Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Quantitative modelling of survivability and controlled release of a double-entrapped Azospirillum formulation

Abitha Benson
Karpaga Vinayaga College of Engineering and Technology
Melvin Joe Manoharan
micromelvin@gmail.com

SRM College of Agricultural Sciences, SRM Institute of Science and Technology
T Ragunathan
SRM College of Agricultural Sciences, SRM Institute of Science and Technology
R. Nagajothi
SRM College of Agricultural Sciences, SRM Institute of Science and Technology
M Selvarani
Denver I. Walitang
Romblon State University
Tongmin Sa
Chungbuk National University

Research Article

Keywords: Azospirillum, double-entrapment, biphasic survival model, controlled release, salt stress, plant growth

Posted Date: August 19th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9875363/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Quantitative modelling of survivability and controlled release of a double-entrapped *Azospirillum* formulation

Authors:

Abitha Benson

Department of Biotechnology, Karpaga Vinayaga College of Engineering and Technology, Chengalpattu, India;

E-mail: abithabenson@gmail.com Orcid id: https://orcid.org/0009-0003-2524-0430

Melvin Joe Manoharan *

Department of Microbiology (Soil Science and Agricultural Chemistry), SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Chengalpattu-603201, India

E-mail: micromelvin@gmail.com Mobile: +91-9361679516 Orcid id: https://orcid.org/0000-0003-3740-4239

Ragunathan T

Mathematics (Department of Basic Sciences), SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Chengalpattu-603201, ragunatt@srmist.edu.in Orcid id: 0009-0000-9799-7924

R. Nagajothi

Section of Biochemistry and Crop Physiology, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai Campus, Chengalpattu-603201, India

Email: nagajotr@srmist.edu.in Orcid id: 0009-0004-0630-7422

Selvarani M

Independent Researcher (Mathematics),Guduvanchery, Tamilnadu-603202, Orcid id: 0009-0000-9799-7924, Email: selvaraniilango@gmail.com

Denver I. Walitang

DNA Barcoding Laboratory and College of Arts and Sciences, Romblon State University, Romblon 5505, Philippines

E-mail: denwing82@rsu.edu.ph Orcid id: https://orcid.org/0000-0001-5469-5838

Tongmin Sa

Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju, Korea

The Korean Academy of Science and Technology, Seongnam, Republic of Korea

E-mail: tomsa@chungbuk.ac.kr Orcid id: https://orcid.org/0000-0001-7444-5852

***Corresponding author**

Melvin Joe Manoharan

Department of Microbiology (Soil Science and Agricultural Chemistry), SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Chengalpattu-603201, India

E-mail: micromelvin@gmail.com Mobile: +91-9361679516

Orcid id: https://orcid.org/0000-0003-3740-4239

Abstract

Background

Bioinoculants also known as biofertilizers suffer from the major limitation of poor survivability during storage, followed by a rapid reduction during their application time and

both these factors limit their plant growth promotion ability. Given study exploits the possibility for developing a double-entrapped (DE) *Azospirillum* based, followed by a mathematical modelling approach and in vivo evaluation of its performance. DE system was developed by entrapping *Azospirillum* containing alginate beads in non-woven paper. Performance and bacterial release of DE system was studied based on various mathematical models, while Weibull kinetics evaluated the bacterial release rate. Efficiency of DE was further evaluated based on pot culture conditions with an comparison on traditional alginate and non-woven carriers. DE achieved lower inactivation rate constant of $K_1 = 0.030$ day^{-1} and $K_2 = 0.003$ day^{-1} compared to alginate and non-woven carriers. Moisture diffusion analysis showed a reduced effective diffusivity of = D_{eff} = 3.47×10^{-10} m^2 day^{-1} supported by a Thiele modulus of 6.84, confirming diffusion-controlled transport. Weibull kinetics exhibited a controlled, sustained bacterial release of $K = 0.135$ day^{-1}. Pot culture studies demonstrated 25-35% higher rhizosphere colonization and higher plant growth with the DE carrier over the other two formulations.

Keywords: *Azospirillum*, double-entrapment, biphasic survival model, controlled release, salt stress, plant growth

1. Introduction

Fertilizer consumption in India has reached an alarming rate of 63.94 metric tonnes during the year 2021-22, as the country is the world's largest producer of cereals, pulses and vegetables (Viswambharan, 2024). Except for the legumes, cereals and vegetable crops heavily rely on nitrogen-based fertilizers. However, N fertilizers are blamed for the increase in nitrogen oxide production ever since the industrial revolution. As an effective alternative to the "N" based fertilizers, plant growth-promoting rhizobacteria have played a major role in improving

nutrient availability and enhancing root development through various direct and indirect mechanisms (Kloepper and Schroth, 1978).

