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Self-Assembled Nanocoatings Protect Microbial Fertilizers for Climate-Resilient Agriculture

Benjamin Burke,[†] Gang Fan,[†] Pris Wasuwanich, Evan B. Moore, and Ariel L. Furst*



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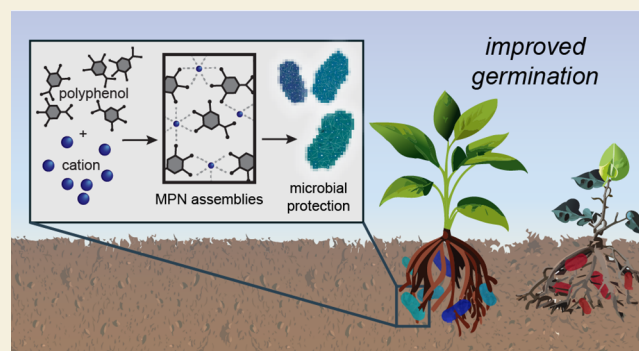


Supporting Information

ABSTRACT: Chemical fertilizers have been crucial for sustaining the current global population by supplementing overused farmland to support consistent food production, but their use is unsustainable. *Pseudomonas chlororaphis* is a nitrogen-fixing bacterium that could be used as a fertilizer replacement, but this microbe is delicate. It is sensitive to stressors, such as freeze-drying and high temperatures. Here, we demonstrate protection of *P. chlororaphis* from freeze-drying, high temperatures (50 °C), and high humidity using self-assembling metal-phenolic network (MPN) coatings. The composition of the MPN is found to significantly impact its protective efficacy, and with optimized compositions, no viability loss is observed for MPN-coated microbes under conditions where uncoated cells do not survive.

Further, we demonstrate that MPN-coated microbes improve germination of seeds by 150% as compared to those treated with fresh *P. chlororaphis*. Taken together, these results demonstrate the protective capabilities of MPNs against environmental stressors and represent a critical step towards enabling the production and storage of delicate microbes under nonideal conditions.

KEYWORDS: Regenerative agriculture, temperature-sensitive microbes, self-assembling nanomaterials, metal-phenolic networks, nanoencapsulation



INTRODUCTION

Chemical fertilizers have been essential to feed the current global population, supplementing overused farmland to support consistent food production. But these chemicals are unsustainable;^{1,2} they damage the environment,^{3,4} and they contribute to greenhouse gas emissions.^{5,6} Further, agricultural runoff containing chemical fertilizers can enter surface and groundwater^{7,8} and damage native ecosystems.^{9,10} In addition to the environmental concerns, chemical fertilizer is costly, representing between 20% and 36% of a farm's operating budget.¹¹ In the past two years, chemical fertilizer prices have nearly tripled, climbing from \$94 to \$232 per acre of corn.^{12,13} This cost impacts consumer food prices and can lead to decreased yields due to sub-optimal fertilizer application.¹¹ These environmental and economic impacts necessitate alternative agricultural solutions; land for agriculture is a finite resource, and its long-term sustainable use is essential for food stability.

Microbes offer sustainable alternatives to chemical fertilizers, especially due to their native role in plant growth.^{14–16} Microbes can efficiently process essential nutrients (*i.e.*, nitrogen, phosphorus, and sulfur) to render them bioavailable for plants, they can manipulate plant hormone signaling,¹⁷ and they can stop invasive pathogens.¹⁸ Because of their importance, researchers have worked to deliver microbes to

soil, but critical species are difficult to produce and transport because of their sensitivity to temperature¹⁹ and humidity.²⁰ Accordingly, a platform to stabilize delicate microbes against stressors during processing, storage, and transport would enable widespread agricultural adoption.

We recently reported metal-phenolic network (MPN) coatings that serve as synthetic spore coatings,^{21–23} protecting microbes from the drying process and anaerobes from oxygen exposure. MPNs are composed of polyphenols and metal ions that assemble into two-dimensional networks.^{24–27} Diverse polyphenols and metal ions have been used to assemble these materials, and the identities of the components have been linked to the physical properties of the final assembled network. Importantly for agricultural applications, the components of these materials are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA).

