3D-printed biological habitats for the protection and persistence of *Rhizobia* species in compacted soils

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Abstract

Microorganisms in soils are responsible for many ecosystem services. However, in degraded soils, microbial abundance and function are limited, compromising several biologically facilitated processes. Inoculating soils with desirable microbes can help to re-instate or initiate a viable functioning microbial community. However, establishment success is reliant on the survival of the microorganism in an adverse environment. In this proof-of-concept study, artificial microbial refugia have been developed using resin and light-emitting diode array (LED) 3D printing technology. We assessed whether the artificial refugia, termed a *Rhiome,* would support better microbial growth in degraded soils. Soil compaction, a form of soil degradation, and *Rhizobium*, an important microorganism for global agriculture, were selected as the use case application for this assessment. Different materials, together with resin, were assessed for their suitability as a 3D printing material and for supporting rhizobial growth. The best result was found in materials constructed with a combination of polylactic acid (PLA) resin, yeast extract, and mannitol. In a soil compaction experiment with inoculation of rhizobia, the addition of *Rhiome* significantly increased bacterium survival in the compacted soil to a level similar to, or higher than, the rhizobial loading in non-compacted soils. In addition, augmentation of the resin with yeast extract and mannitol increased *Rhizobium* growth significantly compared with the *Rhiome* constructed only with resin. These results indicate that the *Rhiome* was highly beneficial for instigating and maintaining significant rhizobia survival and growth in compacted soils. Further work, including near-to-field assessments, is required to assess Rhiome performance in various applications and to refine material properties relative to important context-specific performance metrics such as degradation rate. We propose the *Rhiome* concept as a promising asset in the toolbox for soil ecological restoration as a means of improving soil resiliency.

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Introduction

Soil microorganisms mediate several biogeochemical cycles that are essential for sustaining agroecosystems and therefore play a critical role in addressing many challenges faced by the agricultural sector worldwide (Barrios, 2007). For instance, soil microbial communities improve soil fertility and limit the risk of plant disease and pest outbreak (Thomashow *et al*., 2019; Shi *et al*., 2021). Therefore, maintaining healthy and resilient microbial communities in agricultural soils helps to reduce the dependence on chemicals, *e.g*., fertilisers and pesticides, as well as positively impacting water quality, biodiversity, carbon emissions, and crop yield (Thomashow *et al*., 2019).

In intensive agricultural farming systems, soil compaction caused by animal treading and mechanical equipment significantly reduces the activity and diversity of soil microbes due to its destructive effect on microhabitats (Pengthamkeerati *et al*., 2011). Reduced pore size and pore continuity limit the supply of oxygen and nutrients to plants, and this indirectly affects the functioning of the microbial community due to less root growth and exudation (Głąb, 2014; Tan and Chang, 2007). For instance, in New Zealand, animal treading reduced the abundance of white clover by as much as 85% and annual nitrogen fixation by over 50%, *e.g*., annual N fixation in non-damaged soils was 76 kg N ha/yr compared with 36 kg N ha/yr in compacted soils (Menneer *et al*., 2005). A decline in microbial activity, especially lower carbon and nitrogen metabolisation, further reduces the supply of nutrients to plants, thereby contributing to a spiralling degradation of the soil-plant system and soil health (Siczek *et al*., 2013). Reductive conditions that often prevail in compacted soils (Nawaz *et al*., 2013) favour the growth of anaerobic microbes that facilitate the production of nitrous oxide and produce metabolites with a toxic effect on plant roots (Schnurr-Putz *et al*., 2006; Weisskopf *et al*., 2010; Kaminsky *et al*., 2021; Longepierre *et al*., 2021). It has been shown that a single soil compaction event has a long-lasting impact (*i.e*., four years) on some microbial taxa within community

structures and their activities (Longepierre *et al*., 2021).

