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Core-shell encapsulation formulations to stabilize desiccated *Bradyrhizobium* against high environmental temperature and humidity

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Summary

Engineered materials to improve the shelf-life of desiccated microbial strains are needed for cost-effective bioaugmentation strategies. High temperatures and humidity of legume-growing regions challenge long-term cell stabilization at the desiccated state. A thermostable xeroprotectant core and hydrophobic water vapour barrier shell encapsulation technique was developed to protect desiccated cells from the environment. A trehalose core matrix increased the stability of desiccated *Bradyrhizobium* by three orders of magnitude over 20 days at 32°C and 50% relative humidity (RH) compared to buffer alone; however, the improvement was not deemed sufficient for a shelf-stable bioproduct. We tested common additives (skim milk, albumin, gelatin and dextran) to increase the glass transition temperature of the desiccated product to provide further stabilization. Albumin increased the glass transition temperature of the trehalose-based core by 40°C and stabilized desiccated *Bradyrhizobium* for 4 months during storage at high temperature (32°C) and moderate humidity (50% RH) with only 1 log loss of viability. Although the albumin-trehalose core provided exceptional protection against high temperature, it was ineffective at higher humidity conditions (75%). We therefore incorporated a paraffin shell, which protected desiccated cells against 75% RH providing proof of concept that core and shell encapsulation is an effective strategy to stabilize desiccated cells.

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Introduction

Biotechnology products such as crop and bioremediation inocula, biobanked cell culture starters, and cell-based biosensors require long-term stabilization of bacteria. While liquid inocula are the current standard (Schoebitz and López Belchí, 2007; Tittabutr *et al*., 2007), stabilization of rhizobial crop inocula by desiccation promises a longer shelf-life, decreasing storage and shipping costs as cryogenic storage requirements are relaxed and product weight is reduced. However, the challenges associated with storing a desiccated product at locations with high environmental temperature and humidity remain.

Bacteria have various strategies to overcome desiccation stress, including intracellular accumulation or synthesis of desiccation compatible solutes (e.g. polyols such as glycerol, sugars such as trehalose and amino acid derivatives such as glycine betaine) and production of an exopolysaccharide layer (Siderius *et al*., 2000; Santos and Da Costa, 2002; Lebre *et al*., 2017; Singh *et al*., 2018). Some bacteria and microbes, such as *Arthrobacter*, *Bacillus* and *Clostridium* species, form specialized structures such as cocci or spores, that are desiccation resistant (Mongodin *et al*., 2006; Setlow *et al*., 2014). Others, such as *Deinococcus radiodurans*, employ different strategies, such as an exceptionally efficient DNA repair mechanism, against environmental stresses (Slade and Radman, 2011).

Rhizobial species, nitrogen fixing bacteria that form symbiotic relationships with legumes, have been used for over 120 years as soil inoculum for bioaugmentation to boost yield of crops such as soybeans (Zahran, 1999; Santos *et al*., 2019). Rhizobial strains have been stably desiccated and stored at room temperature at 0% RH (Mugnier and Jung, 1985). Environmental humidity and temperature are easy to control in the laboratory setting. However, during transport, storage and field use of the

desiccated product, the environmental conditions are almost impossible to control, and large quantities of the product may be exposed to the extremes of temperature and humidity. For example, one main challenge facing wide-spread use of desiccation for crop inocula is that desiccated rhizobia, even though stable at low humidity, lose viability when exposed to high humidity (Mugnier and Jung, 1985; Mary *et al.*, 1993; Mary *et al.*, 1994). Our data also indicate that desiccated *Bradyrhizobium* lose viability fast (3 logs of loss in viability in 3 weeks) if the cells are exposed to moderately high-relative humidity (50%) environment. Sensitivity of the desiccated product to high humidity and temperature is especially critical in legume-growing regions of the world where both are high during the growing season, such as Midwest United States and Midwest and Southern Brazil. Therefore, to ensure stability and long shelf-life, desiccated bacteria need to be shielded from the adverse effects of both high humidity (RH > 50%) and high temperatures (T > 28°C).

Taking cues from the natural response of bacteria to desiccation, we aimed to create a multilayer, spore-like structure by encapsulating *Bradyrhizobium* in a xeroprotectant, hydrophilic core matrix coated with a hydrophobic vapour barrier shell to protect the desiccated cells from high temperature and high humidity exposure (Fig. 1). Vitrified trehalose-based matrices offer a promising solution for long-term storage of desiccated bacteria (Potts, 1994; Julca *et al.*, 2012). Because vitrified matrices destabilize above their glass transition temperature, we explored the use of polysaccharide and protein additives to increase the glass transition temperature of the trehalose-based formulations. Both dextran and albumin increased the glass transition temperature of the vitrified core matrix above the target storage temperature of 32°C; and in a matrix consisting of trehalose, sucrose and albumin, desiccated *Bradyrhizobium* were stable for 4 months at 50% RH, 32°C with only 1 log loss of viability. The optimized core matrix alone was unable to protect the desiccated cells from high humidity; however, the application of a water vapour barrier shell of paraffin protected the cells against exposure to high humidity at 75%.