Among the PGPR, members of the genus *Azospirillum* is known to improve growth and yield of numerous crops through various mechanisms such as nitrogen fixation and production of phytohormones such as a gibberellic acid (GA3) and Indole Acetic Acid (IAA) (Piccoli et al. 1999; Thuler et al. 2003; Döbereiner et al., 1976; Bashan & de-Bashan, 2010). The possibility of replacing 25% N-fertilization with the help of *Azospirillum* inoculation, which in turn could reduce the CO_2 emission to the tune of 4.95 million Mg of CO_2 per year, was reported by Hungria et al. (2022).

In spite of this, the global adoption of *Azospirillum*-based biofertilizers remains a major global challenge, with the exception of certain South American countries such as Brazil and Argentina (Bashan et al., 2014). Main reasons attributed to this are the inconsistent field performance, the low shelf life, sensitivity to environmental stress, and the lack of regulatory frameworks that emphasize the functional efficacy rather than mentioning mere cell numbers (Malusá et al., 2012; Herrmann & Lesueur, 2013). On the other hand, poor survivability during storage and rapid decline after soil application remain the major constraints affecting the effectiveness of the PGPR-based formulations, including that of *Azospirillum* (van Veen et al. 1997; Bashan et al., 2014).

To overcome this, the encapsulation technique (also known as immobilization) serves as a way to cover the living cells in a polymer matrix, which in turn protects the cells and the metabolites from adverse environmental conditions by providing them with a microenvironment (Balla et al., 2022). Alginates are the most preferred universal polysaccharide utilized for microbial encapsulation owing to its biocompatibility, low toxicity, low cost, and mild gelation through the addition of divalent cations such as Ca^{2+} (Gombotz and Wee,1998; Bashan et al., 2014).

Alginate-based encapsulated formulations are known for their ability to facilitate slow release of bacteria or other nutrients by degradation or pore-assisted diffusion (Young et al., 2006; Flores et al., 2013).

However, alginate-based formulations exhibit low mechanical strength, limiting their ability to withstand applied forces, limiting their applications in industrial environments (Serrano-Aroca et al., 2017). Additionally, the restricted tolerance to monovalent cations, and presence of macropores enhance the diffusion and release, which in turn restrict their practical use in real-world situations (Córdoba et al., 2013).

To overcome the limited structural integrity and performance of alginate-based formulations, several modifications have been proposed including the incorporation of additives such as polyvinylpyrrolidone and other polymers (Bashan et al., 2014) or the addition of bentonite polymers to promote slow release (He et al. 2015). As an effective alternative, non-woven biodegradable materials are known to offer superior mechanical strength, apart from having high porosity and large surface area, and can act as superior scaffolds (Tan et al. 2020). Although studies have shown the survival of various microorganisms including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans* in non-woven matrices (Liu et al., 2017; Zhang et al., 2019), the application of non-woven materials for PGPR formulation, particularly for *Azospirillum* spp., has not been systematically investigated.

Except for a few studies, there is a lack of quantitative understanding of PGPR and in particular *Azospirillum* survivability and release behaviour using mathematical models. Microbial survival curves often exhibit non-linear behaviour due to resistance variability and mixed microbial population (Panigrahi et al. 2021), which can be effectively described using biphasic survival models (Cerf, 1977; Phaiboun et al. 2015). Moreover, the diffusion-controlled

heterogenous release data can be fitted and characterized using the well-known Weibull model (Weibull, 1951; Peleg and Cole, 1998).

Diffusion–reaction interactions within carrier systems can be explained based on the transport phenomena, including the Thiele modulus, which studies the reaction and diffusion balance, and the Biot number, which evaluates whether internal diffusion or external mass transfer is the limiting step (Driessen et al. 2020). However, such modelling approaches have rarely been applied to PGPR formulations.

Taking all these into account, the present study explores the possibility of developing a double-entrapped carrier system for *Azospirillum* by combining sodium alginate and non-woven biodegradable paper. The survivability of *Azospirillum* was studied during the storage and fitted with the biphasic survival model to study the diffusion-reaction interactions. The survivability of *Azospirillum* sp. during storage was analyzed using biphasic survival models, while the diffusion–reaction interactions were quantified using the Thiele modulus and Biot number analysis. Kinetics of release both under normal and salt-affected conditions were fitted using the Weibull model. Pot culture studies were also conducted to evaluate the performance of the double-entrapped carrier system for its influence on the colonization efficiency, root architecture, and growth.

2. Materials and Methods

2.1. Preparation of *Azospirillum*-based Carrier Formulations

Strains **of** *A. brasilense* CW903/CW903-PLA were from previous studies (Kim et al. 2005; Joe et al. 2013). Strains were maintained in nutrient agar with bimonthly transfer. Alginate beads were developed based on the descriptions of Bashan et al. (2002) with required modifications as described in our earlier studies (Joe et al. 2012;2013). For the double

entrapment, alginate beads (mean diameter approx. 3 mm) were embedded between layers of non-woven paper to form an effective composite structure.