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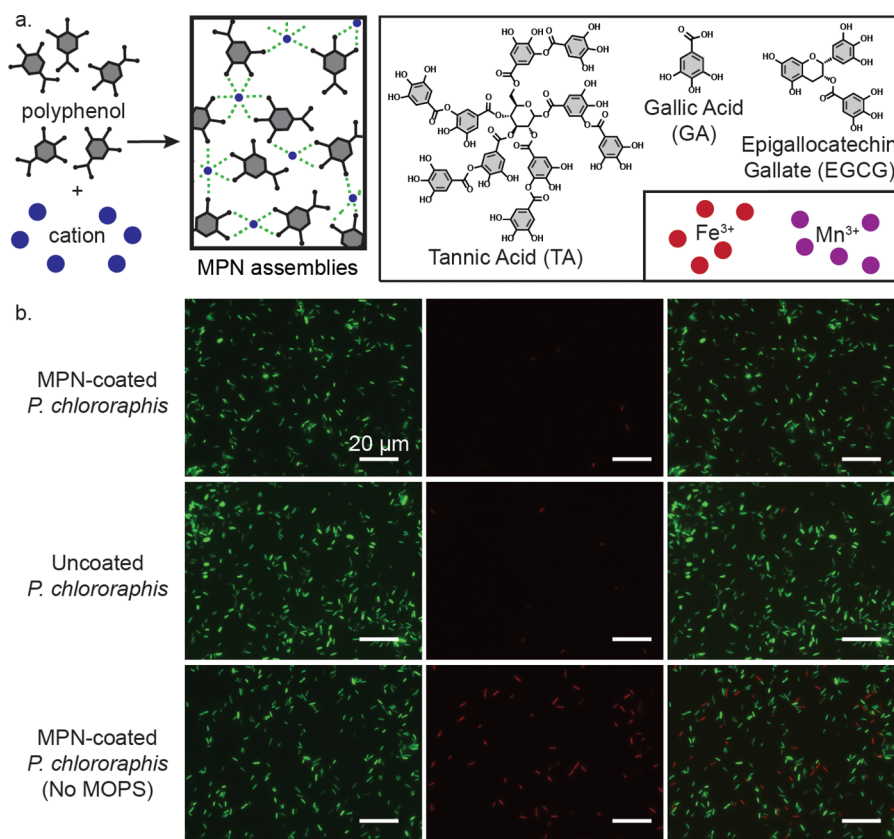


Figure 1. Metal–phenolic network (MPN) assembly on *P. chlororaphis* and its fluorescence images. (a) Cells were coated with a mixture of polyphenols (TA, EGCG, and GA) and metal ions (Fe and Mn). (b) Cells were treated with a live–dead staining kit. Shown are fluorescence images of uncoated and Fe^{III}-TA-coated *P. chlororaphis* in the FITC channel (SYTO-9) and TxRed channel (propidium iodide) and as merged images. Scale bars are 20 μm . The viabilities of *P. chlororaphis* were 98.3% (uncoated), 98.4% (Fe^{III}-TA-coated), and 79.6% (Fe^{III}-TA-coated but without MOPS addition).

Here, we establish the ability of these materials to protect the agriculturally-important nitrogen-fixing bacteria *Pseudomonas chlororaphis*.²⁸ *P. chlororaphis* natively interacts with plant roots and fixes nitrogen at them.^{29–31} It can also protect plants against phytopathogenic fungi and pests.^{32,33} However, *P. chlororaphis* is highly unstable at elevated temperatures, and prior efforts to protect this species have failed.³⁴ We demonstrate the protection of *P. chlororaphis* with MPNs. Importantly, we find that the MPN composition plays a critical role in protective efficacy, based on both the valency of the metal as well as the size and number of chelation sites on the polyphenol. Upon determining the optimal MPN composition for *P. chlororaphis* protection, the optimal compositions were shown to enable these microbes to survive harsh environmental exposure including lyophilization, storage at temperatures as high as 50 °C and humidity of 48% (relative humidity). We further demonstrate that our formulated *P. chlororaphis* is more beneficial to seed germination than even fresh microbes. Together, these results establish that MPN composition directly impacts protective behavior.

RESULTS AND DISCUSSION

We have shown that MPNs readily form on diverse prokaryotic microbes, including *Bacillus subtilis* and *Bacteroides thetaiotaomicron*.^{21–23} *P. chlororaphis* is a nitrogen-fixing species that is natively found in soil ecosystems and has been used as a bioinoculant for agriculture because of its beneficial properties, making it an important target for protection. Additionally, *P.*

chlororaphis is especially useful for studying the MPN protective efficacy because of its well-known temperature instability. In previous work, we have shown that MPNs can significantly improve protection of living biotherapeutics and probiotics to enable their delivery.^{21–23} Here, we find that the components of the MPN significantly impact their protective efficacy against freeze-drying, high temperatures, and high humidity.