Results and Discussion

Design of a vitrified trehalose-based core matrix for high temperature stability

Desiccated bacteria are stable if stored at 0% RH and moderate temperatures; however, it is not practical to store large quantities (e.g. 20 kg bags) of the desiccated product at 0% RH. Therefore, we focused on the challenge of stabilizing desiccated *Bradyrhizobium* at suboptimal storage conditions of high environmental humidity (75% RH) and temperature (32°C).

One possible configuration for the desiccated cell product is small (1–2 mm diameter) granules ready to be applied on the soil. To produce the granules, *Bradyrhizobium* suspended in 5 µl low-sodium phosphate buffer saline (LSPBS) were deposited in individual wells of a 96-well plate and stored at 50% RH and 32°C (Fig. 2). This experiment established a baseline storage stability for the bacteria. Under these conditions, bacteria lost viability very rapidly (3 log loss in CFU in 3 weeks). Note that in these experiments, samples reached their final water content after 8 h and did not dry further (Fig. S1).

There are certain chemicals that can be used extracellularly (and intracellularly) to enhance the desiccation resistance and stability of organisms (Leslie *et al.*, 1995; Sun and Leopold, 1997; Welsh, 2000; Stewart and He, 2019). Ideal candidates for desiccated state stabilization are carbohydrates that form a glass upon drying (Sun and Leopold, 1997; Crowe *et al.*, 1998; Loi *et al.*, 2013). Preferably, for long-term storage stability, the glass transition temperature (T_g) of the dried cells should be higher than the desired storage temperature. Trehalose is a disaccharide with a high glass transition temperature that has previously been used successfully

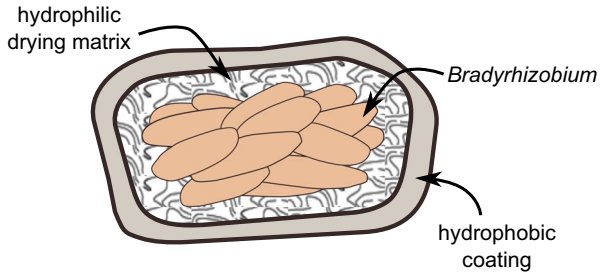

Fig. 1. Schematic of the core-shell encapsulation technology to protect desiccated *Bradyrhizobium* against high humidity and temperature. The hydrophilic matrix stabilizes the dried cells at high temperatures and the hydrophobic outer coating protects them against high environmental humidity.

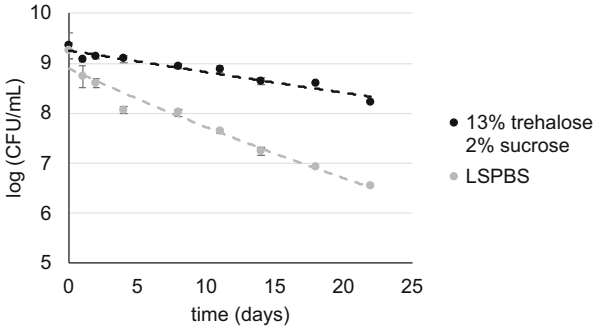

Fig. 2. Extracellular trehalose improves viability of *Bradyrhizobium* stored at 50% RH, 32°C. Cells were suspended in LSPBS or in LSPBS containing 13% trehalose and 2% sucrose.

for stabilization of bacteria (Crowe *et al*., 1998; Chen *et al*., 2000). However, trehalose has some drawbacks; (i) during drying of the cell solution, it is known to form a skin, which slows down desiccation rates significantly (Romdhane *et al*., 2001; Aksan and Toner, 2004), (ii) when used in formulations by itself, it is known to crystallize, losing its xeroprotectant capability (Surana *et al*., 2004), and (iii) it forms a very fragile glass, as its T_g is a very strong function of water content (Chen *et al*., 2000), making trehalose glasses very susceptible to fluctuations in environmental humidity. Therefore, we decided to include sucrose with trehalose in the xeroprotectant formulation. Even though sucrose is a disaccharide very similar to trehalose molecularly and in terms of its physico-chemical properties, it is known to inhibit trehalose crystallization (Sundaramurthi and Suryanarayanan, 2011). At high concentrations, trehalose is more susceptible to crystallization; therefore, we chose a dilute xeroprotectant solution containing 13% trehalose with 2% sucrose to prevent crystallization and skin formation (Romdhane *et al*., 2001, Aksan and Toner, 2004). As expected, in the presence of extracellular trehalose and sucrose, stability of the desiccated *Bradyrhizobium* was enhanced. However, when stored at 50% RH, and 32°C *Bradyrhizobium* still lost viability, albeit at a slower rate (1 log loss in CFU in 3 weeks) (Fig. 2).

