) Effect of salinity in solution.

Silk degradation in soil is not well studied. Initially, the degradation profile of 80µm ± 10µm thickness silk-fibroin films in soil of 25% water content was measured. The water annealing time was varied to vary the beta-sheet content in the films. Changes in silk fibroin film structure were determined by FTIR (Figure 5.8 of the Supporting Information). Figure 5.2 shows degradation profile of SF based material which was similar to silk fibroin degradation in past studies of silk degradation in tissue engineering and drug delivery as the crystallinity (beta-sheet content) increased the slower the degradation rate[284]. Silk materials with amorphous structures commonly degrade faster compared with semi-crystalline structures as the amorphous structures are more loosely packed and thus accessible to the proteolytic enzymes[262,283,285,286]. Thus, the amorphous structures are first degraded, then the crystalline structure. Beta-sheet content of 45%, 50% and

54% were obtained from 3 hours, 6 hours and 9 hours of water annealing at 22℃. The results imply that by increasing beta-sheet content, the degradation rate of silk fibroin films is increased. It is known that the structures of silk materials strongly determine the enzymatic degradability of the silk materials[283]. Thus, degradation properties can be achieved for silk materials through structural control during materials processing or post-treatment (eg., water annealing)[283]. After incubation in soil for 8 weeks the weight loss of the water-annealed silk films was about 5-7% for 3 hour and 6 hour annealed silk and 30% for 9 hour annealed silk. For the first two weeks, the degradation rate appeared to be quicker, perhaps because of the degradation of silk I and noncrystalline silk in the films. After two weeks , the degradation rate tended to slow down, which could be related to the degradation of silk II Beta sheet crystal structures in samples[284].

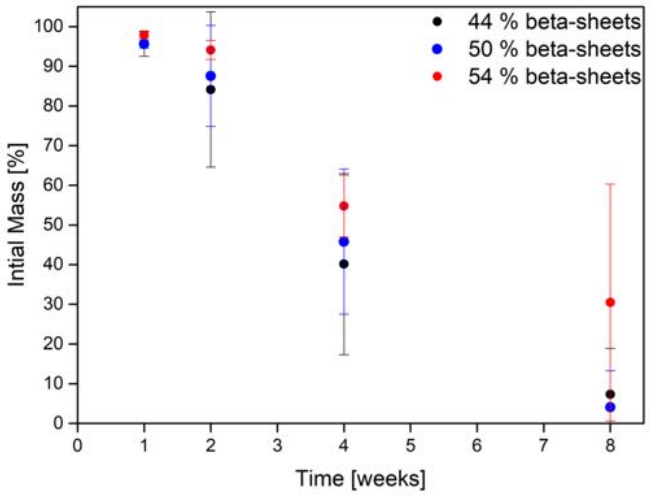

Figure 5.2. Silk film degradation profile with different silk annealing times (Beta-sheets).

We hypothesized silk-fibroin degradation in soil was driven by microbial (enzymatic) activity as silk-fibroin degradation is carried out by proteolytic enzymes (e.g., protease XIV, ***α***-chymotrypsin, proteinase K, papain, collagenase, etc)[283]. The enzymes have different cleavage sites along the silk fibroin chains, which support different degradation effects[283]. Thus, predicting silk fibroin degradation requires knowledge of both the cleavage sites and material structure. Enzymatic

activity is known to be a major indicator of biological activity in soils, which is strongly dependent on moisture, oxygen content, tillage and presence of heavy metals[287,288]. For assessing the degradation impact microbial activity has on silk fibroin film, a degradation study was initiated. Sodium azide solution was mixed into the test soil to retard microbial activity while the control soil had no sodium azide. Figure 5.3 shows after 4 weeks the test hardly had any degradation while the control had about 40% degradation. Enzymatic degradation leads to the breakdown of the silk fibroin into smaller polypeptides and eventually amino acids which are easily absorbed and washed away[283]. The nanostructure of samples was investigated by SEM (Figure 5.3). More interestingly, in our study silk fibroin degrades into fibrillar structures[289]. The Material format plays a critical role as it dictates physical properties such as porosity and surface morphology[283]. Generally, films have longer degradation times due to their denser structure and lower porosities which limits enzyme accessibility and surface erosion features[283,290–292].