Mechanical tillage helps to accelerate the soil recovery process by alleviating the initial constraints caused by soil compaction, thereby promoting plant growth and subsequent re-establishment of those microorganisms, including bacteria and fungi, responsible for soil aggregation. However, the benefit of aeration can be short-lived when the compactive pressures on soils are ongoing, such as under continuous grazing (Laurenson and Houlbrooke, 2014; Laurenson *et al*., 2015).

Microbial inoculants have the potential to reinstate important nutrient cycles. For instance, *Rhizobium* inoculation of clover species is widely used around the world to support pasture systems. Bacterial and fungal inoculants enhance nutrient availability to plants with subsequent positive benefits on carbon availability in the rhizosphere and production of polysaccharide compounds that temporarily bind and stabilise soil aggregates (Tang *et al*., 2011; Mengual *et al*., 2014; Rashid *et al*., 2016). However, success in establishing viable communities within compacted soils is low because their ability to move and access essential nutrients is limited (Canbolat *et al*., 2006). To overcome this challenge, we have employed 3D printing technology for prototyping artificial structures that could provide refugia for microbial species. We refer to these structures as *Rhiomes*. The application of 3D printing technology in the building industry is a relatively new concept but has already proven to be a rapid and affordable mechanism for developing materials and structures such as houses (Lei *et al*., 2020).However, in soil and agricultural science, the application of 3D printing technology into biological systems is few (Dal Ferro and Morari, 2015; Lamandé *et al*., 2021).

In this proof-of-concept study, we seek to demonstrate that the use of the *Rhiome* structures in compacted soils increases the survival rates of microbial inoculants compared with what is achievable without such structures. Here, we used a *Rhizobium leguminosarum* strain, an important and widely used agricultural inoculant, as a case species for testing the efficacy of the *Rhiome* structure. *Rhizobium* is of special interest in agriculture because of its significant economic contribution to global agriculture, largely through N-fixation. Furthermore, N-fixing bacteria also encourage the growth and persistence of other soil microbial groups by increasing the nitrogen available to the soil biota (Singh, 2014). However, the abundance and persistence of *Rhizobium* in compacted soils are low because rhizobia are obligate aerobes and are extremely sensitive to environmental conditions (Irisarri *et al*., 2019; Lowther and Kerr, 2011; Rutten *et al*., 2021). In this context, we hypothesize that the *Rhiome* structure can provide refugia for rhizobia in compact soils, allowing them to grow rapidly within the *Rhiome* and 'release out' into the surrounding compacted soils.

Materials and methods

Rhizobia strains and culture conditions

The rhizobial inoculant used in this experiment was prepared from a commercial strain *R. leguminosarum* bv. *trifolii* TA1 (sourced from Australia Inoculants Research Group). TA1 was first cultured in yeast mannitol agar (YMA) plates at 25°C for 5 days. A liquid inoculant was then prepared by adding a single colony to a 3 mL aliquot of yeast mannitol broth (YMB) made from 1.0 g yeast extract (YE), 10.0 g mannitol (M), 0.5 g K_2HPO_4, 0.2 g $MgSO_4$ and 0.1 g NaCl at pH of 6.8. The inoculated YMB was incubated at 28°C under constant agitation for 24 hours. Next, 1 mL of the YMB broth was used to inoculate a further 100 mL of YMB and again incubated at 28°C under constant agitation for 48 hours. This final broth is referred to as the 'rhizobia inoculant'. Bacteria loading in the rhizobia inoculant was estimated by plate enumeration, for which 10-fold serial dilutions were made using a phosphate buffer (20.2 g of Na_2HPO_4•$7H_2O$ and 3.4 g of NaH_2PO_4•H_2O per L, pH 7.0) and 100 µL aliquots were spread on to YMA plates. Viable cell numbers were estimated by counting the colony-forming units (CFU) after incubating the plates at 25°C for 4 days. Bacterial loading was expressed as colony-forming units per mL of YMB (CFU/mL).