For the double-entrapped carrier, alginate beads containing *Azospirillum* were embedded between layers of non-woven material to form a composite structure. A slightly larger size is preferred for *Azospirillum*, while a size close to 1 mm would be ideal for aerobic bacteria to ensure effective oxygen penetration (Wu et al. 2012). The effective diffusion thickness for *Azospirillum* under microaerophilic conditions is ≃ 2mm (1.79 mm) (8.32 Log Cfu g^{-1} Population), calculated based on the earlier reports of Hill (1928) and Hiemstra (1983).

For the double entrapment (DE) process, non-woven abaca paper (hereafter will be referred to as only non-woven paper) was cut in size of 5 x 8cm. Oxygen diffusion to the bead is restricted by soaking the non-woven paper in 5% glycerol solution. The non-woven paper was made into a cover by pasting the ends with gum Arabic. Alginate beads with entrapped *Azospirillum* were placed inside the cover, represented in Figure 1. The entire setup containing 6 covers were placed in sterile plastic boxes fitted with 0.45um PTFE membrane filter maintained at room temperature.

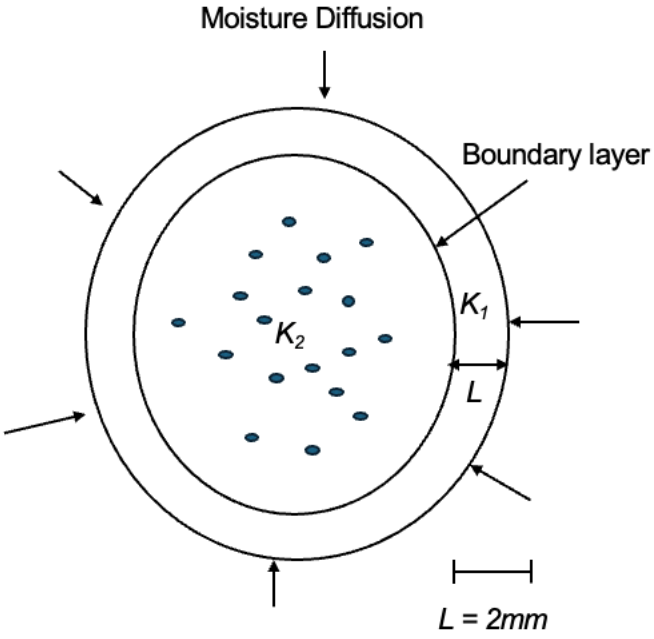


Fig. 1. Schematic representation of diffusion–reaction controlled double-entrapped carrier. Alginate beads (Ø ≈ 3 mm) containing entrapped PGPR cells are embedded within a non-woven support matrix. Associated with the diffusion-reaction controlled system are the effective diffusion path length (L ≈ 2 mm), reaction-dominated surface regions (k_1) and diffusion-limited core regions (k_2).

2.2. Storage Survivability Assessment

The survivability of *Azospirillum* was done periodically in the study period of 180 days. A population estimation was done based in nitrogen-free malic acid medium, and the population was studied based on MPN technique (Cochran et al. 1950).

2.3. Biphasic Survival Modelling

The survivability of *Azospirillum* was modelled based on the biphasic survival model since the experimental data follows two sub microbial populations when subjected to stress under storage condition (Peleg, 1999; Peleg & Cole,1998). The formula below is derived by assuming the populations as sensitive N_1 and resistant N_2 populations.

$$N(t) = N_1 e^{-k_1 t} + N_2 e^{-k_2 t}$$

N(t) represents the bacterial viability at time t, (N_1) and (N_2) represents fast and slow inactivating populations, and (k_1) and (k_2) are their inactivation (day-^{1}) rate constants.

2.4. Model Fitting and Statistical Validation

Model assessment was determined based on the coefficient of determination (R^2) and root mean square errors (RMSE). Model selection was further analysed using Akaike information criterion (AIC).

2.5. Moisture Diffusion and diffusion reaction analysis

The non-dimensional moisture ratio (MR) was calculated based on experimental data as described by Crank (1975) following the formula below:

$$MR = \frac{M_t - M_e}{M_0 - M_e}$$

M_t -moisture content at time t, M_0- initial moisture content experiment time t = 0. M_e is the equilibrium moisture content.

Effective moisture diffusivity (*Deff*) is worked out based on Fick's second law of diffusion (linearized version) applied to porous materials with the equation expressed as (Citation):

$$\ln(MR) = -\frac{\pi^2 D_{eff}}{L^2} t$$

L is the diffusion length and t is the storage or drying time**.**

Microbial inactivation kinetics was integrated into the Thiele modulus (Φ) as described by Fogler (2016):

$$\Phi = L\sqrt{\frac{k_1}{D_{eff}}}$$

In the present study, the effective thickness of the entrapped material is represented as L (≃ 2 mm), the first order reaction rate constant $k(\text{time}^{-1})$ and the fast inactivation rate constant **(k_1)** are obtained from the biphasic survival model.