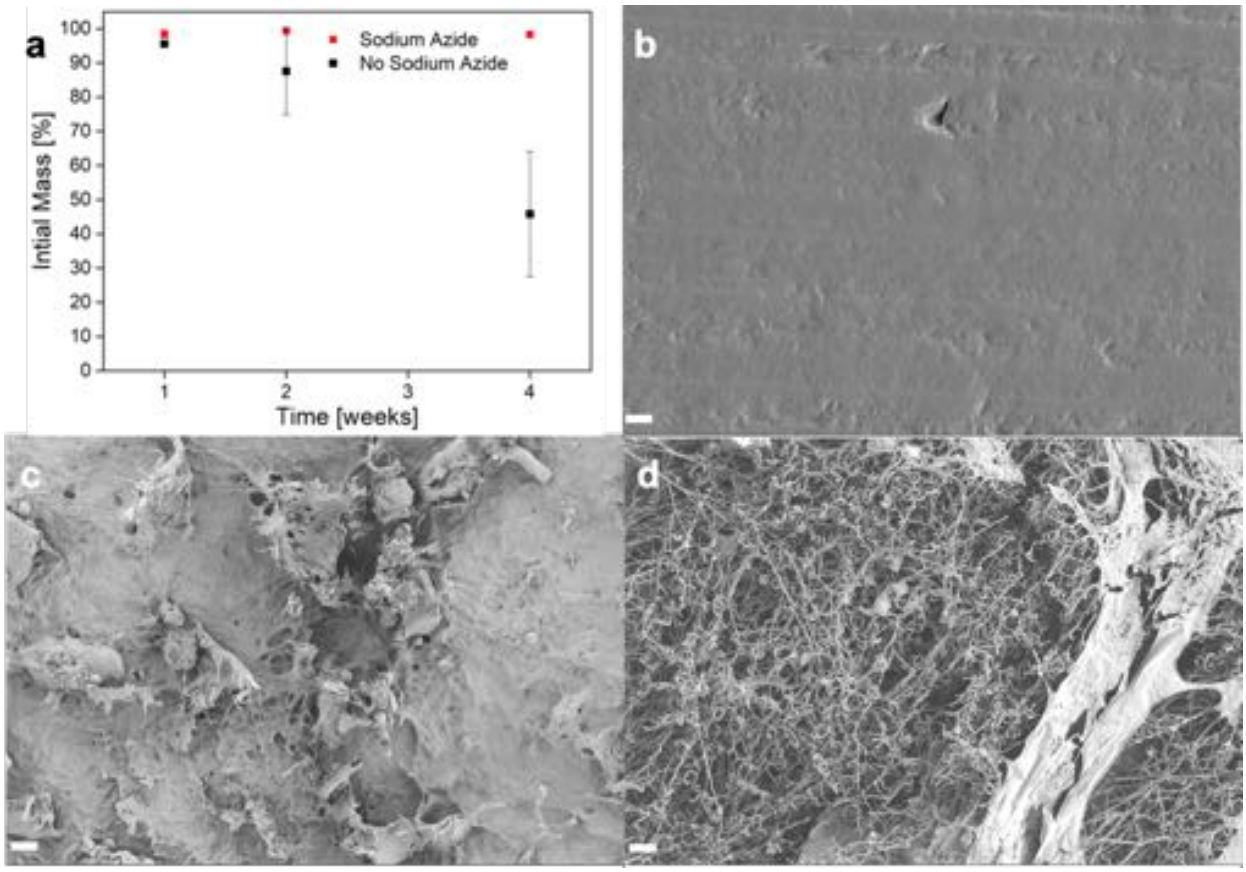

Figure 5.3. Silk film degradation with soil microbial activity vs no activity. A) Mass loss B) 6hr Annealed silk film T=0 C) No microbial activity T=4 weeks D) Microbial activity T=4 weeks. Scale bar 10 μm.

To determine whether soil salinity controlled degradation, silk fibroin films were degraded in soils of 0mM, 50mM, 100mM and 200mM. The data had no statistical significance implying silk fibroin degradation was not controlled by soil salinity (Figure 5.4). Soil salinity however, has an impact on the microbial activity of the soil on different microbes thus the degradation likely was controlled by different types of soil microbes at the varying salinities. With embedded *R.tropici* in the films, films were 100μm ± 15μm, the degradation rates were comparable with no *R.tropici* embedded in films. This implies native soil microbes control the degradation of silk fibroin films and the addition of microbes in silk fibroin have a negligible impact. SEM shows fibril degradation in silk fibroin degraded films (Figure 5.2 and 5.3 of the Supporting Information). The more degraded the silk fibroin film was the less evident the fibrils become as the non-degraded material is less eroded.

The silk fibroin films also were leached by iron thus appeared orange-brownish and pink in color (Figure 5.9 of the Supporting Information). ICP confirmed the soil containing iron and manganese (Figure 5.10 of the Supporting Information). The degraded films in soil also had distorted cubes on the surface. Since iron was the element that leached onto the silk fibroin films, the cubes could have been iron crystals of around 20μm in size or spores and bipyramidal cystals of Bacillus thuringiensis (Bt) as Bt is occurs naturally in aquatic environments, insect-rich environments and grain-storage facilities[293] (Figure 5.3 of the Supporting Information). Further, microbe spores can also be seen on the degraded silk fibroin films confirming the impact of microbial activity on degradation (Figure 5.9 of the Supporting Information). On silk fibroin films degraded in solution the iron crystals were not present as iron was non-existent in large enough quantities to have an effect. More interestingly, extracellular polysaccharides can be viewed on the degrading silk. Three FTIR bands (1000-1200band, ~2900 band and 3300-3500 band) confirm the presence of carbohydrates in the silk fibroin films, the bands are very wide as extracellular polysaccharides are constituted of numerous types of polysaccharides[294].