Assessment of the printing material effects on rhizobial growth

A screening experiment was carried out to assess the potential toxicity of PLA printing resin on the *Rhizobium* bacteria. Spheres (5 mm dia.) were printed using an *Elegoo Saturn 3D printer* (https://www.elegoo.com/) and a clear PLA resin (eResin-LC1001: 30% min polyurethane acrylate CAS 25035-69-2, 30% min monomer CAS 13048-33-4, 5% max photoinitiators CAS 947-19-3 and 5% max colour pigment, manufacturer eSun; https://www.esun3d.net/). The printed spheres were rinsed in Isopropyl Alcohol for 10 seconds. Then the printed support material laid down during the printing (an artifact of the printing process) was removed. Spheres were then dried and UV cured for 6 minutes using a wash and cure machine ('*ANYCUBIC'* - Anycubic Wash & Cure Plus Machine www.anycubic.com). The spheres were dried again and cured for another 6 minutes. Three candidate materials (additives) were assessed for their beneficial effect on bacterial growth. These additive materials included 2 carbon sources; corn starch (CS; particle size < 65 µm; CAS 68525-86-0) and D-mannitol (Sigma-Aldrich M4125; CAS 69-65-8), and one nitrogen source, YE (CAS 8013-01-2). The YE was combined with each of the carbon sources at concentrations that achieved the same final carbon-to-nitrogen ratio (C:N) as the YMB (6:1) once combined with the PLA resin.

In total, six treatments were designed to determine resin toxicity and additive release/accessibility properties when immobilised in resin structures (Table 1). Three replicates were included per treatment. Treatments consisted of beads manufactured from PLA resin (*PLA control*), PLA resin with YE and M (PLA+YE+M: bead composition comprised PLA 59.0%, YE 20.5%, and M 20.5%), and PLA resin with YE and corn starch (PLA+YE+CS: PLA 59.0%, YE 20.5 %, and CS 18.4%). For these first three treatments, beads were added to 100 mL of sterile salt solution (0.5g K_2HPO_4, 0.2 g $MgSO_4$ and 0.1 g NaCl at pH of 6.8, termed 'salt solution'). The following three treatments were prepared using the salt solution but did not include PLA beads. These treatments included the following salt solution without PLA beads (*Control*), salt solution supplemented with YE and M (*YE+M*), and salt solution supplemented with YE and CS (*YE+CS*). For each treatment, 100 mL of salt solution was prepared without additives, with YE and M (2

Table 1. Treatments used to assess the effects of poly-lactic acid (PLA) printing resin and resin additives on bacterial growth. Resin additives included yeast extract (YE), mannitol (M), and corn starch (CS).

Treatment	3D printed beads	Liquid medium*
PLA control	PLA beads	Salt solution
PLA +YE + M	PLA beads + YE + M	Salt solution
PLA +YE + CS	PLA beads + YE + CS	Salt solution
Control	-	Salt solution
YE + M	-	Salt solution + YE +M
YE +CS	-	Salt solution + YE +CS

*Salt solution (0.5g K_2HPO_4, 0.2g $MgSO_4$, 0.1g NaCl, pH 6.8).

g/L, 20 g/L, respectively) or YE and CS (2 g/L, 18 g/L, respectively; Table 1). The experiment was carried out in a series of 250 mL flasks, each inoculated with 1 mL of the same rhizobia inoculant prior to being incubated at 28°C for 72 hours under continuous agitation (150 rpm). Plate count determined bacteria loading following the methodology described above.

Rhiome development

The *Rhiome* structure was modelled using a computer-aided design (CAD) programme (*OnShape*; www.onshape.com). *Rhiome* structures were designed to have a high internal surface area relative to soil displacement volume, sufficient water retention and percolation, and adequate ratios of additives without compromising structural integrity. The structure was conical, with a base diameter of 33 mm, height of 37 mm, and an internal surface area of approximately 5600 mm^2 (Figure 1). The conical shape enabled the *Rhiome* to be inserted easily into soil and provide an exposed surface for receiving irrigation (not tested in the current study). In addition, twelve holes (2 mm diameter) were located around the outer wall of the *Rhiome* to allow water flow from the structure.