MPN Formation on *P. chlororaphis* and Lyophilization Protection

MPNs are higher-dimensional coordination complexes of metal ions and polyphenols that form through self-assembly. Though a diverse array of polyphenols and metal ions can be used for MPN formation, we have focused on three well-studied polyphenols that are GRAS approved by the FDA (Figure 1a). These three polyphenols, tannic acid (TA), epigallocatechin gallate (EGCG), and gallic acid (GA), span the range of molecular diameters and number of chelation sites, with TA being both the largest with the most catechols and GA being the smallest with the fewest catechol groups. In the presence of a di- or trivalent cation, the polyphenols chelate and generate an extended coordination network. We have generally observed more structurally rigid assemblies with trivalent cations, and we want to focus on metals that are beneficial and have low risk of cytotoxicity.^{25,35–37} We therefore focused on Fe³⁺, Mn³⁺, Al³⁺ and Zn²⁺ as metal components of the MPN. Upon transition of the MPN formation solution to a

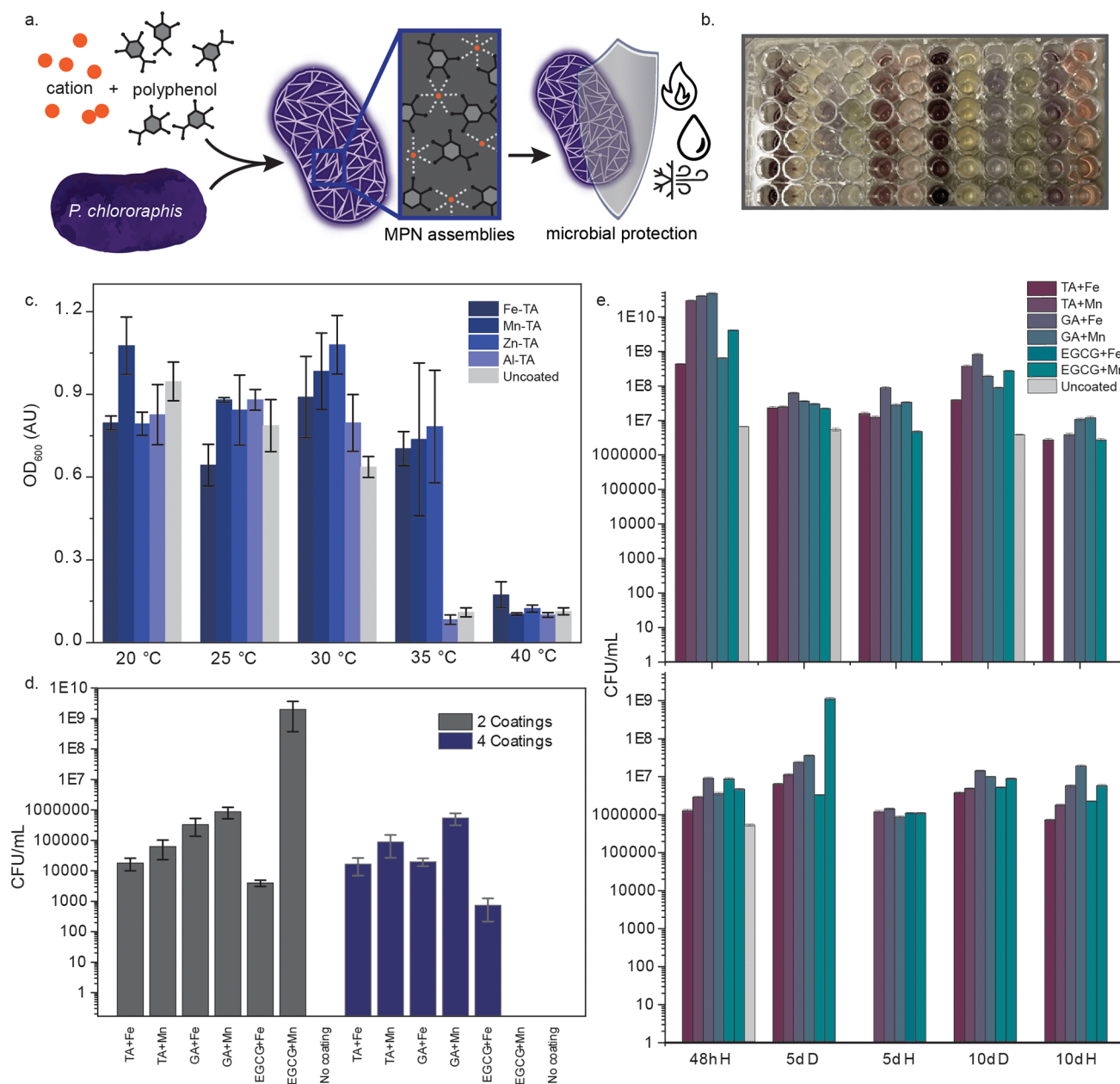


Figure 2. MPN protection of lyophilized *P. chlororaphis* under extreme temperatures. (a) Overview of workflow for microbial protection with MPNs, followed by exposure to stressors. (b) Photo of 96-well plate containing *P. chlororaphis* coated in MPNs composed of diverse metal ions and polyphenols. The bright colors are indicative of the ligand-to-metal charge transfer band that emerges. (c) OD₆₀₀ of lyophilized *P. chlororaphis* following 48 h of growth in the presence and absence of tannic-acid-based MPNs. The lyophilized cells were stored at various temperature (20, 25, 30, 35, and 40 °C) and 43% relative humidity (RH) for ten days before inoculations. (d) CFU counts from serial dilution plating of MPN-coated lyophilized *P. chlororaphis* after storage under 50 °C and 48% RH over five days (The coating thickness was controlled by repeating the coating step multiple times). Error bars represent standard deviations of $n = 3$ replicates. (e) CFU counts from serial dilution plating of lyophilized *P. chlororaphis* under extreme conditions (top 37 °C, bottom 50 °C, relative humidity varies) using a mixture of polyphenols (TA, EGCG, and GA) and metal ions (Fe and Mn). (h = hours, d = days, H = 43% RH under 37 °C or 48% RH under 50 °C, and D = dry condition.) Error bars represent standard deviations of $n = 50$ replicates.

pH above 8.0, the metal ions chelate the phenolic groups of the polyphenols, forming well-defined coordination centers.

Through UV–vis spectroscopy, we readily established the formation of MPNs on *P. chlororaphis* through the appearance of a well-defined ligand-to-metal charge transfer (LMCT) band upon full deprotonation and coordination of the network. We have previously demonstrated that the appearance of this LMCT band corresponds to the formation of contiguous core-shell coatings on microbial substrates (Figure 2b).²² In