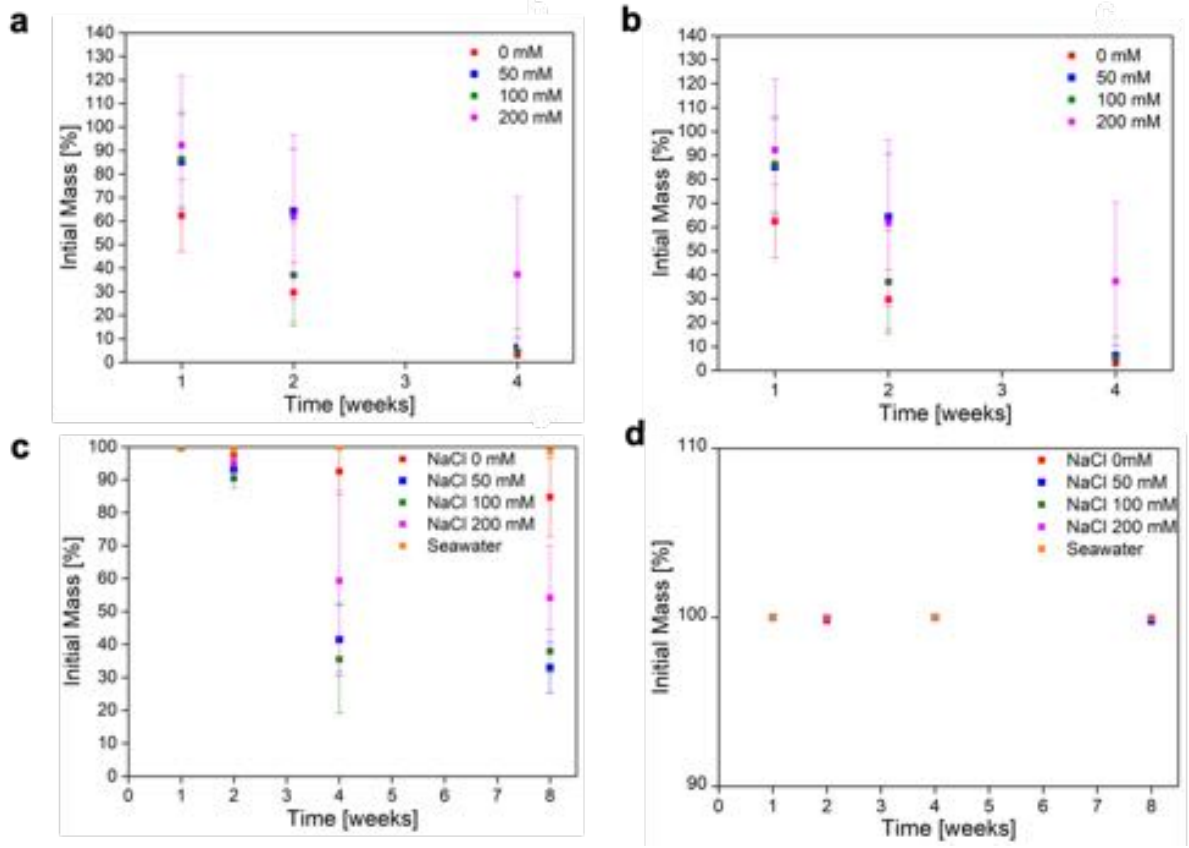

Figure 5.4. Silk film degradation profile in Soil and Solution. A) Soil with varying salinity with *R.tropici* embedded in films B) Soil with varying salinity with no *R.tropici* embedded in films C) Solution with varying salinity with *R.tropici* embedded in films D) Solution with varying salinity with no *R.tropici* embedded in films.

Degradation in solution

In previous studies the degradation of water -annealed silk films after 2 weeks in PBS solution show no weight change[262]. As no relationship between varying soil salinity and silk degradation was confirmed, silk degradation in varying NaCl concentrations was conducted to limit the parameters found in soil. Initially, silk fibroin films were degraded in 0mM, 50mM, 100mM, 200mM and seawater. After 8 weeks hardly any degradation had occurred (Figure 5.4). Total protein content in solution was used as a proxy for degradation. The silk fibroin films have a fixed quantity of protein and once fully dissolved in solution this becomes the total protein content. Surprisingly, seawater had no degradation impact on the silk fibroin as it has sea microbes in it.

The enzymatic degradation of biomaterials is a two step-process. The first step is adsorption of the enzyme on the surface of the substrate through surface binding domain and the second step is hydrolysis of the ester bond[281]. We can assume either seawater microbes do no secret proteolytic enzymes that can degrade silk or the concentration of microbes thus secreted enzymes is too little to have an impact in the timescale or the enzymes fail to adhere to the surface of the silk fibroin films. A morphological analysis was conducted (Figure 5.7). The SEM revealed very minor surface erosion on the films after 8 weeks. The silk fibroin film in 200mM looked the most degraded then the 100mM with some fibril patterns beginning to be seen on the surface of the material. The silk fibroin film in seawater looked very similar to silk fibroin film at T=0. Similar to the HMS Royal George which sunk in 1782 and other silk textiles discovered after more than 350 years in seawater, the silk fibroin films were unimpacted[274,295]. However, the mechanical properties were affected by the varying NaCl solutions. The young modulus and hardness decreased after being in solution (Figure 4 of the Supporting Information). The hardness decreased as the NaCl concentration increased. The young modulus also decreased as the NaCl concentration increased with NaCl 100mM not experiencing much decrease. Please note this study had differing results from Tsuchiya et al., who used 30 min boiled silk fibroin versus our 45 min boiled silk fibroin and achieved biodegradability in seawater[296]. This maybe due to lack of microbial activity in the seawater used in our experiments.

Silk fibroin, *R.tropici* embedded films were then incubated for 8 weeks. Total protein content increased over the 8 week period (Figure 5.4). The rate of increase in the first four weeks was faster than the last 4 weeks in total protein content, which could be related to the degradation of silk I and noncrystalline silk in the films in first 4 weeks and silk II Beta sheet crystal structures

last 4 weeks[284] In seawater the silk fibroin films have no degradation. We assume the seawater lysis the *R.tropici* thus the *R.tropici* can not secret the proteolytic enzymes required for degradation. As the NaCl concentration varies the optimal growth salinity for the *R.tropici* is reached thus optimized for microbe degradation. NaCl 50mM appears to be the most optimal environment for *R.tropci* thus degradation of silk fibroin films. This highlights that silk fibroin film degradation in the environment is controlled by microbial (enzymatic) activity. After 8 weeks no films were leftover in the solutions besides for silk fibroin films placed in seawater.

5.5. CONCLUSION

The degradation of silk fibroin in the environment was studied based on the films with different crystallinity and working environments (i.e, salinity, soil, microbial activity and seawater). As expected Beta-sheet content controlled silk fibroin film degradation rate. The higher the Beta-sheet content the longer the degradation rate. Microbial (Enzymatic) activity is the driving factor for silk fibroin film degradation in soil. We show the negligible degradation present when silk fibroin films are in a non-microbially active soil. It is not only about microbial activity, however it also ensures the microbes are secreting proteolytic enzymes that can degrade silk fibroin protein. In our seawater study, silk fibroin films fail to degrade in seawater with *R.tropici* and without highlighting to us that the concentration of enzymes released by seawater microbes are not enough or none at all or fail to interact with silk fibroin surface for enzymatic cleavage. Based on the degradation data silk fibroin films can be deployed for agriculture use. Within 8 weeks silk fibroin with 50% beta-sheet content will be almost completely degraded which falls in line with E.U regulations on biodegradable materials to be used in their soils.