Biot numbers (Bi) were calculated to assess internal mass-transfer limitations as described by Fogler (2016) and the Biot number expression is:

$$\text{Bi} = \frac{h\,L}{D_{eff}}$$

h is the mass-transfer coefficient of biological porous materials in nature under natural or low-airflow conditions and *L* is the characteristic diffusion length ($L \approx 2$ mm in this study).

$$h = 1 \times 10^{-6} \text{ to } 5 \times 10^{-6} \text{ m s}^{-1}$$

2.6. Release Kinetics Under Normal and Salt-Affected Conditions

Weibull equation is used to study the bacterial release kinetics under both normal and salt-affected conditions as described by Peleg (1999):

$$\frac{M_t}{M_\infty} = 1 - \exp[-(kt)^n]$$

$M_t$is the cumulative amount of bacteria released from the carrier at time t. M_∞ is the maximum amount of bacteria released under the experimental conditions. M_t/M_∞ is the fractional release**,** representing the amount of bacteria released at time t, k is the release rate constant and n is the shape parameter representing the heterogeneity of the release.

2.7. Pot Culture Experiment

Pot culture experiments were conducted under greenhouse conditions to evaluate the effect of double-entrapped *Azospirillum* formulations on the plant growth both under normal and salt affected conditions. The experiment was carried out using rice (*Oryza sativa* L.) cultivar Green World Pusa PR-130. The soil used for the experiment was clay loam**,** pH 7.6, EC-1.71 dS m^{-1}, Soil organic C (%)- 0.56, N-121.3 Kg ha^{-1}, P-13.9 Kg ha^{-1}, and K- 172.6 Kg h^{-1}. The soil was homogenized and sieved to remove stubbles and larger particles prior to filling in pots. Pots were arranged in a completely randomized design (CRD) with six replications per treatment. The experimental treatments include uninoculated control, free PGPR inoculation, alginate-based PGPR formulation, and double-entrapped PGPR formulation.

Soil salinity was induced by irrigation with salt water containing 100 mM NaCl**,** while other treatments received 0 mM irrigation. Soil fertilization used recommended dose of fertilizers (RDF) calculated per pot basis following standard agronomical practices. Pot culture was maintained at natural lighting conditions and the standard agronomic practises were followed. Different plant biometrics such as root length (cm), root area (cm^2 $plant^{-1}$), plant height (cm), plant dry weight (unit), leaf area (cm^2) and grain yield (unit) were measured using standard methods.

Nitrogenase activity of soil was estimated as per the methods of Hardy et al. (1968) with the modifications described by Madhaiyan et al. (2006). In brief fresh soil samples of 10 g quantity

are mixed with 0.4% (w/w) glucose and 4 ml of distilled water to maintain adequate moisture. Soil was transferred into a rubber stoppered bottle and sealed with paraffin wax to make it air tight. Acetylene gas of 10 % w/w was injected into the bottle and incubated at room temperature for 24 h. After incubation 100 µL of gas was withdrawn and injected into gas chromatograph fitted with flame ionisation detector (FID) for the estimation of ethylene.Sub-surface pellicle formation in the semi-solid nitrogen-free malic acid medium was observed as described by Döbereiner (1980). The visualization of *Azospirillum pLA-lacZ* plasmids in roots was done through staining with X-Gal (30 µg ml^{-1}) and electron microscopy as described earlier (Joe et al. 2013; 2022).

2.8. Statistical and Software Analysis

Data was analyzed statistically using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT), with significance calculated at $P < 0.05$. Survivability, transport, release and plant growth parameters were standardized and evaluated for their relationships using Principal Component Analysis (PCA) with Python. Interpretation was based on the first two principal components (PC1 and PC2). Graphs and model fittings were produced using Python libraries (Matplotlib, Seaborn, and SciPy).

3. Results and Discussion

3.1. Survivability of *Azospirillum* during Storage and Biphasic Kinetics

In the present study, a progressive decrease in *Azospirillum* populations across all the carrier systems were observed. However, there were notable variations among the different formulations (Figure 2). Among the carriers tested, the non-woven biodegradable paper-based carrier recorded the fastest reduction in terms of bacterial viability. On the other hand DE based carrier system showed better survivability visualized via pronounced tailing behaviour