addition to the UV–vis verification of MPN formation, we imaged cells using a fluorophore-BSA complex that specifically interacts with MPNs (and not uncoated cells, Figures S1, S2). We confirmed through these images that the MPNs form a contiguous coating on the microbes that is not present on the uncoated cells. The consistent fluorescence across the surface of the coated cells confirms that uniform core-shell structures are formed on these microbes, as they have for us in previous reports.^{21–23} Moreover, as with other strains we evaluated, the

viability of MPN-coated *P. chlororaphis* (98.4%) is comparable to that of the uncoated controls (98.3%) immediately following coating. This was assessed by live and dead staining with fluorescence microscopy (Figure 1b). We also found that the addition of a MOPS buffer was critical for maintaining the viability of MPN-coated *P. chlororaphis*. In contrast, loosely formed MPNs and low pH were found to negatively affect cell viability.²¹⁻²³ Both of these results are critical, as the components of the MPNs on their own are associated with toxicity, which we observe when they are not set by using a high pH buffer. Thus, we demonstrate that setting the MPN coating with a basic MOPS buffer is necessary to observe the MPN protective effects.

Following confirmation of MPN formation on the bacteria and validation of viability maintenance, we evaluated MPN protection against key stressors that *P. chlororaphis* would encounter during formulation and production. After growth, the first step in processing microbes is to enable their effective transport and delivery via drying. However, such processes cause significant stress and damage to cells, leading to decreases in viability of between three and six orders of magnitude. Thus, we evaluated the impact of the MPNs on protecting *P. chlororaphis* from the freeze-drying (lyophilization) process. Lyophilization generally requires freezing a sample in liquid nitrogen, followed by sublimation of the ice under vacuum. Both steps cause significant stress to microbes. However, we previously demonstrated that MPNs significantly improve survival following lyophilization, even without additional cryoprotectants, which we also observe here.

For evaluation of stress tolerance, we coated *P. chlororaphis* in either two or four layers of MPNs composed of the aforementioned components. Two and four layers of MPNs were selected because we find these to be the best balance between protection and nonhindered microbial growth. Two layers are the minimum required to observe protection with many strains, and four layers provide additional protection in many cases but do not significantly hinder microbial division. Additional coatings beyond 4 layers significantly inhibit microbial division and often do not offer additional protective benefits.