Associated Content

Supporting information. FTIR images, ICP and SEM of degraded silk films

Supplementary

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Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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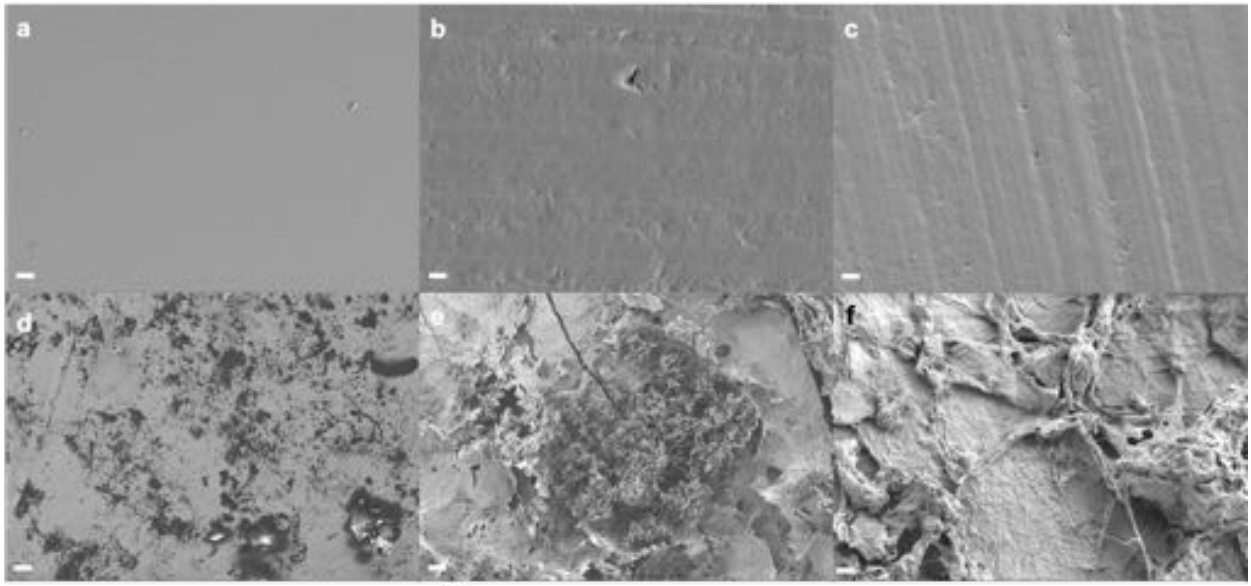
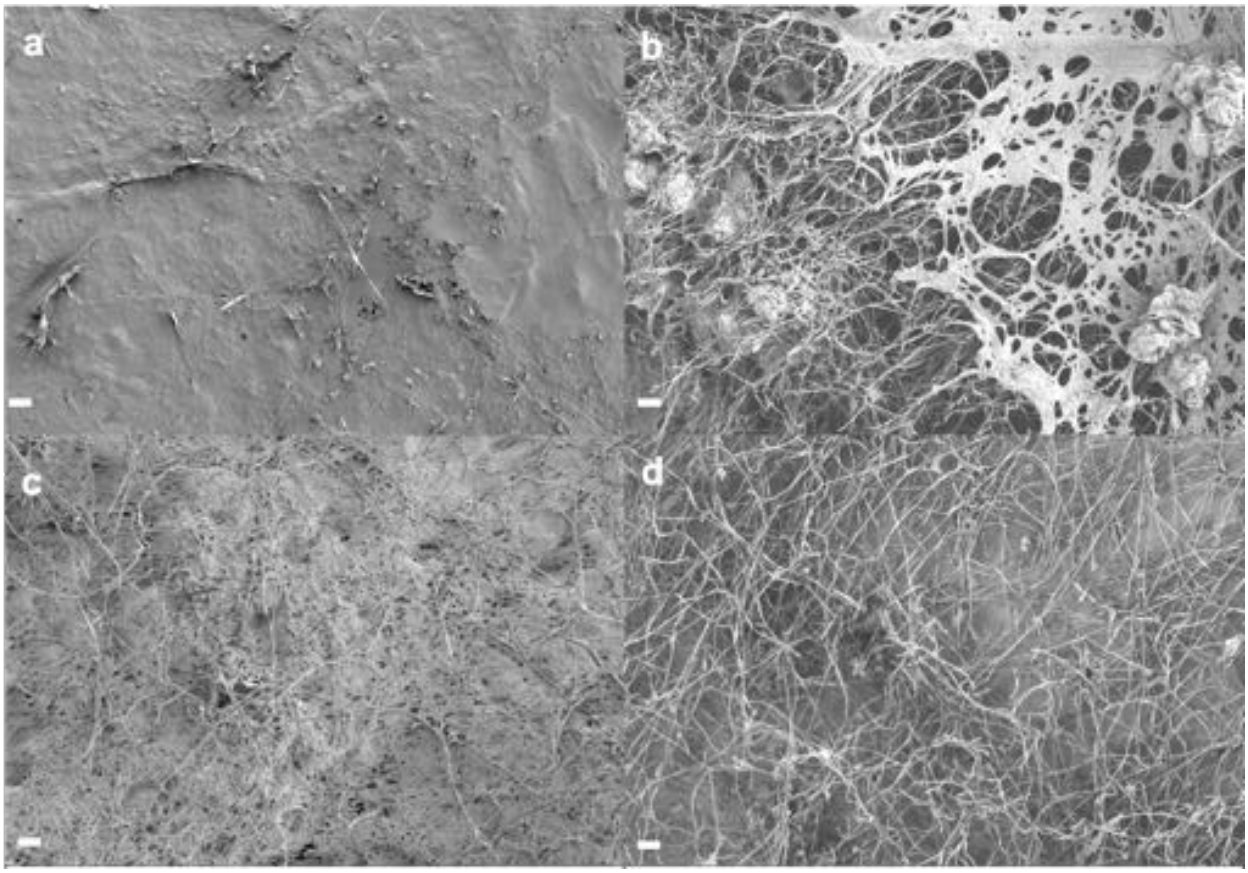
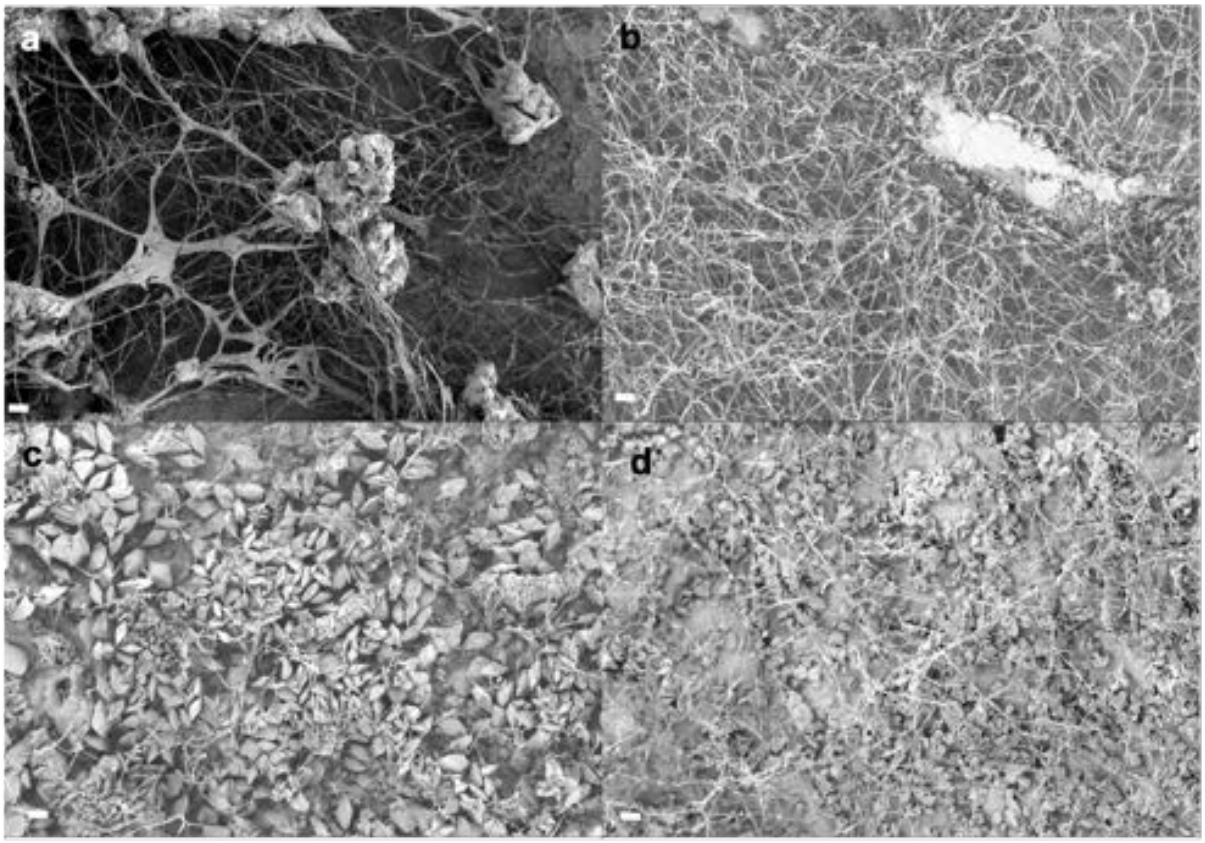
Supplementary Information



