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Stress, Phages, and EPS: Factors That Enhance *Bradyrhizobium* Biofilm Formation

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ABSTRACT

Biofilms and their exopolysaccharide (EPS) matrix help soil rhizobia ride out stress, colonize roots, and ultimately succeed in symbiosis. We asked whether bacteriophages and common abiotic stressors modulate EPS-linked biofilm traits in *Bradyrhizobium* S3.9.2GM. Using a validated crystal violet (CV) microtiter assay for biomass and Congo Red (CR) colony phenotyping for matrix-linked traits, we found that phage and sublethal stressors (osmotic, oxidative, temperature shock) generally increased biofilm biomass, with the largest effects under combined phage + stress conditions. One-way ANOVA showed a strong treatment effect on OD570 (biofilm biomass; $p < 0.001$), while mucoid score and CR halo intensity trended upward in combined treatments but were not significant at $\alpha = 0.05$. These patterns are consistent with literature showing stress-induced EPS in rhizobia and context-dependent phage–biofilm interactions, and they suggest that simultaneous biotic and abiotic pressures can amplify EPS-associated phenotypes in the rhizosphere^{1,15,7}.

Introduction

Biofilm formation in rhizobia is more than a structural adaptation; it is a survival strategy that underpins the success of their symbiotic partnerships with plants. Within these multicellular communities, bacterial cells are embedded in an extracellular matrix that shields them from environmental hazards, anchors them to root surfaces, and facilitates the complex signaling events that lead to nodulation and nitrogen fixation. A central component of this matrix is exopolysaccharide (EPS), which not only cements cells together and to surfaces but also prevents desiccation and contributes to host recognition during the early stages of symbiosis. In *Bradyrhizobium*, a genus of slow-growing, nitrogen-fixing bacteria, EPS biosynthesis is unusually complex, producing polymers such as succinoglycan, glucomannan, and the genus-specific sugar bradyrhizose¹⁸.

Environmental signals are powerful regulators of EPS production. Numerous studies have shown that osmotic, oxidative, thermal, and nutrient-limiting stresses can each activate EPS biosynthetic pathways and trigger biofilm formation as a defensive response^{5,1}. In the laboratory, these responses often manifest as visibly mucoid colonies on agar plates and increased retention of crystal violet in microtiter plate biofilm assays. For example, *Bradyrhizobium* grown under high

salt or oxidative conditions often develops viscous colony morphology and binds more red dye, both indicators of elevated polysaccharide output⁵. Such adaptations likely improve persistence on root surfaces, where fluctuating conditions can be severe.

More recently, attention has turned to the influence of bacteriophages on biofilm phenotypes. Phage infection can act as both a threat and a signal. In some bacteria, phage attack triggers EPS overproduction, creating a physical barrier that impedes viral penetration or traps phage particles within the matrix⁹. In other cases, phages carry enzymes such as depolymerases that degrade biofilm polymers, reducing matrix integrity^{7,17,2}. While this interplay is well documented in clinical and aquatic systems, its ecological significance in plant-associated microbes remains largely unexplored. Given the abundance of phages in the rhizosphere, such interactions may be an underappreciated factor shaping rhizobial biofilm structure and function.

To study EPS-linked phenotypes, microbiologists often use Congo Red (CR) agar plates to score colony mucoidity and dye halo formation. CR is a convenient probe for extracellular matrix components, binding both amyloid fibers and certain polysaccharides^{6,8,12}, but this versatility means that its readouts are composite signals. For robust interpretation, CR scoring is best paired with a quantitative biomass assay such as the crystal violet (CV)

microtiter method, which measures total surface-attached biomass under controlled conditions^{10,13}.

Here, we examine how bacteriophage exposure interacts with environmental stress to influence EPS production in *Bradyrhizobium* strain S3.9.2GM. Using both the CV microtiter assay and a CR-based mucoidity assay, we evaluate biofilm biomass and visible EPS phenotypes under control, phage-only, stress-only, and combined phage plus stress conditions. We hypothesize that phage exposure will not merely mimic known stress responses but will amplify them, leading to additive or synergistic effects when combined with osmotic, oxidative, or thermal stress. Understanding how biotic and abiotic pressures intersect to shape EPS production in *Bradyrhizobium* has implications for symbiotic efficiency, microbial competition in the rhizosphere, and the design of phage-based interventions in agricultural systems.