One possible explanation for the continued loss of viability in the presence of trehalose sucrose solution is that to be fully protective, the xeroprotectant chemicals should be present on both sides of the membrane (Elbein *et al*., 2003; Crowe, 2007). As both sucrose and trehalose are membrane impermeable, they need to be uploaded intracellularly by reversibly permeabilizing the bacterial membrane. We used a variety of approaches to transiently permeabilize the cell membrane using thermocycling through the membrane phase transition temperature, electroporation, thermal shock and chemical permeabilization (Helander *et al*., 1997; Satpathy *et al*., 2004; He *et al*., 2006; Gurtovenko and Anwar, 2007; He *et al*., 2012; Vaessen *et al*., 2019), but neither the internal concentration of trehalose nor the storage stability of the cells could be increased further (Tables S1–S6). The resistance to reversible permeabilization is likely due to the structure of the *Bradyrhizobium* membrane as hopanoids, sterol-like compounds that contribute to low fluidity and permeability, can constitute up 40% of the inner membrane lipid content (Kannenberg *et al*., 1995; Belin *et al*., 2018).

Another possible explanation for the poor stability of *Bradyrhizobum* desiccated in 13% trehalose, 2% sucrose is that the glass transition temperature (T_g) of the dried solution was not higher than the storage temperature (Hancock *et al*., 1995), and/or the sucrose concentration in the solution did not fully inhibit trehalose crystallization. To test these possibilities, we measured the T_g of the dried solution with and without cells using differential scanning calorimetry (DSC). Equilibrated at 0% RH, T_g of the 13% trehalose, 2% sucrose solution was measured to be around 18°C; however, in the presence of cells T_g increased to 60°C (Table 1), suggesting that stability of the cells stored at 0% RH should be high. Also at 0% RH, we observed that addition of 2% sucrose to the trehalose matrix increased the T_g slightly from 51 to 60°C, potentially by sucrose disrupting trehalose skin formation and enabling lower water content, supporting the decision to add sucrose to the xeroprotectant solution. In contrast, when equilibrated at 50% RH, the humidity at which the cells were stored in the long-term stability test (Fig. 2), T_g of the cells dried in the presence of 13% trehalose and 2% sucrose was only 20°C (at a state with only 2% of the initial water content of the sample remaining). We therefore concluded that at 32°C and 50% RH, the desiccated cells were stored more than 10°C above the glass transition temperature of the solution,

Table 1. Differential Scanning Calorimetry determination of glass transition temperatures for cells in desiccation solutions.

Sample[a] 0% Relative Humidity	T_g °C[b]	T_g onset °C	T_g end-set °	Water remaining[c]
15% trehalose (no cells)	20.4 ± 0.1	14 ± 1	28 ± 1	0.4%
15% trehalose	51 ± 2	48 ± 3	54 ± 1	0.3%
13% trehalose, 2% sucrose (no cells)	18 ± 3	12 ± 3	25 ± 2	0.3%
13% trehalose, 2% sucrose	60 ± 3	56 ± 5	63 ± 3	0.4%
13% trehalose, 2% sucrose, 10% dextran	109 ± 2	104 ± 1	114 ± 1	0.5%
13% trehalose, 2% sucrose, 10% albumin	101 ± 3	92 ± 1	108 ± 1	0.5%
13% trehalose, 2% sucrose, 10% milk	46 ± 5	41 ± 6	52 ± 7	0.7%
13% trehalose, 2% sucrose, 5% gelatin	75 ± 9	67 ± 6	90 ± 20	0.5%
50% Relative humidity				
13% trehalose, 2% sucrose	20.2 ± 0.1	15.8 ± 0.4	23 ± 2	2%
13% trehalose, 2% sucrose, 10% dextran	29 ± 1	22 ± 1	37 ± 1	3%
13% trehalose, 2% sucrose, 10% albumin	30 ± 3	22 ± 2	41 ± 2	3%

a. All samples contained *Bradyrhizobium* at 10^{10} CFU ml^{-1} unless otherwise noted.
b. Average of triplicate T_g measurement ± standard deviation.
c. % of initial water content of the 250 μl sample.

explaining their instability during storage. Furthermore, the large change observed in T_g in response to higher environmental relative humidity proved the fragile nature of the trehalose/sucrose glass formed (i.e., compare 13% trehalose and 2% solution stored at 0% RH (T_g = 60.3°C) vs. 50% RH (T_g = 20.2°C) with only 1.6% difference in sample water content). This finding suggested that additives to increase the T_g in the dried state may be needed.

It is common practice to design desiccation formulations with glass transition temperatures higher than the target storage temperature to ensure that the formulation is stable against fluctuations in storage temperature and humidity (Hancock *et al.*, 1995). Therefore, we explored the feasibility of incorporating a higher T_g polysaccharide (dextran) and common high molecular weight proteins (albumin, milk protein and gelatin) in the desiccation solution formulation (Palmfeldt *et al.*, 2003; García, 2011). All of these proteins are well known for their cryoprotectant capabilities (Hubálek, 2003; Jerez *et al.*, 2016; Burnham *et al.*, 2021), but have found limited use in desiccation formulations. The glass transition temperatures of the formulations that contain these additives are also presented in Table 1. Each compound was combined with the 13% trehalose, 2% sucrose solution at a final concentration of 10% except for gelatin where its solubility limited the final concentration to 5%. Each additive increased the T_g of the xeroprotectant solution upon drying. The addition of albumin and dextran resulted in the greatest increases ($T_{g,albumin}$ = 101°C, $T_{g,dextran}$ = 109°C).