indicating better survivability by providing cells with a better protection. Survival data fitted on the biphasic survival model showed a fast inactivation rate constant (k_1) of 0.070 day^{-1}, 0.050 day^{-1}, and 0.030 day^{-1} and slow inactivation rate constant (k_2) of 0.010 day^{-1}, 0.006 day^{-1}, and 0.003 day^{-1} for non-woven paper, alginate and double entrapped formulations, respectively (Table 1). A lowest diffusivity value of 2.57×10^{-9} m^2 day^{-}, relative Deff value of 1.0, Thiele modulus (Φ) value of 6.84, Biot number (Bi) value of 6.73×10^5, and a diffusion-controlled transport regime is observed in the DE formulation. Reductions in the fast inactivation population by 29% in the alginate and 57% in double-entrapped carrier system compared to the non-woven carrier was observed. A similar trend was also observed with slow inactivation population, in which double-entrapped carrier recorded a 70% reduction compared to the non-woven carrier and 50% reduction compared to alginate carrier. This slow inactivation rates in DE carrier system may be attributed to the deeper location of the bacteria in the composite matrix, which in turn might have reduced their exposure to environmental stresses. The survivability of *Azospirillum* under storage conditions can be explained based on the biphasic model survival as they exhibit a gradual decline followed by a slower decay (Phaiboun et al., 2015). This may be due to the fact that a fraction of the cell population may enter a slow metabolising state with higher stress tolerating efficiency compared to that of the actively growing cells (Patra and Klump, 2013). The major challenge that any carrier material would encounter is the loss of viability of the entrapped bioinoculum during the short term storage in the warehouse and the long term storage in growers' house in which the storage temperature may range from −5 to 30 °C (Bashan et al. 2014). The improved survivability observed in alginate and DE formulations can be attributed to the protective microenvironment with enhanced mechanical stability provided by non-woven paper to the alginate matrices in the DE carrier system. Numerous authors (Gaumann et al., 2000; Chang et al., 2002) have reported that the multilayer coatings using polyethyleneimine (PEI) and polyacrylic acid have

enhanced stability, mechanical strength, elasticity, and maintained the pore structure and degradation under stress. The lower relative diffusivity observed in the double-entrapped system shows a stronger diffusion barrier enhance protection at the cost of internal mass transfer limitations as reported by Chen et al. (2003). The boundary layer plays a major role in carrier behaviour and other properties such as higher voidage and favourable geometry which may play major role in diffusion and O_2 transfer (Dias, 2018). Together, with a higher Φ and Bi values observed in the present study, diffusion-limited transport regime was established in DE carrier system.

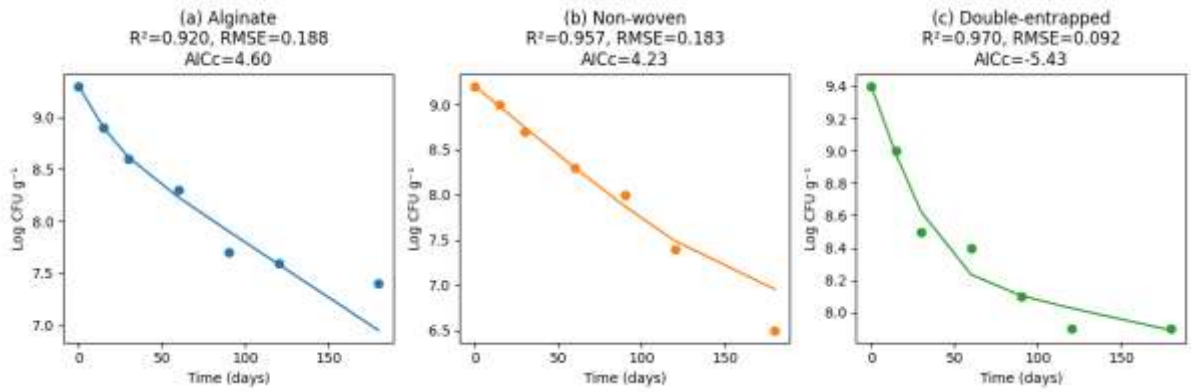


Fig. 2. Biphasic survival behaviour of *Azospirillum* formulated in different carrier systems during storage. (a) Alginate (b) non-woven paper, and (c) double-entrapped carrier. Symbols represent the actual experimental survivability population data (Log Cfu g^{-1}), while solid lines represent fits of the biphasic survival model.

Table 1. Integrated biphasic survival and transport parameters for different carriers

Carrier	k_1 (day^{-1})	k_2 (day^{-1})	$D_e$ff (m^2 day^{-1})	Relative $D_e$ff	Thiele modulus (Φ)	Biot number (Bi)	Transport regime
Non-woven	0.070	0.010	3.47×10^{-10}	0.14	7.10	1.25×10^{6}	Reaction-controlled
Alginate	0.050	0.006	9.43×10^{-10}	0.37	7.28	9.16×10^{5}	Mixed control
Double-entrapped	0.030	0.003	2.57×10^{-9}	1.00	6.84	6.73×10^{5}	Diffusion-controlled

k_1 and k_2 represent the fast and slow inactivation rate constants obtained from biphasic survival model fitting. $D_e$ff denotes the effective moisture diffusivity estimated from experimental data using a linearized Fickian diffusion model. Relative $D_e$ff was obtained by normalizing $D_e$ff with respect to the maximum value among carriers. The Thiele modulus (Φ) was calculated as $\Phi = L \sqrt{(k_1 / D_e\text{ff})}$, where L is the characteristic carrier dimension. The Biot number (Bi) was calculated based on the formulae $\text{Bi} = \frac{h L}{D_{eff}}$.