The *Rhiome* was printed using a clear water-washable resin (eResin-LC1002: 30% min polyurethane acrylate CAS 25035-69-2, 30% min monomer CAS 13048-33-4, 5% max photo-initiators CAS 947-19-3 and 5% max colour pigment, from eSun). This was a different resin from that used in the manufacture of the beads (*i.e.*, e-Resin-LC1001). However, resin substitution was necessary due to supply constraints.

Two types of *Rhiome* were created, including one with resin only and another with nutrients added to the resin matrix (described in the proceeding section). For the resin with nutrients, 2.05 g of yeast and 20.45 g of mannitol were mixed into 75 g of resin for 2 minutes until the resin-powder mixture was uniform. All *Rhiome* were printed (see above) using a vertical resolution 60 µm and a print exposure time of 10 seconds. Every 10 minutes, the print process was paused, and the resin solution in the printer reservoir was stirred for approximately 30 seconds until all powder precipitates were resuspended in the resin. The same procedure was undertaken for consistency in the solution comprised of resin alone. After printing was completed, support material laid down during printing was removed before the *Rhiome* was rinsed in water for 20 seconds and dried. Finally, the *Rhiome* were cured in the wash and cure machine (ANYCUBIC) for 6 minutes with the flat face downwards, dried, and cured again for another 6 minutes, with the rounded face downwards.

Soil compaction experiment

This experiment tested whether the *Rhiome* can support rhizobial growth and establishment in compact soils. Four treatments were included (Table 2), a control soil that was not compacted and did not include a *Rhiome* (*non-compacted control*) and a further three soils that were compacted, with the following treatments imposed: i) *Rhiome* (resin only; '*Rhiome'*); ii) *Rhiome* manufactured from resin, YE and M ('*Rhiome+additives'*) and compacted soil with no *Rhiome* added and no nutrients ('*compacted-control'*).

The soil used in the incubation experiment was a Udic Haplustept, locally known as a Templeton silt loam (pH 5.8; carbon and nitrogen content 1.0% and 0.06%, respectively). Soils were air-dried (48°C forced draft) to a constant moisture content (~5-7%$_{v/v}$) and sieved (<4 mm). Four replicate samples were prepared for each treatment by repacking a known dry weight of soil (549.79 g soil ±4.38 g soil) into glass containers (vol. 5.27×10^{-4} m^3). For the treatments; *Compacted control*, *Rhiome,* and *Rhiome+additives*, soils were repacked to a target bulk density of 1.41 Mg/m^3±0.02 Mg/m^3, which represents the bulk density typical of compacted soils in New Zealand (Laurenson and Houlbrooke, 2014). For the *non-compacted control*, the placement of the sieved soil into the containers, without compaction, resulted in a bulk density of 1.26 Mg/m^3±0.01 Mg/m^3, which is within the expected range for non-compacted mineral surface soils in New Zealand (Sparling and Schipper, 2002). For those treatments inclusive of *Rhiome* structures, 14.87 g of soil was extracted prior to inserting the *Rhiome* to account for the displacement volume (1.055×10^{-5} m^3) of the structure that was then inserted into the centre of the container. No *Rhiome* was inserted in the *non-compacted control* or the *compacted-control* treatments.

Soils and containers were pasteurised by incubating at 80°C for 24 hours (to reduce microbial content and eliminate *Rhizobium* population already present in soils), then allowed to cool before 20 mL of sterile water was applied across the entire soil surface. All treatments were then inoculated with 10 mL of rhizobia inoculant (1.1×10^9 CFU/mL) that was applied via pipette into the aperture at the top of the *Rhiome* or dispensed directly onto the centre of the soil surface for those treatments without *Rhiome*. Inoculated containers were then incubated at 30°C for 15 days, during which time 10 mL of sterile water was added daily to the soil surface to maintain adequate soil moisture for biological growth.