Methods

Bacterial strain and growth conditions

Bradyrhizobium strain S3.9.2GM was revived from glycerol stock in MAG broth and incubated at 30 °C with shaking at 180 rpm for 72 h. Starter cultures were

diluted to an OD600 of 0.05 in fresh MAG (control) or MAG modified with one of three stressors: 0.5 M NaCl (osmotic), 1 mM H₂O₂ (oxidative), or temperature shock (42 °C for 3 h followed by return to 30 °C). For phage treatments, bacteriophage was added at a multiplicity of infection (MOI) of approximately 1. Each condition was prepared in triplicate (n = 3) and incubated with shaking at 30 °C; static incubation was used for biofilm formation.

Crystal violet biofilm assay

Biofilm biomass was quantified using a 96-well microtiter plate assay following established protocols^{10,13}. Briefly, 200 µL of culture was incubated statically in flat-bottom plates for 48 h, washed with PBS, stained with 0.1% crystal violet (CV), rinsed, and destained with ethanol. Absorbance was measured at 570 nm on a microplate reader.

Congo Red colony phenotyping

EPS-linked colony phenotypes were assessed by spotting 10–3 dilutions onto MAG agar supplemented with 0.1% Congo Red (CR) and incubating for 72 h at the appropriate treatment temperature. Colonies were scored on a standardized 1–3 scale for mucoidy (dry to glossy, EPS-rich) and separately for CR halo intensity (width and saturation of the dye ring). As CR binds multiple matrix components, including amyloid fibers and certain polysaccharides, these scores represent a composite proxy for matrix characteristics^{6,8,12}.

Statistical analysis

For OD₅₇₀ values and CR-derived traits, one-way ANOVA was performed across eight treatment conditions: control, phage, NaCl, phage + NaCl, H₂O₂, phage + H₂O₂, temperature shock, and phage + temperature shock. Bonferroni-corrected pairwise t-tests identified significant differences between conditions. Pearson correlation was used to test the relationship between mean mucoid score and OD₅₇₀. Statistical significance was set at α = 0.05. Data collection and reporting followed microtiter assay classification and quality control guidelines^{14,10}.

Results

TABLE 1. CRYSTAL VIOLET ASSAY (OD₅₇₀ VALUES)

Condition	Replicate 1	Replicate 2	Replicate 3	Mean OD ₅₇₀
Control	0.38	0.36	0.4	0.38
Phage	0.46	0.5	0.48	0.48
0.5 M NaCl	0.42	0.45	0.43	0.43
Phage + NaCl	0.53	0.55	0.56	0.55
1 mM H ₂ O ₂	0.43	0.46	0.44	0.44
Phage + H ₂ O ₂	0.53	0.54	0.52	0.53
Temperature Shock	0.39	0.38	0.41	0.39
Phage + Temp Shock	0.47	0.49	0.46	0.47

TABLE 2. CONGO RED ASSAY (MUCOID SCORE AND RED HALO SCORE)

Condition	Mucoid Score (Mean ± SD)	Red Halo Score (Mean ± SD)
Control	1.00 ± 0.00	1.00 ± 0.00
Phage	1.25 ± 0.50	1.25 ± 0.50
0.5 M NaCl	1.25 ± 0.50	1.50 ± 0.50
Phage + NaCl	1.50 ± 0.50	1.75 ± 0.50
1 mM H ₂ O ₂	1.25 ± 0.50	1.50 ± 0.50
Phage + H ₂ O ₂	1.75 ± 0.50	2.00 ± 0.50
Temperature Shock	1.00 ± 0.00	1.00 ± 0.00
Phage + Temp Shock	1.25 ± 0.50	1.50 ± 0.50

Biofilm biomass increased under all phage and stress conditions compared to the control (mean OD₅₇₀: control = 0.38, phage = 0.48, phage + NaCl = 0.55, phage + H₂O₂ = 0.53). To assess EPS production, Congo Red assays were performed by plating serial dilutions of each culture onto MAG agar supplemented with 0.1% Congo Red and incubating for 72 hours at the appropriate treatment temperature. Colonies were visually inspected and scored for mucoidy on a standardized 1–3 scale, where 1 represented dry, non-mucoid colonies and 3 indicated highly mucoid, glossy colonies with visible exopolysaccharide halos. Red halo intensity was scored separately on the same 1–3 scale, based on the width and saturation of the Congo Red ring

surrounding each colony. The highest mucoid and halo scores were generally observed in phage plus stress treatments, especially under oxidative and osmotic conditions. Statistical analysis confirmed that treatment condition had a highly significant effect on biofilm biomass (OD₅₇₀; one-way ANOVA, p < 0.001), while differences in mucoid score (p = 0.185) and red halo intensity (p = 0.099) were not statistically significant. These results indicate that phage exposure and abiotic stress can enhance EPS-linked phenotypes in *Bradyrhizobium* S3.9.2GM, with the strongest and most consistent effect detected in biofilm biomass.