Following coating and drying the microbes, we evaluated their viability using both optical density for growth analysis at 600 nm (OD_{600}) and serial dilution plating for colony-forming unit (CFU) counting. We additionally evaluated *P. chlororaphis* growth following coating, lyophilization, and rehydration using a viability stain. The MPN-coated *P. chlororaphis* reached exponential phase after incubation for 15 h, whereas exponential growth of the uncoated control was delayed to 35 h (Figures S3, S4). This improvement in growth lag indicates that MPN-coated *P. chlororaphis* samples have more viable cells remaining than their uncoated counterparts. Because of the success of MPN protection against these initial stressors for *P. chlororaphis*, we investigated additional critical stressors.

Temperature Stability of MPN-Coated *P. chlororaphis*

A major challenge with *P. chlororaphis*, among many other living microbial agriculture products, is their long-term stability at temperatures above refrigeration. Cold storage and transport are the standard for biologics, but these processes are costly and energy-intensive and require centralized facilities for storage. Additionally, over one billion people globally live farther than 20 km from an all-weather road. This limitation

prevents access to large transportation vehicles required for most cold transport, which is currently essential for transport of agricultural products.^{38,39} Because of the temperature sensitivity of *P. chlororaphis* combined with the need for methods of storage and transport that do not require a cold chain or temperature control, we next evaluated the efficacy of diverse MPN coatings to protect these microbes from high temperatures and humidity.

At 4 °C, the temperature of refrigerated cold-chain transport, even uncoated bacteria survive (Figure S5). However, the advantage of MPNs lies in their ability to protect microbes at temperatures at which uncoated cells do not survive. In our previous work, we had observed that the composition of the MPN coating alters the efficacy of protection.²¹ Yet, the specific components that would enable optimal survival against specific stressors cannot yet be determined *a priori*. We therefore screened different MPN compositions composed of metal ions and polyphenols for *P. chlororaphis* stability. As can be seen in Figure 2a, at low temperatures, there is no statistical significance between the survival of microbes coated with the different MPNs. However, at the transition temperature of 35 °C, no uncoated cells survive, while some of the coated microbes do. This difference is statistically significant ($p < 0.005$) and demonstrates the large difference in survival afforded by optimized MPNs. The marked survivability of *P. chlororaphis* at 35 °C with only certain MPNs further confirms that the MPN composition remains an essential factor in protection.

The lack of a difference in the protective efficiency of MPNs across metal ions evaluated with TA motivated further screening of polyphenols (Figure 2d). In prior work, the rigidity and thickness of the MPN coating has been determined for a variety of polyphenols and metal ions. Both the number of chelation sites (the number of catechols in the molecule) and the cation valency impact the thickness and morphology of coatings, ranging from somewhat rigid coatings 10 nm thick (usually found with TA and a trivalent cation) to very rigid and thin coatings only about 2 nm thick (usually observed with GA and a trivalent cation such as Fe^{3+} and Mn^{3+}). Thus, we expected the physical properties of the MPNs to differ more significantly upon variation of the polyphenol.

As expected, much larger differences in protective efficacy were observed upon variation of the polyphenol as compared to the metal (Figure 2d). In fact, these results highlight the critical importance of evaluating each of the three variables tested: metal ion used in the MPN, polyphenol used in the MPN, and the number of coating layers used. As is evidenced from the two-coating data for EGCG and Mn, this coating offers significantly more effective protection than the other coatings, with statistical significance of $p < 0.05$ for the other coatings. In contrast, this same coating yielded no viable cells if it was applied four times to cells (Figure 2d, right). We attribute this to the rigidity of this coating, which provided protection at two layers but inhibited growth to the point where no cells grew with four coatings. While these experiments demonstrated that some MPN combinations provided little to no protection of cells, the ability of some MPNs to protect against the initial exposure challenges demonstrated the need for further investigation.

Following the initial MPN testing, a screen of post-lyophilization storage conditions was performed. The variables selected for screening are based on the harsh conditions to which these microbes could be exposed during transport and