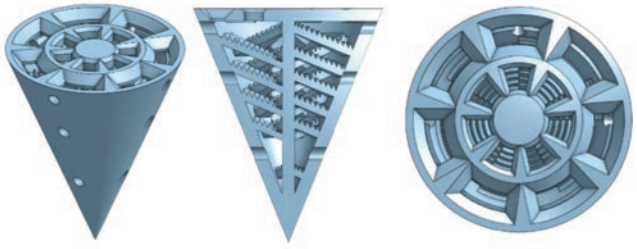

Figure 1. (Left) Rhiome design, (centre) cross-sectional view of *Rhiome*, (right) top view of *Rhiome*.

Table 2. Description of four treatments and four variables that were included in the soil compaction experiment. For each treatment, four replicates were included.

Treatment name	Soil compaction	*Rhiome*	Nutrients in *Rhiome*	*Rhizobium*
Non-compacted control	No	Not added	No	Yes
Rhiome	Yes	Added	No	Yes
Rhiome + additives	Yes	Added	Yes	Yes
Compacted control	Yes	Not added	No	Yes

Soil sampling methodology for rhizobia

After 15 days of incubation, two soil samples were removed from each container using a core sampler (5 mm diameter) to a depth of 20 mm. Samples were extracted 20 mm from the edge of the *Rhiome*, which was approximately halfway between the *Rhiome* and the edge of the glass container. Two samples were extracted per treatment from opposing sides of the *Rhiome* and were combined and weighed. The concentration of live *Rhizobium* in each soil sample was determined by serial dilution and plate counting following the methodology previously described.

Statistical analysis

In the first experiment, *Rhizobium* growth in each treatment was compared against the null hypothesis of no growth (mean growth =0, when compared with rhizobial loading immediately after inoculation) with a one-sample t-test. Prior to each test, the growth values were log10(x+1)-transformed to stabilise variation. For both experiments, final *Rhizobium* bacterial loadings were compared between the treatments using an analysis of variance (ANOVA) consisting of a single-factor treatment. For each experiment, a pair-wise treatment comparison was made using Fisher's least significant difference (LSD) method. All analyses were carried out with the statistical software Genstat (20th edition).

Results

The effect of PLA resin and additive on rhizobial growth

For all treatments, *Rhizobium* loading after 72 hours of incubation was significantly higher ($P<0.001$) than the initial concentration (6.55×10^6 CFU/mL). The lowest rhizobial loading was detected in the treatment 'No additives' (1.11×10^7 CFU/mL), containing only the salt solution as a liquid medium. In comparison, PLA beads in salt solution (*PLA control*) presented significantly higher rhizobial counts (1.74×10^7 CFU/mL) than in the *Control*, suggesting that PLA resin was non-toxic to the bacterium. Rhizobial counts in *PLA+YE+M* and *PLA+YE+CS* (PLA beads with additives) were significantly higher than those in *PLA control*, suggesting these additives positively affected rhizobial growth. Similar rhizobial loadings ($P>0.05$) were found between *PLA+YE+M* and *YE+M* as well as between *PLA+YE+CS* and *YE+CS*. The loading in *PLA+YE+M* was not significantly different from *YE+M,* while rhizobial loading in *PLA+YE+CS* was significantly lower ($P<0.001$) than its pair treatment *YE+CS*. Based on this result and considering that *PLA+YE+M* and *YE+M* presented the highest rhizobial loadings (beads made of PLA resin with YE and M), these materials were selected to be used in further steps of this study.

Soil compaction experiment

The rhizobial loading was significantly ($P<0.001$) higher in the *Rhiome + nutrients* treatment (1.83×10^7 CFU/g soil; Figure 2) compared with all other treatments. The rhizobial loading in the *Rhiome* treatment (9.46×10^6 CFU/g soil) was not statistically ($P=0.443$) different from that of the non-compacted control (2.52×10^6 CFU/g soil) despite the compaction that was imposed. The lowest ($P<0.001$) bacterial loading was measured in the compacted control, which does not contain the *Rhiome* structure (1.58×10^5 CFU/g soil).