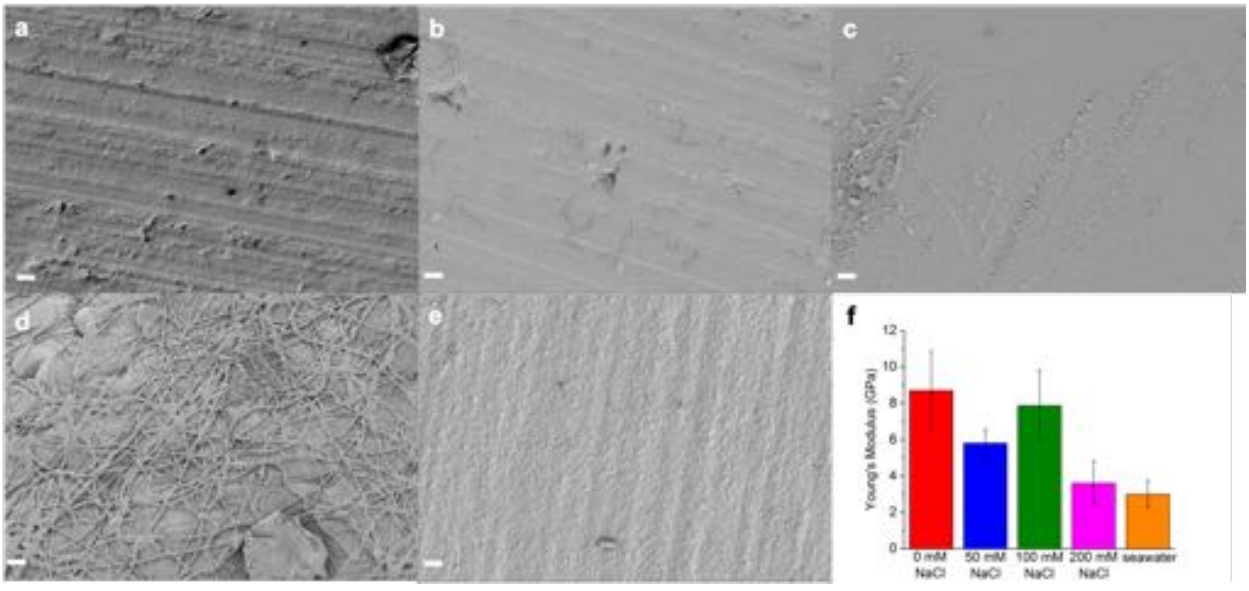
Supplement Figure 5.1. SEM of Silk film degradation with varying annealing times. A) 3hr Annealed silk film T=0 B) 6hr Annealed silk film T=0 C) 9hr Annealed silk film T=0 D) 3hr Annealed silk film T=4 weeks E) 6hr Annealed silk film T=4 weeks F) 9hr Annealed silk film T=4 weeks. Scale 10 μm.