3.2. Bacterial Release Kinetics

The Weibull model for non-linear release was employed to study bacterial release as soil heterogeneity introduces limitations on the bacterial movement. In this study, the Weibull model represented the bacterial release of different carriers including the double-entrapped

carrier under different conditions such as in PBS, normal soil, and salt-affected soil conditions (Figure 3 and Table 2). Weibull shape parameter (n) remained less than 1, with ranges extending from 0.416-0.709 across the different carrier systems showing a decelerating release for a diffusion-controlled-heterogeneous release. In PBS conditions, the double-entrapped carrier system showed a release rate constant of k = 0.216 day^{-1}, which was 24% slower than alginate and 19% lower than non-woven paper. The same trend was noted under non-stressed soil conditions where the release in double-entrapped system was 0.135 day^{-1}, which was 18% slower than alginate and 52% slower than non-woven carrier. Under salt affected conditions, the double-entrapped formulation recorded a k value of 0.235 day^{-1}, which was 39% and 13% lower than non-woven paper, respectively. Weibull-based kinetics have been widely employed to describe controlled release systems with internal mass transfer limitation (Albert and Mafart, 2005). Avsaroglu et al. (2006) reported that due to environmental complexity, the microbial survival or release patterns often deviate from the normal log-linear patterns. The Goodness-of-fit with R^2 values ranging from 0.84–0.94 confirms the accuracy of Weibull model in describing the release behaviour. The maintenance of lower release by double-entrapped system under non-stress and salt-affected conditions indicate the robustness of the carrier as Weibull model accounts for system variability under diverse heterogeneous and variable environmental conditions (Membré and Leguérinel, 2024).

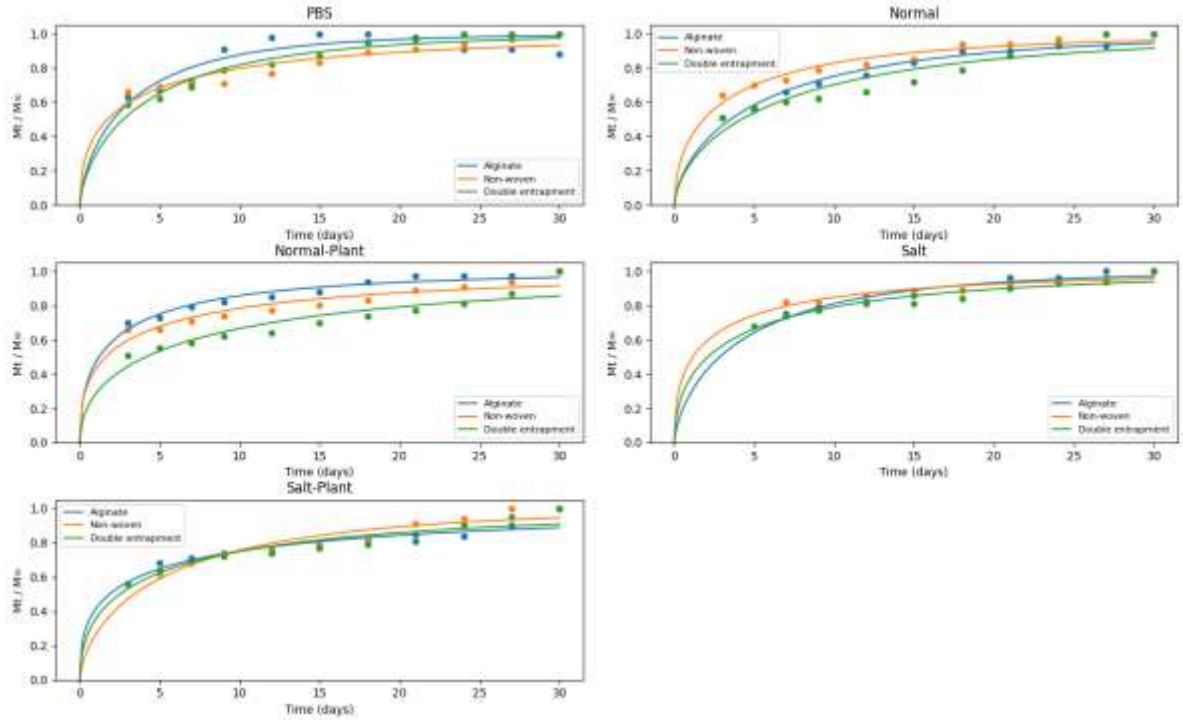


Fig. 3. Comparative Weibull model fitting of bacterial release from alginate, non-woven biodegradable paper, and double-entrapped matrices under different environmental conditions: PBS, normal soil, normal soil with plant, salt-affected soil, and salt-affected soil with plant. Symbols represent experimental data and solid lines represent Weibull model fits.