Discussion

In this study, we demonstrated the potential of the *Rhiome* structure to increase the survival rate of soil microbial communities on compacted agricultural soils. We used *Rhizobium* as a model microbe for assessing the performance of microbial refugia that was 3D printed from a resin. 3D printing is a cost-effective way to rapidly prototype devices in small batches in an on-demand manner, providing the opportunity to easily fabricate complex structures for biological applications (Walsh *et al*., 2016). For example, 3D printed structures that mimic the 3D morphological and spatial architecture of soil have been used to understand interactions among soil microbes (Otten *et al*., 2012). However, from our knowledge, 3D printed structure has not been previously assessed as a delivery system and refugia for plant growth-promoting rhizobacteria.

Understanding which materials are suitable for developing these microbial refugia is an important step in the prototyping process, as some polymers used for constructing 3D structures could negatively affect microbial growth. For example, Black Soft PLA (MatterHackers) and 3D PLA silver colour (3D Solutech) can inhibit the growth of *Pseudomonas aeruginosa* and *Escherichia coli* (Hall *et al*., 2021) and Shapeways Elasto Plastic® and Stratasys Tango Plus® can inhibit *Escherichia coli* growth (Walsh *et al*., 2016). Our study showed that the PLA resin we used did not affect rhizobia growth, as similar or higher loading of rhizobia was detected in the paired treatments (*PLA control vs Control*, *PLA+YE+M vs YE+M*, *PLA+YE+CS vs YE+CS*; Figure 3). The addition of the carbon and nitrogen source significantly increased the growth of rhizobia compared to using the PLA resin only. We assume that the mechanism causing improved survival is the remobilisation of nutrients as a result of resin decomposition in the presence of water. These results strongly suggested the importance of selecting an essential growth substrate to stimulate microbial growth in the printing material.

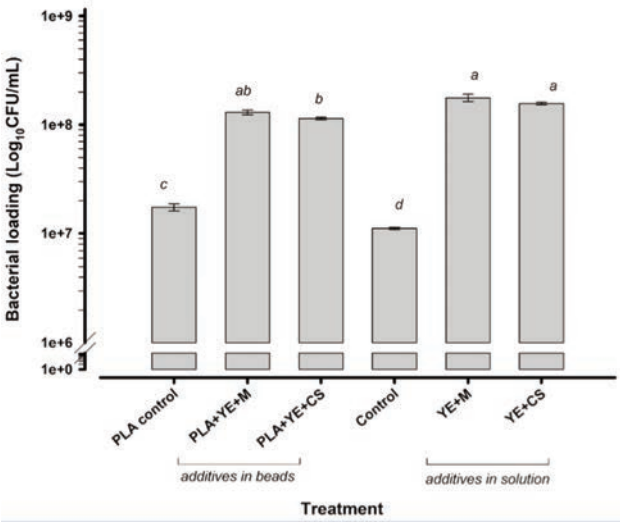

Figure 2. Effect of polylactic acid (PLA) resin and PLA supplemented with additives on rhizobia growth after 72 hours of liquid fermentation. All treatments were prepared in the same salt solution. Treatments included PLA beads in salt solution (*PLA control*), PLA beads supplemented with yeast extract (YE) + mannitol (M) (*PLA+YE+M*), PLA beads supplemented with YE and corn starch (CS; *PLA+YE+CS*), and salt solution without PLA (Control), salt solution supplemented with YE and M (*YE+M*) or YE + CS (*YE+CS*).