To determine the effect of the additives on storage stability, *Bradyrhizobium* cells were equilibrated in each solution at the target temperature of 32°C at both 0% and 50% RH (Fig. 3). Storage stability was measured over 5 months. Each additive improved *Bradyrhizobium* stability during storage at 32°C, 50% RH; however, albumin far outperformed the others. At 4 months of storage, the cells desiccated with albumin in the drying solution lost only 1-log CFU ml^{-1} compared to a 4-log loss of viability for cells without albumin. The addition of albumin, dextran and skim milk all resulted in similar stability for the first 40 days of storage at which point the viability of the cells stored in albumin stabilized, remaining essentially unchanged for the next 3 months. However, one log decrease in viability was observed at 4 vs. 5 months. This was attributed to crystallization of the xeroprotectant solution.

In the absence of polysaccharide or protein additives, *Bradyrhizobium* desiccated in trehalose-sucrose solution lost viability faster at 50% than 0% RH (Fig. 3 dashed vs. solid blue line) suggesting that higher environmental humidity is detrimental for storage stability as it accelerates degradation kinetics. These findings are consistent with previous reports of decreased stability for desiccated rhizobial strains stored at high humidity (Mugnier and Jung, 1985; Mary *et al.*, 1993; Mary *et al.*, 1994). Specifically, viability of desiccated *Bradyrhizobium* USDA 138 stored at 52% RH dropped 5 log CFU g^{-1} in 8 days while the viability of cells stored at 6% RH was unchanged (Mugnier and Jung, 1985).

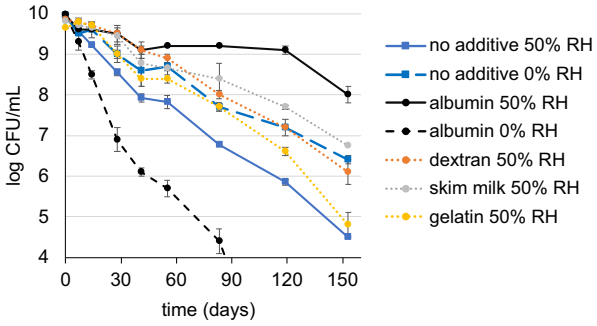

Fig. 3. Viability of *Bradyrhizobium* during storage at 50% RH (solid lines) and 0% RH (dashed lines) at 32°C in xeroprotectant matrices containing 13% trehalose, 2% sucrose with or without 10% albumin. *Bradyrhizobium* viability data in drying matrices with 10% dextran, 10% milk and 5% gelatin at 50% RH are shown as dotted lines, and the data at 0% RH were omitted from the plot for clarity. They are included in Fig. S2.

It is expected that at lower environmental humidity, stability of the dried product increases. However, even though albumin protected the cells very well when stored at 50% RH, the presence of albumin in the matrix was detrimental to cell viability during storage at 0% RH (Fig. 3 dashed vs. solid black line) so much so that viability dropped even below the no-additive matrix. In contrast to albumin, the stability of *Bradyrhizobium* in matrices containing dextran, milk and gelatin changed very little when the dried cells were stored at 0% vs. 50% RH (Fig. S2). We considered the possibility that albumin might have a high water-binding capacity at 0% RH, preferentially retaining the remaining water in the matrix, which would be detrimental to the cells. To test this possibility, water binding capacities of the xeroprotectant solutions equilibrated at 0 and 50% RH was determined gravimetrically: At 0% RH albumin lost 100% of the initial water content after 1 week of equilibration whether mixed with sugars or not (Table S7). Therefore, it was concluded that high water binding by albumin at low RH conditions was not the reason behind significantly decreased storage stability of *Bradyrhizobium* at 0% RH when dried in the albumin drying solution.

It was noted that during storage, physical appearance of the matrix samples that contained albumin changed. The samples equilibrated at 50% RH remained clear but those at 0% RH were opaque (Fig. S3). It is therefore plausible that trehalose crystallized during storage at 0% RH; however, FTIR analysis of the equilibrated samples that contained albumin is consistent with vitrified samples at both 0% and 50% RH (Fig. S3). It was also

noted that presence of albumin significantly increased the T_g of the trehalose, sucrose solution (Table 1) at both 0% (by about 50°C) and 50% RH (by about 10°C). Therefore, T_g alone cannot fully explain the cell stability difference as the T_g of the drying solution as both dextran and albumin increased the T_g to similar extents, but dextran was not as protective. Anti-oxidant or other properties of albumin likely contribute to its effectiveness in stabilizing desiccated *Bradyrhizobium* in moderately high environmental RH (Halliwell, 1988; Anraku *et al*., 2001). Loss of albumin protective capability at 0% RH requires further research.