Table 2. Weibull kinetic parameters describing bacterial release from alginate, non-woven biodegradable paper, and double-entrapped matrices under PBS, normal soil, normal soil with plant, salt-affected soil, and salt-affected soil with plant conditions

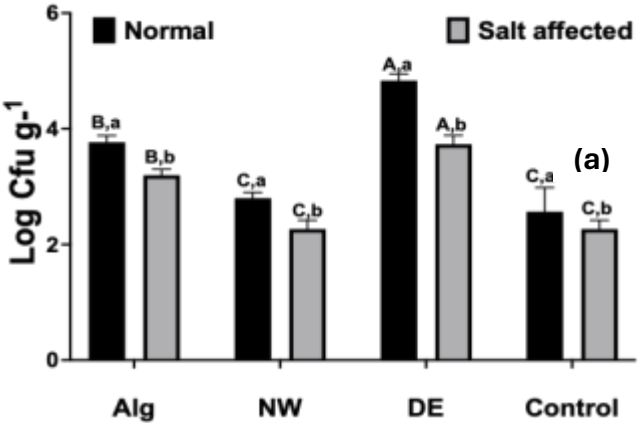
Condition	Carrier	n (β)	k (day^{-1})	R^2
PBS	Alginate	0.709	0.285	0.741
	Non-woven paper	0.479	0.267	0.855
	Double entrapment	0.692	0.216	0.939
Normal soil	Alginate	0.672	0.165	0.969
	Non-woven paper	0.555	0.279	0.926
	Double entrapment	0.646	0.135	0.853
Normal soil + plant	Alginate	0.490	0.389	0.932
	Non-woven paper	0.436	0.265	0.860
	Double entrapment	0.518	0.119	0.842
Salt-affected soil	Alginate	0.698	0.207	0.918
	Non-woven paper	0.466	0.387	0.878
	Double entrapment	0.525	0.235	0.903
Salt-affected soil + plant	Alginate	0.416	0.222	0.764
	Non-woven paper	0.663	0.169	0.899
	Double entrapment	0.501	0.192	0.891

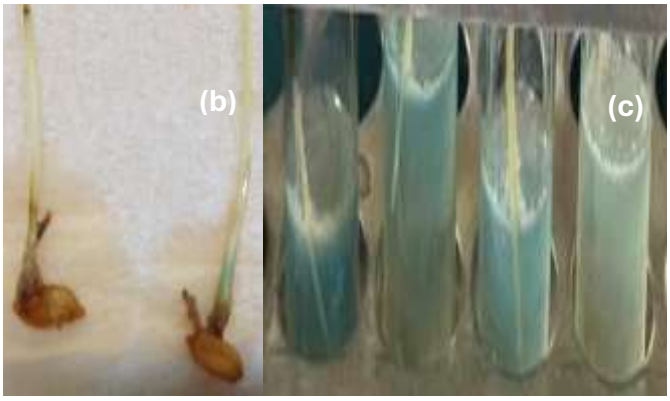
The Weibull model to describe bacterial release kinetics under different environmental conditions is expressed as$M_t/M_\infty = 1 - \exp[-(kt)^n]$, where $k$is the release rate constant (day^{-1}) and n(β) is the shape parameter. R^2 represents the coefficient of determination used to evaluate the goodness-of-fit of the Weibull model.

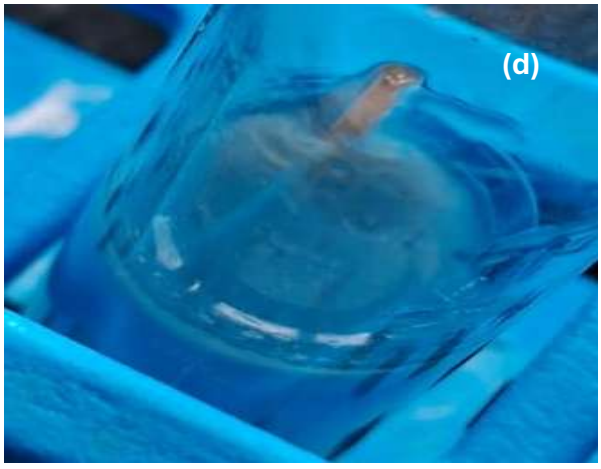
3.3. Rhizosphere Colonization of *Azospirillum* and its Effects on Rice Root Architecture