As expected, the compaction condition significantly reduced the rhizobial survival, assessed at 15-days post-inoculation, when no *Rhiome* was present. This could be due to the adverse soil conditions (*e.g.*, reduced macroaggregates, low oxygen availability, low water flow, *etc.*), which have been shown in many studies to reduce microbial activities (Głąb, 2014; Pengthamkeerati *et al*., 2011). Interestingly, the presence of *Rhiome* structure resulted in a greater increase of rhizobial survival in compacted soils when compared to the compacted control without *Rhiome*. This result strongly indicates that the *Rhiome* structure can better support rhizobial growth and establishment in compacted soils. More promisingly, the *Rhizobium* population in the compacted soils was significantly higher or similar to that of the non-compacted control. This data strongly supports our hypothesis that the *Rhiome* structure can act as microbial refugia for rhizobia, allowing growth in the *Rhiome* where conditions are more favourable (especially in terms of oxygen supply, pore size, and water flow) and the gradual release out to surrounding soils (as soil samples were collected 20 mm away from the *Rhiome* structure).

Significantly higher rhizobial loading was detected in the *Rhiome* + nutrient treatment compared to the *Rhiome*-only in the compacted soils, indicating the rhizobial growth was faster in the *Rhiome* with additional nutrients that were slowly released from the structure. This is complemented by the results found in the material screening experiment (Figure 3), where treatments with PLA plus additives had higher rhizobia numbers than PLA resin alone, and the growth rate in treatments with beads created with PLA resin supplemented with YE + M was similar to the rhizobial growth rate in the liquid medium of the salt solution containing YE + M. Although the release rates of additives into the liquid medium were not measured in this study, both experiments indicate the release of nutrients was not a bottleneck for *Rhizobium* growth. Root exudates are an important carbon source and support a high abundance and diversity of soil microbes within the rhizosphere (Schimel and Schaeffer, 2012). However, when compaction limits plant growth, the supply of carbon in soils also becomes limited, thereby slowing down microbial growth and their activities. The refugia we have created using the *Rhiome* structure and supply of carbon and other key nutrients (*e.g.*, nitrogen) have enabled significant bacterial growth despite the adverse environmental conditions. As a proof-of-concept, we have conducted only one sampling event 15 days post-inoculation. Therefore, it is unclear how long the positive effect of additives would have lasted. However, the initial boost of rhizobial growth could be important to ensure their establishment in soils.

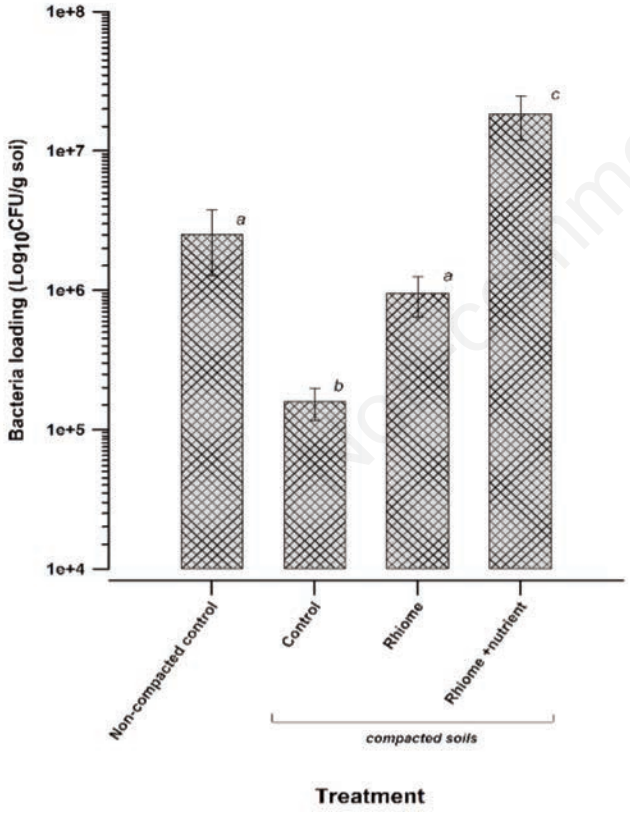

Figure 3. Rhizobial loading (CFU/g $soil_{dwt}$) in soils 15-days after inoculation. Treatments include a non-compacted soil without *Rhiome* (*non-compacted control)* and compacted soils without *Rhiome* (compacted control), with Rhiome (*Rhiome*), and *Rhiome* with nutrients included in their structure (*Rhiome+nutrients*). Treatments that share a common letter do not differ statistically significantly at a 5% significance level.