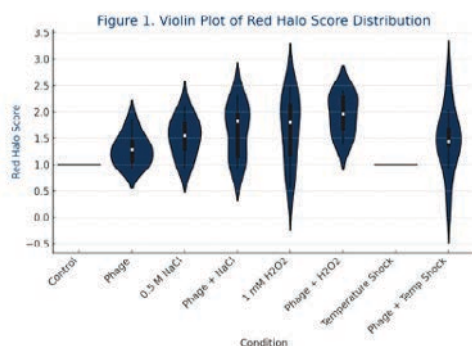


Figure 1: Red halo scores, used as a proxy for EPS production, were generally higher in phage + stress conditions, especially under oxidative and osmotic stress. However, differences across treatments were not statistically significant (one-way ANOVA, $p = 0.099$).

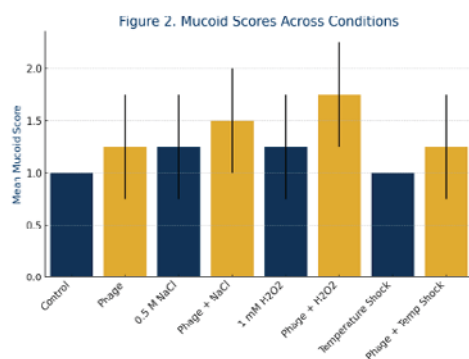


Figure 2: Mean mucoid scores were highest in phage + NaCl and phage + H₂O₂, suggesting increased EPS secretion under dual stress. Variation across treatments was not statistically significant (one-way ANOVA, $p = 0.185$). Error bars represent $\pm$ SD

Figure 3: Heatmap of Mean OD570 Values

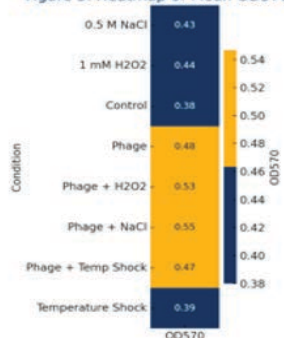


Figure 3: Biofilm biomass increased under all phage and stress treatments compared to the control, with the highest values in phage + NaCl and phage + H₂O₂. Differences between groups were highly significant (one-way ANOVA, $p < 0.001$).

Figure 4: Interaction Plot of OD570 by Stress and Phage Treatment

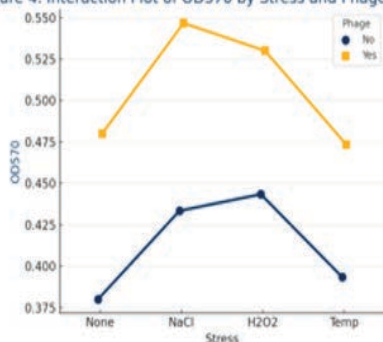


Figure 4: Across all stress categories, phage exposure consistently elevated OD570. The separation between phage and no-phage lines indicates additive or synergistic effects on biofilm formation when both biotic and abiotic stressors are present.

Figure 5: Correlation Between Mucoid Score and OD570

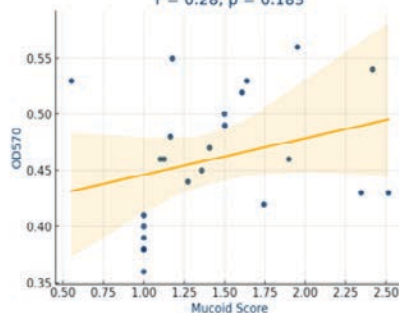


Figure 5: A weak positive correlation ($r = 0.28$, $p = 0.183$) was observed between EPS production (mucoid score) and biofilm biomass (OD570). This relationship was not statistically significant.

Discussion

Our results demonstrate that *Bradyrhizobium* S3.9.2GM biofilm biomass increased under all phage and stress treatments relative to the control, with the strongest effects observed in combined phage + stress conditions such as phage + NaCl (OD570 = 0.55) and phage + H₂O₂ (OD570 = 0.53). One-way ANOVA confirmed that treatment condition had a highly significant effect on biomass ($p < 0.001$), supporting the idea that both abiotic stress and phage exposure can promote surface-attached growth. This pattern aligns with prior work showing that osmotic and oxidative stress upregulate EPS production and favor a

biofilm lifestyle in rhizobia^{15,51}.