Taken together the results of the storage experiment (Fig. 3), and the T_g measurements (Table 1) provide strong evidence that *Bradyrhizobium* desiccated in a vitrified storage solution with a T_g sufficiently above the storage temperature can be stored long term. Specifically, we observed that *Bradyrhizobium* desiccated in a xeroprotectant solution consisting of 13% trehalose, 2% sucrose and 10% albumin remained relatively stable at the target storage conditions of 32°C at 50% RH for up to 4 months with only one log loss in CFU. This formulation protected the cells against high temperatures and moderate RH a product might encounter, but the question remained if it would protect the desiccated cells against even higher environmental humidity. This is what we explored in the next section.

Design of a hydrophobic shell coating against high humidity in the environment

To determine the effect of increased humidity on *Bradyrhizobium* stability desiccated in the trehalose-sucrose-albumin solution, we quantified the effect of a step increase in environmental humidity from 50% to 75%. *Bradyrhizobium* was equilibrated in the optimized xeroprotectant solution at 50% RH for 12 days with 0.5 log loss in viability. Half of the samples were then moved to a 75% RH environment while the other samples were held at 50% RH and kept isothermal (Fig. S4). Upon shifting from the low to high humidity environment, cells started to lose viability at a faster rate; potentially due to degradation accelerated by adsorption of water into the core matrix. We reasoned that the desiccated cells need protection from fluctuations in relative humidity as a decrease to 0% RH diminished the protective effects of albumin (Fig. 3) killing the cells rapidly, while an increase to 75% had the same result (Fig. S4). Coating the product stabilized at 50% RH with a hydrophobic shell with low water vapour permeability appeared as one possible method for protecting the cells against fluctuations in environmental humidity.

An effective shell coating will need to withstand changes in environmental humidity, have low water vapour permeability and easy, cytocompatible application process and efficient removal (potentially by granulation before application in the field). Two polyvinylalcohol polymers (PVA) of different degrees of hydrophobicity and polyvinylpyrrolidone were tested. These polymers were selected as potential shell coating candidates as they are water soluble, yet upon drying they form gas barriers (Johansson *et al*., 2012; Schmid *et al*., 2014). Because silica is also generally cytocompatible, we chose to test a hydrophobic silica and phenyltriethoxysiloxane (PTES) (Rao *et al*., 2003; Benson *et al*., 2018). We also tested a solid and liquid hydrocarbon coating, paraffin and mineral oil, because hydrocarbon waxes are commonly used in food coatings to provide a vapour barrier (Donhowe and Fennema, 1993; Baldwin *et al*., 1995).

First, to aid choice of coating material, water vapour permeability of potential coatings was determined. A standard ASTM water vapour test was used to determine vapour permeability of films with <1 mm thickness. The permeability of the PTES film was an order of magnitude higher than the PVA and paraffin films (Table 2).

Second, the cytocompatibility and feasibility of using the polymers and the PTES silica were tested. Cells were dried at 32°C, 0% RH for 5 days, coated with thin layers of each compound and shifted to 50% RH environment (Fig. S5). Except for cells coated with PTES, stability of the coated cells within the first 8 days of storage at 50% RH did not differ significantly from the uncoated controls, indicating good recovery of cells from the coatings. The testing was continued for up to 64 days and for all coatings, 2–3 log decrease in CFU were noted. The relatively fast decrease in viability in PTES-coated product (as compared to uncoated product) was attributed to the PTES silica coating forming a very hard layer that was not easily physically disrupted. It is possible, perhaps even likely, that the cells embedded in the PTES layer could not be recovered for an accurate test of viability. Because PTES does not fit the criteria of an easily removable coating and the water vapour permeability was poor, it was not further pursued.

The cytocompatible polymers (PVA HR3010, PVA 18–88 and PVP) and the hydrocarbons (mineral oil and paraffin) were then tested for protection of desiccated *Bradyrhizobium* from high humidity. Cells were suspended in 5 µl of 13% trehalose and 2% sucrose solution and

Table 2. Water vapour permeability of coating materials.

Thin film	Total water vapour permeability g/(s·m·Pa) × 10^{12}
PTES	160 ± 50
10% PVA 18–88	9 ± 2
8% PVA HR3010	6 ± 3
Paraffin	11 ± 5
No film	660
Parafilm	4