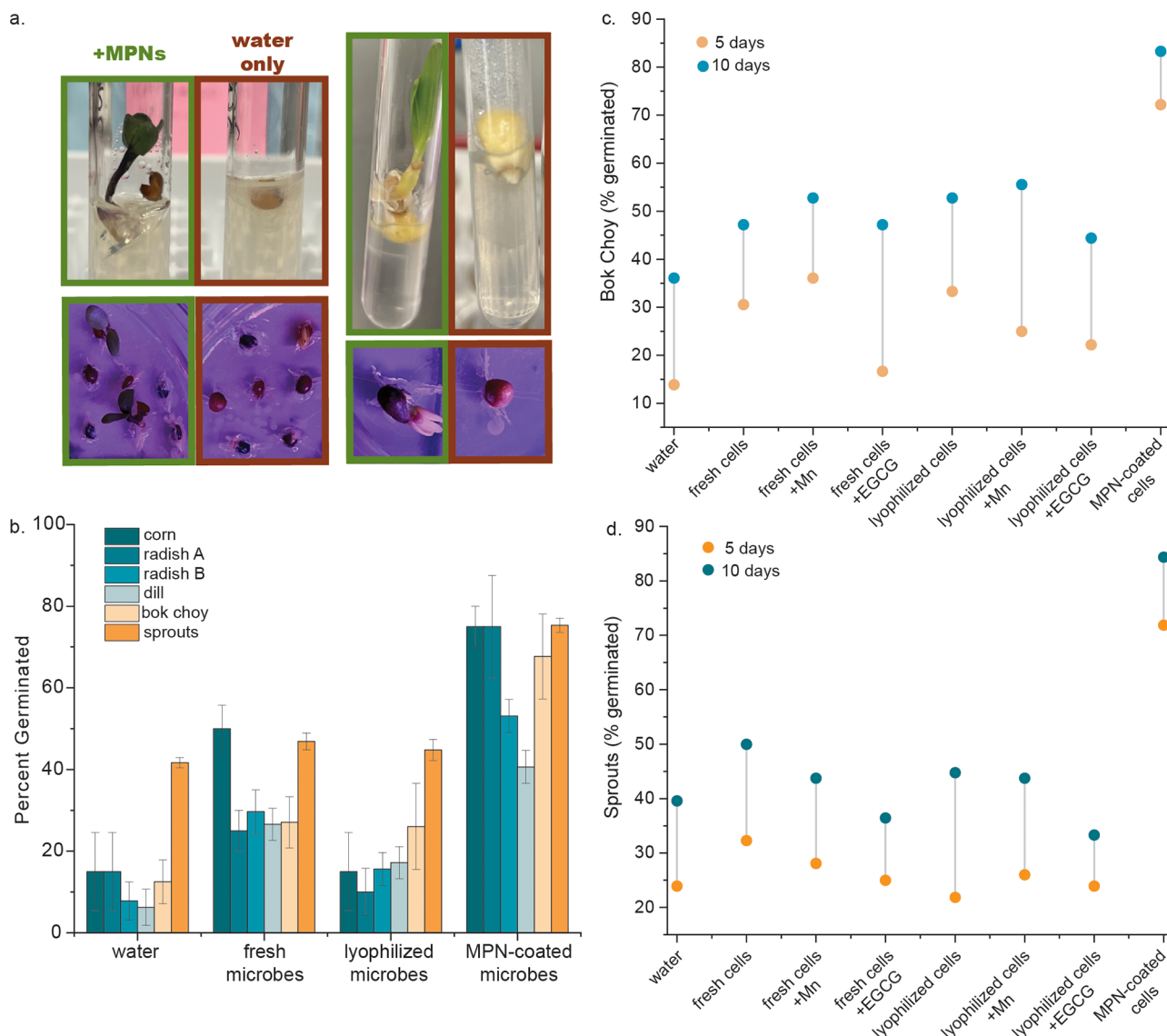


Figure 3. Impact of MPN-coated *P. chlororaphis* on seed germination. (a) Images of seeds treated either with MPN-coated, lyophilized *P. chlororaphis* (green box) or with water only (red box). (b) Percentages of seed germination after applying water-only, fresh live, lyophilized, Mn^{II}-EGCG-coated lyophilized *P. chlororaphis* in agar plates for five days. Error bars represent standard deviations of $n = 3$ replicates. Percentages of (c) bok choy seed and (d) soy bean germination after applying water-only, fresh live cells only, fresh live cells with Mn^{II}, fresh live cells with EGCG, lyophilized cells only, lyophilized cells with Mn^{II}, lyophilized cells with EGCG and Mn^{II}-EGCG-coated lyophilized *P. chlororaphis* in agar plate for five or ten days. The lyophilized cells (both coated and uncoated) were stored at 50 °C and 48% RH for five days before incubation with seeds.

storage to farms where they would be distributed. Critically, many of these farms are not accessible by paved roads and do not have temperature-controlled storage for their seeds and fertilizers, so necessitating cold storage would be a non-starter. Thus, we focused on temperature and humidity preservation to ensure that the microbes survive under conditions under which they could be stored in farmland in South America and South Asia. As we previously identified differences in MPN efficacy based on the polyphenol and metal ions used for assembly on these microbes, we anticipated the observation of similar trends here in the more expansive study. Variables for stress testing of the cells included high temperatures and humidity for incubation times on the order of production and transport times to potentially challenging agricultural locations. We therefore evaluated incubation times ranging from 48 hours to 10 days. The upper temperature limit for the experiment was set at the estimated temperature a storage shed in Brazil would

reach during a warm day in the planting season, where outdoor temperatures reach 43 °C and 70–80% humidity.