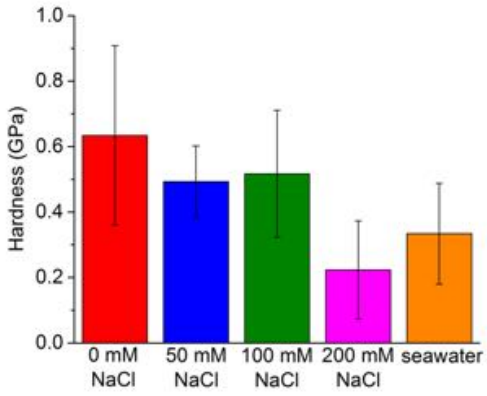
Supplement Figure 5.2. Mass loss profile and SEM of Silk film degradation in varying soil salinity with *R.tropici* embedded in film Week 4. A) 0mM NaCl B) 50mM NaCl C) 100mM NaCl D) 200mM NaCl. Scale bar 20 µm



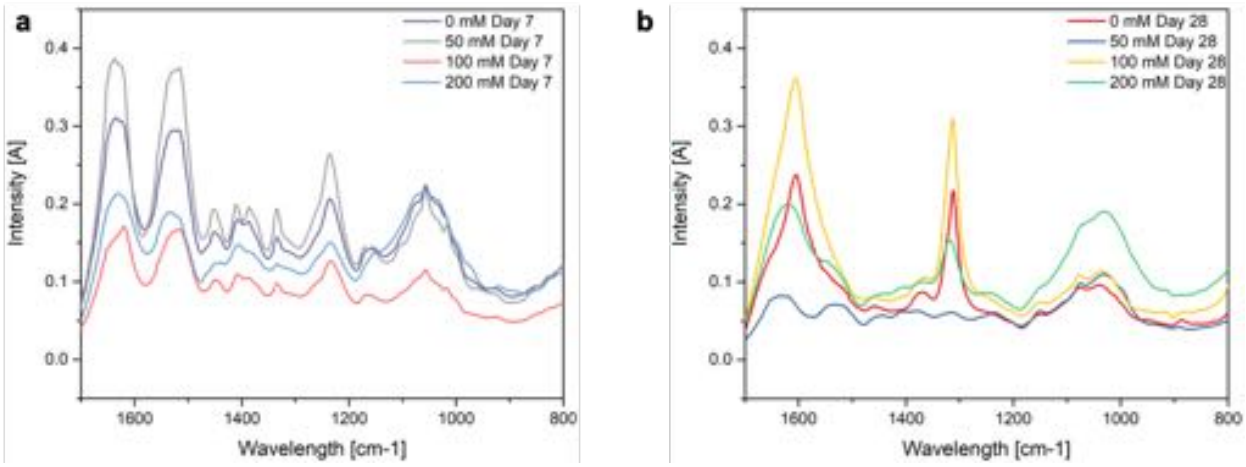
Supplement Figure 5.3. Mass loss profile and SEM of Silk film degradation in soil with no *R.tropici* embedded in film week 4. A) 0mM NaCl B) 50mM NaCl C) 100mM NaCl D) 200mM NaCl. Scale bar 20 µm.



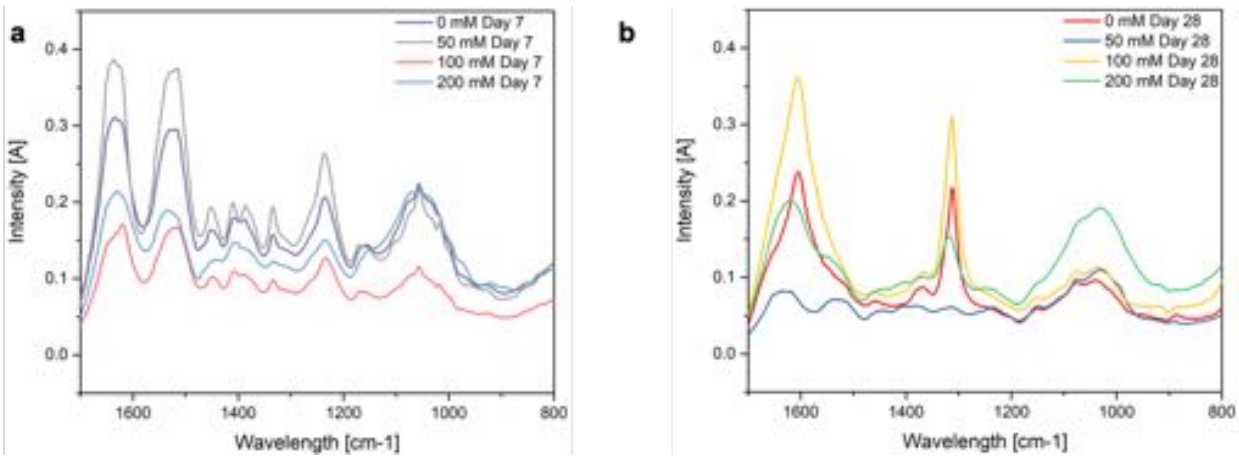
Supplement Figure 5.4. SEM of silk film degradation profile in solution with no *R.tropici* week 8. A) 0mM NaCl B) 50mM NaCl C) 100mM NaCl D) 200mM NaCl E) Sea water. Scale 10 µm F) Young's modulus (Nano-indentation).