equilibrated at 50% RH, 32°C for 5 days prior to coating with the selected materials (60 μl) (Fig. 4 dashed lines). Equilibration humidity at 50% was chosen for this experiment based on the observation that albumin added to the xeroprotectant solution decreased stability at 0% RH and because this would likely be a less energy intensive industrial process. No cells were recovered from samples coated with PVP or PVA HR3010 prior to the humidity shift, and therefore, these data are not included in Fig. 4. Because thinner coatings of both polymers did not decrease viability of *Bradyrhizobium* (Fig. S5), it was concluded that the thickness of the coating rather than the polymers is detrimental to the cells. These coatings were not further investigated. In contrast at day 7, similar amounts of cells were recovered from samples that were coated with PVA, mineral oil or paraffin as from uncoated samples, indicating that these coatings are cytocompatible and easily physically disrupted for cell release. After 7 days at 50% RH, all remaining samples were shifted to 75% RH to determine if the coatings provide protection from the high humidity (Fig. 4 marked with arrow). Strikingly, both hydrocarbon coatings provided protection, and most notably, degradation rates of the samples coated in paraffin were unaffected by the change in humidity, demonstrating that a hydrocarbon vapour barrier shell can efficiently protect desiccated cells against increase in humidity. In contrast, uncoated cells and cells coated with PVA 18–88 lost viability rapidly within 24 h. We note the poor performance of the PVA coating is consistent with previous reports of the failure of PVA gas barriers at high humidity (Schmid *et al.*, 2014; Nyflött *et al.*, 2017).

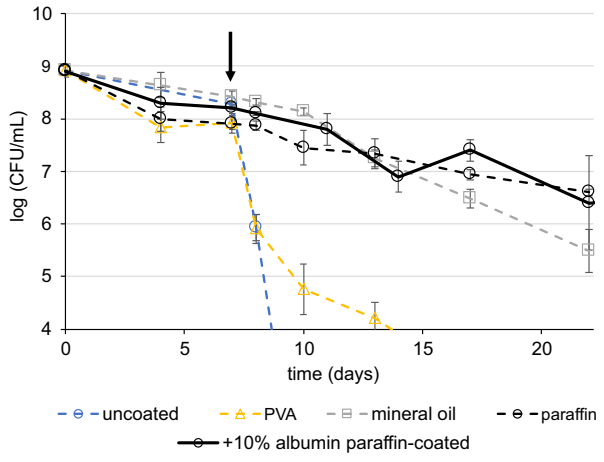

Fig. 4. Hydrophobic coating of desiccated *Bradyrhizobium* protects the cells from a subsequent transition to high relative humidity (75% RH). *Bradyrhizobium* (5 μl) were desiccated in 13% trehalose, 2% sucrose at 50% RH without albumin (dashed lines) or with 10% albumin (solid black line), 32°C for 5 days. The cells were coated with 60 μl of each coating (day 0 in graph): PVA Mowiol 18–88, PVA HR3010, PVP, paraffin and mineral oil. After 7 days, all samples were shifted to 75% RH (marked with an arrow).

In a final experiment, storage stability (50–75% RH, 32°C) of the final product paraffin-coated *Bradyrhizobium* desiccated in the optimized xeroprotectant solution (13% trehalose, 2% sucrose and 10% albumin) was determined (Fig. 4 solid black line). After humidity was shifted from 50% to 75% RH at 7 days as in the previous experiment, as seen with cells desiccated in xeroprotectant solution without albumin, the degradation rate of the samples with albumin was also unaffected. Taken together, the experiments presented here provide proof of concept that a temperature-stable xeroprotectant core and a humidity-barrier coating are effective for protecting desiccated rhizobia. While paraffin derived from crude oil is not considered a sustainable coating, improvements in gas barrier properties of polymeric thin films in high humidity and the use of multi-layer coatings offer a promising future for the development of desiccated cell products (Johansson and Clegg, 2015; Anukiruthika *et al.*, 2020).

In summary, desiccated rhizobial crop innocula require stabilization at both high temperature and high humidity for a minimum of three months for their use to be practical in many growing regions of the world. We examined the use of vitrified trehalose matrices for storage stability at high environmental temperatures. *Bradyrhizobium* was stable for 4 months with less than 1 log loss of viability in a hydrophilic, vitrified matrix of 13% trehalose, 2% sucrose and 10% albumin at 32°C at 50% RH. The stability of cells desiccated in this xeroprotectant matrix decreased with increased humidity; therefore, a hydrophobic vapour barrier shell was developed to protect the desiccated cells from high humidity. As a proof of concept, we observed that hydrocarbon coatings such as paraffin and mineral oil make good vapour barriers during storage at 75% RH. Shortcomings of hydrocarbon-based coatings remain to be addressed in the future, and more environmentally friendly coatings will have to be developed. In summary, in this study, we demonstrated the effectiveness of a core and shell formulation for protecting desiccated rhizobia against fluctuations in both heat and humidity.

Experimental procedures

To maintain uniformity, a single batch fermentation of a *Bradyrhizobium* species was carried out by BASF (Tarrytown, NY) using the normal industrial process. The cells (aged 13–20 months) maintained high viability (10^{10} to $10^{9.5}$ CFU ml^{-1}) stored at 4°C. Trehalose was from TCI America (Portland, OR USA). Bovine serum albumin (Fraction V, fatty acid free) was purchased from Calbiochem (La Jolla, CA USA). Polyvinylpyrrolidone (PVP) 380 000 MW, polyethylenimine (PEI) 800 MW linear and 25 000 MW branched, Polydiallyldimethylammonium

chloride (polyDADMAC), polyvinyl alcohol (Mowiol 18–88), triethoxyphenylsilane (PTES), mineral oil and dextran from *Leuconostoc mesenteroides* 9–11 000 MW were purchased from Sigma-Aldrich (Sigma-Aldrich Corp. St. Louis, MO, USA). Polymyxin B was from Alfa Aesar (Ward Hill, MA, USA) Skim milk powder was purchased from Accumedia (Lansing, MI USA), sucrose and gelatin (100 bloom) was purchased from Fisher Scientific. Low melting lab-grade paraffin wax was purchased from Carolina Biological Supply (Burlington, NC USA). and Drierite™ was purchased from Beantown Chemical (Hudson, NY USA). Polyvinylalcohol (Exceval) HR3010 was purchased from Kuraray America (Houston, TX, USA).