In contrast, mucoid score and red halo intensity both colony-level EPS indicators, showed similar directional trends but were not statistically significant ($p = 0.185$ and $p = 0.099$, respectively). The highest values for both traits were typically found in phage + stress combinations, particularly phage + H₂O₂ (mucoid score 1.75 ± 0.50 ; halo score 2.00 ± 0.50) and phage + NaCl (mucoid score 1.50 ± 0.50 ; halo score 1.75 ± 0.50). These increases, although not significant at $\alpha = 0.05$, suggest possible biological relevance that may require larger sample sizes or more sensitive compositional assays to detect statistically. Given that Congo Red binds multiple matrix chemistries including amyloids and certain polysaccharides^{6,8,12}, these scores should be interpreted as composite indicators of EPS-linked traits rather than direct measures of biomass.

The consistency between the biomass data and the directional changes in EPS traits suggests that biofilm enhancement under combined stress and phage exposure could involve both increased matrix production and shifts in matrix composition. This interpretation is supported by observations in other bacteria, where phage infection can stimulate EPS overproduction as a defensive strategy to immobilize or block viral particles^{9,747}. While such mechanisms are well described in clinical pathogens, our findings extend their potential relevance to slow-growing, nitrogen-fixing soil bacteria.

From an ecological perspective, EPS-mediated biofilm reinforcement under multitrophic and environmental stress could influence rhizosphere community dynamics, root colonization efficiency, and symbiotic performance. In agricultural systems, this has dual implications: enhanced resilience of beneficial rhizobia could improve plant-microbe symbiosis, but it could also complicate the use of phage as biocontrol agents if non-target mutualists are affected⁹.

Methodologically, this study highlights the value of pairing crystal violet microtiter assays with plate-based EPS scoring to capture both quantitative and qualitative aspects of biofilm formation. The CV assay provided robust detection of biomass changes across conditions, while the CR assay offered additional insight into colony

morphology and matrix characteristics. However, the lack of statistical significance for CR traits underlines the need for increased replication or more specific matrix-targeted approaches, such as polysaccharide-specific staining or EPS composition profiling¹⁰.

Overall, the strongest and most consistent treatment effect detected here was an increase in biofilm biomass under phage + stress conditions, with EPS-associated phenotypes showing similar but statistically non-significant trends. These findings indicate that phage exposure can act additively with abiotic stress to promote biofilm development in *Bradyrhizobium*, with potential consequences for rhizosphere ecology and agricultural microbiome management.

Conclusion

This study shows that when *Bradyrhizobium* S3.9.2GM is exposed to both phage and environmental stress, biofilm biomass increases more than with either factor alone. While this pattern is clear, we still do not fully understand the underlying mechanisms. The increases we observed could be due to more EPS being produced, changes in the types of matrix components, or both. Since Congo Red scoring alone cannot distinguish between these possibilities, the next step should be to combine our phenotypic assays with biochemical approaches. Methods such as EPS monosaccharide profiling, phenol-sulfuric acid quantification, and targeted staining for specific matrix components (for example, cellulose-specific dyes or amyloid-binding probes) would help reveal whether the response is primarily about quantity, composition, or a combination of both.

The relationship between phages and biofilms in rhizobia is likely to vary depending on both the bacterial strain and the type of phage. Expanding this work to include multiple *Bradyrhizobium* isolates and phages with different infection strategies, including those that produce depolymerases capable of degrading biofilm matrices, could provide important insight¹⁷. Using genomic and transcriptomic analyses during combined phage and stress exposure could also

uncover whether key genes for EPS biosynthesis, stress response, or host defense are being switched on or off.

Because our experiments were done in defined lab conditions, the next challenge is to see how these findings translate to more realistic environments. Testing in soil microcosms or directly in plant root systems would show whether the biofilm and EPS changes we see here actually improve root colonization, nodulation, or nitrogen fixation in the rhizosphere.

Finally, the agricultural implications deserve attention. If phage exposure in the soil can unintentionally boost the biofilm resilience of beneficial rhizobia, this could affect the design of phage-based biocontrol strategies aimed at plant pathogens^{9,17}. On the other hand, if used strategically, this phenomenon might be leveraged to help rhizobia persist in stressful conditions such as saline or drought-affected soils. Greenhouse and field trials that track plant performance, soil health, and microbial dynamics will be essential to test these possibilities.

By following these directions, future research can move beyond just identifying the pattern we saw here to understanding exactly how and why it happens, and how it might be applied to support more sustainable agricultural systems.

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