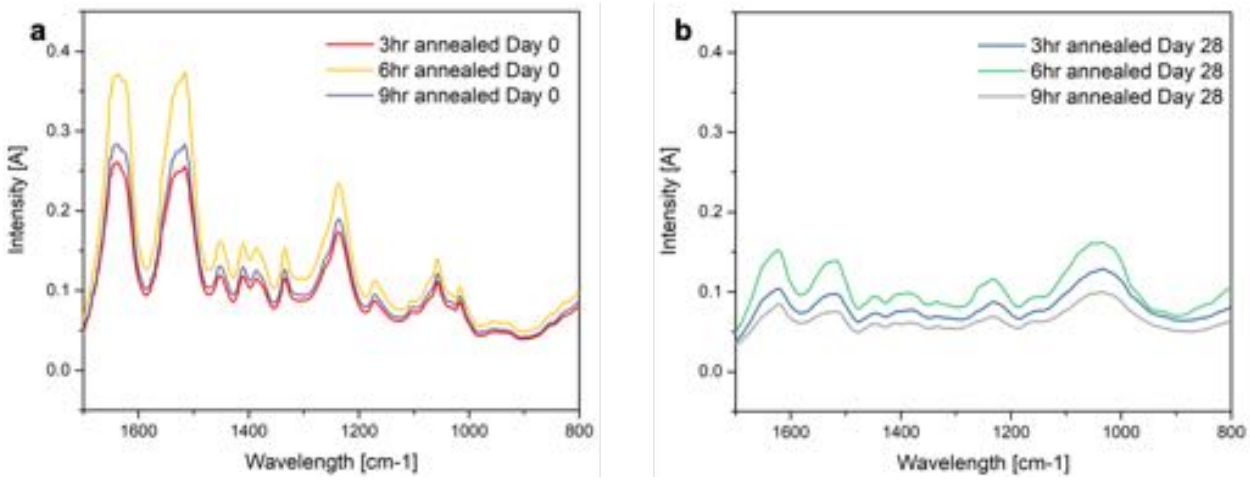
Supplement Figure 5.5. SEM of silk film degradation profile in solution with no *R.tropici* week 8. Hardness (Nano-indentation).



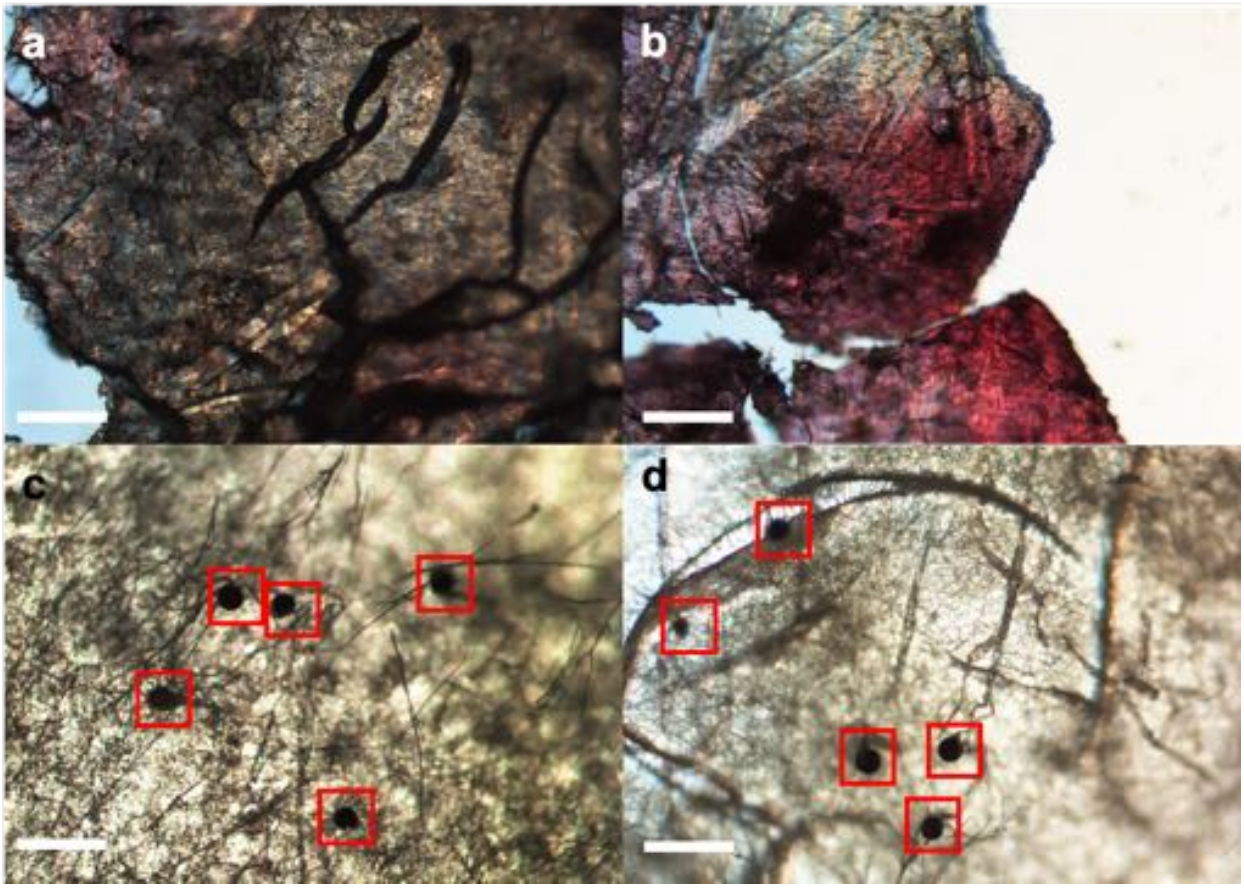
Supplement Figure 5.6. FTIR of degraded silk film with *R.tropici* in soil of varying salinity. A) Day 0 B) Day 28