Measurement of the glass transition temperature (T_g) and water retention. Samples were prepared for differential scanning calorimetry (DSC) by desiccating 125 μl of each matrix in 2.5 cm petri dishes for 1 week sealed in plastic tubs at 32°C 0% RH (with Drierite™) or at 50% RH (with a saturated solution of K_2CO_3). The percent water remaining was determined gravimetrically using a Mettler AE100 balance with 0.1 mg accuracy before and after equilibration and after baking at 45°C. The temperature of baking was limited to 45°C to ensure that albumin did not denature releasing its structural water, which would complicate interpretation of the results. The glass transition temperature (T_g) of the trehalose-based drying matrices was measured using a Q1000 (TA Instruments Inc., New Castle, DE, USA) differential scanning calorimeter. 2–10 mg of the sample to be tested was loaded into a Tzero hermetical aluminium pan and sealed by a Tzero Press. The sample was then rapidly cooled down to −60°C at a rate of 50°C min^{-1}, equilibrated at that temperature for 5 min and then warmed to 140°C at a rate of 2°C min^{-1}. The heat capacity of the sample during the cooling/warming processes was recorded as a function of temperature using TA Universal Analysis. A baseline shift in heat capacity observed during warming of the sample corresponds to glass to amorphous transition, and therefore the midpoint temperature of the shift is recorded as T_g.

Measurement of intracellular trehalose concentration. Concentration of intracellular trehalose was determined after washing the cells twice with low-sodium phosphate saline buffer (LSPBS) (per litre: 0.2 g NaCl, 1.78 g Na_2HPO_4-$2H_2O$, 0.24 g KH_2PO4; pH 6.8) to remove the extracellular trehalose. Cell pellets (5–10 μl) were lysed by boiling in 50 μl phosphate buffer, and the trehalose concentration was determined using Megazyme assay kit (Wicklow, Ireland) following the manufacturer's instructions. The total volume of the cell pellet prior to lysing was used to determine the intracellular trehalose concentration.

Bradyrhizobium desiccation and hydrophobic coating. Cells were dried in 5 μl droplets in 96-well plates inside plastic boxes where relative humidity was kept constant. 0% relative humidity (RH) was achieved with Drierite™, 50% and 75% relative humidity with saturated solutions of K_2CO_3 and NaCl, respectively, at 32°C. Cells were dried in 13% trehalose, 2% sucrose in LSPBS unless otherwise noted. Cells were desiccated at 50% RH 5 days prior to coating with 60 μl paraffin. Paraffin was melted at 90°C and applied with pre-warmed pipet tips to the desiccated cells.

Measurement of Bradyrhizobium viability. Counts of viable *Bradyrhizobium* cells were determined by rehydration of samples of dried cells for 20–30 min (1 h rehydration did not increase viability) in peptone-tween buffer (per litre: 1 g peptone, 1.2 g K_2HPO_4, 0.3 g KH_2PO_4 and 0.1 g Tween 80; pH 7.0) followed by serial dilution in the same buffer and plating on CRYMA media with agar (per litre: 1 g dextrose, 9 g mannitol, 0.4 g yeast extract, 0.2 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$-7H20, 0.2 g NaCl, 5 ml 0.25% congo red; pH 6.8) to determine colony forming units (CFU). Cells were grown at 28°C. When coated with hydrophobic materials, coatings were physically disrupted with a spatula tip prior to rehydration.

Measurement of water vapour permeability. A modified ASTM E96/E96M water vapour transmission test standard was followed to measure the water vapour permeability of the coatings used (ASTM, 2016). Each coating material was pipetted (300 μl) onto a nitrile sheet and allowed to solidify into a film at ambient conditions 15% RH and 22°C. Film thickness was determined using ImageJ (Schneider *et al.*, 2012) analysing an image of the film cross-section. Vacuum grease was used to seal the film over a 0.95-cm hole drilled at the centre of a 0.076 cm thick galvanized steel plate 10.16 cm in diameter. The plate was placed over a 10-cm diameter glass dish containing approximately 30 g of drierite (0% RH), sealed with vacuum grease spread over a nitrile gasket and secured using four 1.90 cm nylon spring clips (Fig. S6). The test setup was placed in an environmental box kept at 22°C, 75% RH. Gravimetric measurements of the glass dish were conducted daily for a week. The following equations were used to calculate the water vapour permeability (WVP) of the thin films:

$$WVP = C\frac{x}{A \cdot \Delta P}.$$

In the equation, C is the slope of weight gain of the dish over time (g/s), x is the film thickness (m), A is the

area of exposed film (m^2), and ΔP is the water vapour pressure differential across the film (Pa).