In all compacted treatments, degradation of the *Rhiome* structure was visually apparent after the 15 days of incubation yet not measured (not shown). This result was not surprising given the 'water-washable' properties of the resin used. The degradability of PLA-based resin has previously been shown to occur due to hydrolysis following the uptake of water (de Jong *et al*., 2001), photodegradation (Tsuji and Nakahara, 2002), and biodegradation. Although the drivers of the degradation (*i.e.*, biological, chemical, or photo) were not defined in this experiment, we expect the breakdown to be beneficial for the product's end-of-life management. However, the speed of degradation and any pollution risk posed by breakdown products should be assessed with future research.

This study assessed the proof-of-concept of using the artificial microbial refugia to assist in establishing the beneficial microbial inoculant in compact soils, using rhizobium as a model inoculant. However, the application of the *Rhiome* technology is not limited to this application and can be applied to a larger diversity of microbial species and environmental contexts. Soil biota abundance and diversity are critical for most alternative agriculture practices, such as regenerative agriculture (Schreefel *et al*., 2020) and agroecology (Gianinazzi *et al*., 2010). With worldwide concerns about the environmental threats generated by conventional agriculture and the need for a sustainable transition, biodegradable structures that help promote specific soil biota can be a promising asset in the toolbox for soil ecological restoration. The results from our work demonstrate that the refugia provided by *Rhiome* structures have the potential to accelerate the recovery of soil biota and their functions within degraded soils. The *Rhiome* could act as microbial refugia when delivering microbial inoculants onto the farm, because it can protect inoculants from the stressful environment (*e.g.*, UV, dry conditions, limited nutrients in soils) and support their growth by slowly releasing the nutrients from the biodegradable structure. Effective delivery of a high quantity of active inoculants has been a huge challenge in bio-inoculant research (Jambhulkar *et al*., 2016), and this *Rhiome* concept could have large potential in this space. Combined with current research on soil microorganism enhancement and engineering, the *Rhiome* concept could promote specific microbial 'bundles' and, therefore, increase the delivery of specific ecosystem services from soil (*i.e.*, soil fertility, soil carbon, pest control). The ability to maintain a healthy soil microbial life will also be challenged in a warmer world where short and sharp changes in environmental conditions, such as drought events, are expected more frequently than they currently are. In this context, the *Rhiome* concept provides protec-

tion for microbial communities and, in doing so, has the potential to support soil resiliency and mitigate the impacts of climate change on crop yields.

Conclusions and future possibilities

Soil microorganisms are essential for sustaining agroecosystems yet are vulnerable to soil disturbance (such as compaction). In this proof-of-concept research, we have created microbial refugia using emerging 3D printing technologies and biodegradable resin products. Using a strain of Rhizobia as a model inoculant, we have demonstrated that the *Rhiome* structure was effective in supporting inoculant growth in compacted soils despite adverse environmental conditions. While this study shows promise for the microbial refugia concept, future work should seek to optimise the *Rhiome* structure with respect to its design and the materials with which it is made from a degradation rate and pollution perspective. In addition, this proof-of-concept study was conducted using pasteurised soils (to eliminate *Rhizobium* in soils for an accurate measure of inoculated rhizobia in the experiment). This pasteurising step removes many other soil microbes, which could have helped enable the easy establishment of inoculated rhizobia. Therefore, future assessments should evaluate *Rhiome* performance in fresh soils. Near-to-field assessments are also required to test the *Rhiome* concept under a wider range of environmental considerations than those included in this study. For instance, this might include bioremediation of contaminated soils or combatting desertification. Future applications should consider a diversity of temperature and moisture regimes and help define optimised installation numbers to support a desired biological outcome, whether to alleviate an environmental constraint or support improved biological performance.

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