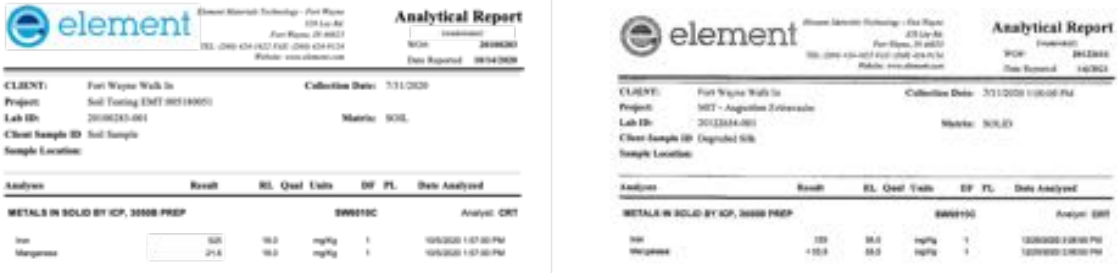
Supplement Figure 5.7. FTIR of degraded silk film in soil of varying salinity. A) Day 7 B) Day 28



Supplement Figure 5.8. FTIR of degraded silk film week 4. A) Day 7 B) Day 28



Supplement Figure 5.9. Microscope images of degraded silk film week 4. A) Silk B) Silk embedded with *R.tropici* C) Silk D) Silk embedded with *R.tropici* . Red boxes highlight microbial spores. Scale 100 µm.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Supplement Figure 5.10. ICP of degraded silk film week 4. Soil ICP shows presence of Iron and Manganese and silk ICP shows presence of Iron.

Chapter 6

Conclusions and Future Directions

6.1 Conclusions

Climate change and population growth will bring numerous challenges, and sustainable and resilient food production is one of them. The use of microbes as solutions to boost crop yields and lower environmental impact by using nitrogen fixing microbes and phosphate and potassium solubilizing microbes is currently underway. However, the delivery of microbes was not well studied and is key in the adoption of current and future use of microbe based biofertilizers. Spore forming microbes are very sturdy while non spore forming microbes are delicate and do not survive well outside the soil. The delivery (storage and administration) of non spore forming rhizobacteria on a seed surface, encompasses five tasks 1. Encapsulation 2. Desiccation 3. Preservation 4. Release 5. Colonization. The soil is very complex as numerous indigenous microbes (fungi, archaea and bacteria) are already well established. Utilizing silk fibroin (protein) and trehalose (disaccharide) we were able to push the boundary of knowledge on these key challenges in microbe delivery in the soil. We engineered the seed microenvironment.

The work of the dissertation was conducted through five different tasks listed below:
In Task 1, 2 and 3 (Encapsulation, desiccation and preservation), using silk and trehalose we were able to show rhizobacteria encapsulation, desiccation protection and preservation by the interaction of silk and trehalose by vitrifying our rhizobacteria (rhizobium tropici CIAT 899) for 4 weeks at room conditions. Chapter 3 of the dissertation highlights these studies. Further, the

impact of the delivered microbes in a controlled environment was carried out in saline soil and showed the benefits of the technique. Using the seed coating we were able to show increase in seed vigor and alleviate salinity effects.

In Task 4 and 5 (Release and colonization), using a bilayered approach highlighted in chapter 3 we show the ability to revive and culture microbes in situ for a more concentrated microbe release and better colonization of the plant. Using Phaseolus vulgaris as our model the seeds were water stressed in Morocco and with our collaborators we were able to show proliferation of microbes in situ, better colonization and alleviation of water stress during plant germination and early growth. Chapter 4 then detailed the degradation and payload release of silk based materials in the environment. This was important as most studies of silk fibroin degradation are focused on degradation in drug delivery and the human body. However, silk fibroin has the potential to have a large impact in environmental use.

The work conducted had some limitations and one of them was field testing. Technologies developed for agriculture require to be systematically tested in uncontrolled environments to ensure their efficacy in the real world. Some field trials however were conducted by our collaborators in Morocco and continue to be worked on. The Covid-19 pandemic slowed our progress.

6.2 Future research directions

We evaluated the delivery (storage and administration) of non spore forming rhizobacteria on a seed surface, encompasses five tasks 1. Encapsulation 2. Desiccation 3. Preservation 4. Release

5. Colonization. A highly interdisciplinary approach was utilized for this dissertation, providing insights into future research in various fields.

In agriculture, we require systematic field trials on 40 acres or more to understand the universal and uncontrolled environmental use of the technologies developed in this dissertation. Soil salinity and drought alleviation were targeted by the seed coatings. Therefore regions with saline soils and experiencing drought in Morocco should be studied. The control should use current rhizobacteria products and compare them with our product.

In material science, the seed coating parameters can further be optimized and this can be done in greenhouse conditions to understand the coating thickness and water volume required for germination in each region. Empirical model's can be derived and these substantiated by the experimental data. Empirical model's would increase the universal use of the technology and reduce the cost (time and financial) for deploying the technology in regions of varying climatic conditions. This will prove important as climate change effects make the climate more unpredictable thus systemic and scalable solutions are important. Furthermore, optimization of microbe delivery through microparticles should be investigated. Ultrasonic spraying and microemulsion technologies can be used to obtain monodispersed microplastics.

In biology, how our delivery technology affects the plant microbiome will be important to study. The technology developed delivers rhizobacteria into the plants microbiome, however how the microbiome structure is affected was not studied and this would provide better understanding on how the plant colonization is occurring and how long term the process is. In addition, how the seed coatings affect gene expression will be important in understanding how to better mitigate plant stressors.

In conclusion, this dissertation explores biopolymer assembly with bacteria during dehydration. Further, we studied how these biopolymers (silk and trehalose) can be used as a seed coating material to deliver rhizobacteria (Rhizobium tropici CIAT 899). In the future studying material interaction at the nanoscale level will be important. The obtained knowledge and interdisciplinary approach utilized in this study can be extended to various fields of study and used to develop different engineering applications.

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