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1

Fig. S1. Water loss from 5 μl solutions of 13% trehalose, 2% sucrose in LSPBS at 50% relative humidity (RH). 5 μl of solution were placed in each well of a 96-well plate and the weight loss of the entire plate was monitored gravimetrically.

Fig. S2. Viability of *Bradyrhizobium* in matrices containing 13% trehalose, 2% sucrose without an additive at 0% RH (gray) and at 50% RH (blue) and 0% RH (orange) with 10% dextran (A), 10% skim milk (B), and 5% gelatin (C).

Fig. S3. (A) *Bradyrhizobium* (10^{10} CFU ml^{-1}) stored in different xeroprotectant solutions indicated above the pictures at 32°C, 0% RH (top row) or 50% RH (bottom row) for 1 week. (B) Transmitted light microscopy images of dried samples. Scale bar = 100 μm. (C) IR spectra of samples dried at 0% RH. D) IR spectra of samples dried at 50% RH.

Fig. S4. Stability of desiccated *Bradyrhizobium* decreases rapidly after a step change in environmental relative humidity from 50% to 75% RH (marked with arrow). The cells

were desiccated in 13% trehalose, 2% sucrose, and 10% albumin xeroprotectant solution and kept at 32°C.

Fig. S5. Cytocompatibility of hydrophobic coatings. Cells were dried at 0% RH, 32°C as 5 μl droplets in 13% trehalose, 2% sucrose, 10% dextran, LSPBS in a 96 well plate. After drying for 5 days, cells were coated with: hydrolyzed PTES, 10% PVA Mowiol 18-88, 8% PVA HR3010, or 15% PVP (molecular weight = 380 kDa). PTES was hydrolyzed before coating by stirring for 3 hours as a mixture of 1 PTES: 0.43 water: 0.006 1N HCl (v/v/v). Samples were then stored at 50% RH, 32°C.

Fig. S6. Experimental setup used for measuring water vapor permeability. Thin films were placed over an aluminum disc that had a 30 mm diameter hole in its center and sealed with vacuum grease. The disc and film were clamped onto a petri dish that contained Drierite™ and placed into a 75% RH chamber (maintained by exposure to a saturated solution of sodium chloride).

Table S1. Intracellular trehalose concentration of *Bradyrhizobium*.

Table S2. Stability of EDTA-treated *Bradyrhizobium*. The cells were resuspended at 10^8 CFU ml^{-1} in 12% trehalose in LSPBS ± EDTA and dried as 5 μl aliquots at 0% RH and 32°C. For the samples where EDTA was removed prior to desiccation, cells were incubated with EDTA 30 minutes and then washed with 12% trehalose in LSPBS.

Table S3. Stability of *Bradyrhizobium* electroporated in the presence of trehalose. Prior to electroporation, to remove salt, the cells (10^{10} CFU ml^{-1}) were washed twice in water and then resuspended in 10% trehalose in phosphate buffer at pH 6.8. Cells were electroporated in 40 μl aliquots in a 0.2 cm gap cuvette chilled on ice before pulsing. Samples were dried as 5 μl aliquots at 0% *RH*, and 32°C.

Table S4. Stability of *Bradyrhizobium* electroporated and/or thermocycled in the presence of trehalose. Cells (10^{10} CFU ml^{-1}) were electroporated as detailed in Table S3, except in 100 μl aliquots. After electroporation, the drying matrix was adjusted to 9.5% trehalose and 2% sucrose and desiccated as 5 μl aliquots at 0% relative humidity and 32°C. Thermocycling of 1 ml cell suspensions in cryovials was achieved in Mr. Frosty™ cooler (Nalgene) with isopropanol in the reservoir for controlled temperature cycling at ~ 1°C min^{-1}. A temperature cycle consisted of cooling to -25°C and warming to 25°C monitored with a thermocouple. Samples were thermocycled twice prior to desiccation. Duplicate samples were analyzed at each time point.

Table S5. Stability of *Bradyrhizobium* exposed to positively charged polymers. *Bradyrhizobium* cells (10^{10} CFU ml^{-1}) were suspended in 15% trehalose in LSPBS. Branched polyethylenimine 2500 Da (PEI) or polydiallyldimethylammonium chloride (pDADMAC) were added at 1% final concentration. Cells were desiccated as 5 μl aliquots at 0% RH, and 32°C.

Table S6. Stability of *Bradyrhizobium* in the presence of polymyxin. B. *Bradyrhizobium* cells (200 μl) were suspended in 7.5% trehalose in LSPBS. Polymyxin B was added to 20 mg ml^{-1} final concentration and 5 μl aliquots were desiccated at 0% RH, and 32°C.

Table S7. Water Binding Capacity of Albumin.