Because of the differences in recovery observed with two and four coatings in our initial experiments (Figure 2d), for our larger evaluation of stressors, we evaluated only two coatings. As seen from CFU data (Figure 2e), MPNs are capable of protecting *P. chlororaphis* under the harshest transport and storage conditions, even enabling growth following multiple days at the highest temperature and humidity (50 °C, 48% RH for ten days, Figure 2e). Importantly, the uncoated microbes demonstrated little to no survival under similar conditions (Figure 2e). In fact, it is critical to emphasize that the uncoated microbes did survive some conditions at 37 °C storage, but in every case, 1–2 orders of magnitude fewer uncoated cells survived as compared to the coated cells. Additionally, the uncoated cells fared significantly worse under humid conditions. At 37 °C storage with humidity, no uncoated

cells survived. Similarly, beyond 48 h of exposure to 50 °C, no uncoated cells survived, while many combinations of MPNs effectively protected cells. Finally, we observed significantly improved survival of coated microbes at elevated temperatures (30 °C) for extended lengths of time (two months) due to MPN coatings (Figure S4). These results emphasize the importance of MPNs for microbial protection.

Interestingly, for the majority of the stressor studies, no specific MPN significantly outperforms the rest. Based on the optimal coatings, which are GA and Fe, GA and Mn, and EGCG and Mn, we anticipate that protection against heat and humidity is dependent on the rigidity of the coatings, as the thinner, more rigid coatings formed from GA and EGCG were favored for protection in these cases.

Impact of Microbes on Seed Germination

Though the protection of microbes is important, we additionally sought to investigate whether the impressive recovery results would translate to seed germination rates, the ultimate goal of *P. chlororaphis* use. We applied fresh live, lyophilized and MPN-coated, or just lyophilized *P. chlororaphis* to a variety of seeds, including corn, bok choy, sprouts, two types of radishes, and dill (Figure 3b). The MPN employed for seed germination studies was the one found to be optimal in the initial studies (Figure 2d) and continued to be one of the best coatings across the expanded stressor experiments (Figure 2e), which was EGCG and Mn. Initial studies on a variety of seeds were important to establish whether any observed germination effects were seed-dependent. As can be seen, MPN-coated cells, despite their harsh storage conditions at 50 °C and 48% RH, yielded higher rates of germination than any other condition, including seeds treated with the fresh cells. Within each type of seed, statistically significant differences were observed between the conditions ($P < 0.05$). These initial studies provided support for our hypothesis that seeds growing in nutrient-depleted environments would benefit from the presence of nitrogen-fixing bacteria, such as *P. chlororaphis*. However, these initial studies did not enable clarification of why the MPN-coated cells were superior even than to fresh cells.

This lack of clarity as to whether improvements were due to the cells themselves, the components of the MPNs, or a combination of the two led to further investigation of the germination rate that incorporated additional controls. To further investigate the observed improvement, we studied germination with bok choy and sprout seeds because they are relatively rapid germinators (Figure 3c,d). For bok choy, after five days, the fresh cells improved germination over the water-only cells by 16.7%, while the lyophilized bacteria exhibited slightly smaller improvements (13.7%). Importantly, MPN-coated cells improved germination by 58.3%, significantly more than under any other conditions, including fresh cells with the individual MPN components added (Figures 3c, S7).

After ten days, overall germination increased, but the relative percentages of germinated cells remained consistent. Similar results were observed with sprout seeds. After five days, the fresh cells had 8.3% improved germination over water-only, while applying lyophilized cells had slightly lower germination than the water alone, although this difference is not statistically significant (2.1% less) (Figure 3d, S7). In contrast, MPN-coated cells had 47.9% higher germination than that of water only. Additionally, combining freeze-dried uncoated *P. chlororaphis* with alginate beads (500 nm diameter) coated in

the MPN did not show statistically significant differences relative to the seeds treated with only freeze-dried cells (Figure S8). These improvements confirm that it is the combination of the MPN coatings with the microbes that leads to improvements, even when the microbes are stored under exceptionally harsh conditions. The results further demonstrate the immense power of MPNs to maintain microbial viability and directly translate that to improved seed germination.

CONCLUSIONS

Taken together, these results demonstrate the power of some MPN assemblies to protect temperature-sensitive microbes from high temperatures and humidity. Critically, the microbes evaluated are capable of fixing nitrogen in soil and could be used in regenerative agriculture applications. As expected, MPNs protected *P. chlororaphis* from lyophilization, as we have shown with other strains previously. Importantly, though, upon screening combinations of polyphenols and metal ions, we found MPN combinations that protect microbes from exceptional heat and humidity. Importantly, these are not necessarily the types of MPNs that we have previously identified as optimal, as they are assembled with smaller polyphenols that form thinner, more compact shells on microbes that seem to be more rigid than some of the larger polyphenols that we have tested. Following the optimization of the coatings, we demonstrate long-term protection. Our ability to protect *P. chlororaphis* for 10 days at extremely high temperatures (50 °C) and humidity as well as slightly lower temperatures (30 °C) for months pushes the protective ability of our coatings into the realm of utility for regenerative agriculture by negating the need for either microbial growth in bioreactors on-site or employing cold chain transport to enable the microbes to reach their destination. Further, we demonstrated that the microbes protected by MPN coatings yielded significantly higher germination rates for multiple types of seeds and, more specifically, bok choy and sprouts, as compared to seeds treated with fresh microbes. These results are promising for the application of these microbes as replacements for chemical fertilizers at scale. Long-term, we anticipate these coatings will enable the storage and transport of critical microbial species without cold chain transport and enable microbes to be viable, sustainable alternatives to chemically-produced fertilizer.

METHODS

Encapsulation of Bacteria with MPNs

P. chlororaphis were coated in MPNs as previously published.¹ Briefly, 125 μL of aqueous solution of phenol (TA-1.6 mg mL^{-1} ; GA-1.5 mg mL^{-1} ; EGCG-2.7 mg mL^{-1} , 50% from tea extract) and 125 μL of aqueous solution of the cation to be used (iron chloride (0.24 mg mL^{-1}), zinc sulfate heptahydrate (0.42 mg mL^{-1}), aluminum chloride (0.19 mg mL^{-1}), or manganese sulfate monohydrate (0.72 mg mL^{-1})) were added sequentially to an aqueous suspension of cells (250 μL , OD_{600} of 3.0). The resulting suspension was mixed vigorously for 10 s. A volume of 0.5 mL of MOPS buffer (20 mM, pH 7.4) was then added to form a stable MPN shell. The resulting encapsulated cells were washed with ultrapure water three times to remove any residual starting materials. The coating process can be repeated as many times as desired; here, we either used two total coatings or four total coatings.

Germination Studies

Seed germination studies were performed in 0.5% agar plates made with ultrapure water. Seeds were inserted in the agar to halfway down the seed, and 10 μ L of the relevant microbial sample was added to each seed. Samples were either ultrapure water or microbes and MPN components in ultrapure water. For samples containing cells, 1×10^7 bacteria per seed were added. To add seeds, *P. chlororaphis* was diluted to concentrations of 1×10^9 bacteria/mL in ultrapure water. Seeds were allowed to incubate for five days with the Petri dish cover on at ambient temperature under grow lights. Germinated seeds were then counted. For ten-day studies, the lids of the dishes were removed for the second half of the incubation time (the final five days). Following ten days of incubation, the germinated seeds were counted.

■ ASSOCIATED CONTENT

Data Availability Statement

All data are available in the main text or the [Supporting Information](#).

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacsau.3c00426>.

Additional methods and results (Figures S1–S8) ([PDF](#))

■ AUTHOR INFORMATION

Corresponding Author

Ariel L. Furst – Department of Chemical Engineering and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States; orcid.org/0000-0001-9583-9703; Email: afurst@mit.edu

Authors

Benjamin Burke – Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

Gang Fan – Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States; orcid.org/0000-0002-4185-5692

Pris Wasuwanich – Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States; orcid.org/0000-0003-4590-3863

Evan B. Moore – Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

Complete contact information is available at: <https://pubs.acs.org/10.1021/jacsau.3c00426>

Author Contributions

[†]B.B and G.F. contributed equally

Notes

The authors declare the following competing financial interest(s): A patent on MPNs has been filed through MIT by A.L.F.

Safety Considerations: No unexpected or unusually high safety hazards were encountered.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on October 30, 2023, with an error in Figure 3a. The corrected version was reposted November 9, 2023.