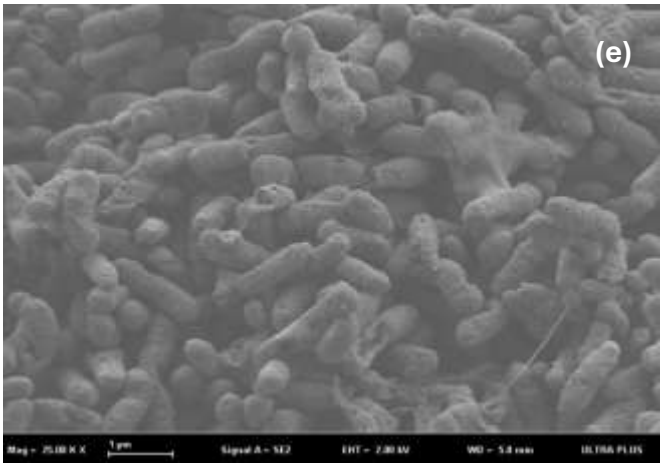
The higher root colonization coupled with better root length and root area were observed DE carrier system compared to the other two formulations both under normal and salt-affected conditions (Figure 4). Double-entrapped carrier system showed the highest survival rates 4.8 Log CFU g^{-1} under normal conditions and 3.7 Log CFU g^{-1} under salt-affected conditions. The DE carrier recorded 26% higher population compared to alginate, 50% higher than non-woven and 92% higher compared to control under normal conditions. Under salt-affected conditions, DE recorded 19% higher population compared to alginate, 40% higher than non-woven and 68% higher population compared to control under-salt affected conditions. Under normal conditions, the highest root length of 29.4 cm was observed with DE carrier system, which recorded 16% higher than alginate, 30% higher than non-woven and 53% higher than control. A similar trend was observed under salt-affected conditions which exhibited 5% higher root length compared to alginate, 20% higher than non-woven and 40% higher root length compared to the control. Double-entrapped carrier system recorded an increased root area of 4.8 cm^2 in normal conditions, which recorded 30% higher root volume than alginate, 45% higher than non-woven, and 78% higher than the control. Under salt-affected conditions, double-entrapped carrier recorded 33% higher root area compared to alginate, 40% higher than non-woven, and 75% higher root area than the control. Earlier observations by van Veen et al. (1997) reported that the introduced populations reduced rapidly by 0.2-1.0 log units owing to the diverse environment, biological stresses, and the unpredictable soil conditions. Bashan et al (2014) and Bashan and Holguin (1995) stressed the importance of effective inoculant-carrier formulations in overcoming major barriers such as soil heterogeneity and microbial competition, thereby promoting successful colonization and improving plant growth. On the other hand, the use of *Lac*Z-marked strain has helped in the identification of the inoculated *Azospirillum* strain from

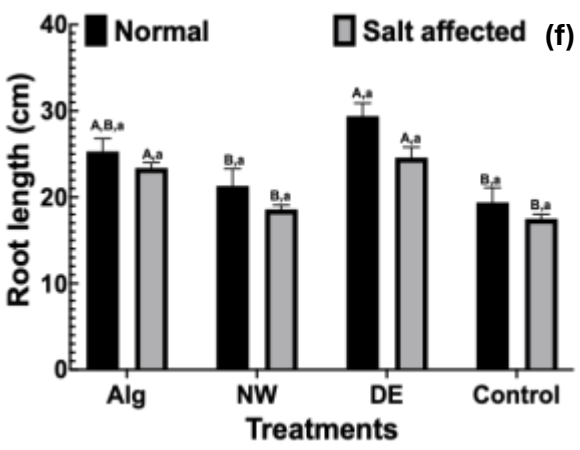
indigenous *Azospirillum* isolates. The metabolic activity of the inoculated strains were confirmed based on the qualitative observations that includes subsurface pellicle formation and colour changes in N-free malic acid medium from yellowish green to blue colour. SEM analysis reveals dense bacterial aggregation of *Azospirillum* isolates around the root surfaces of rice.











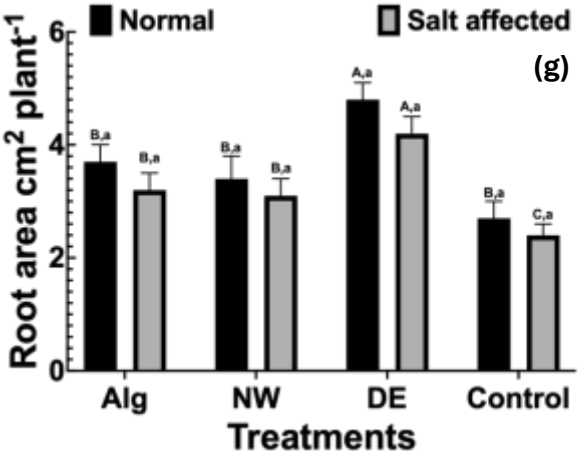


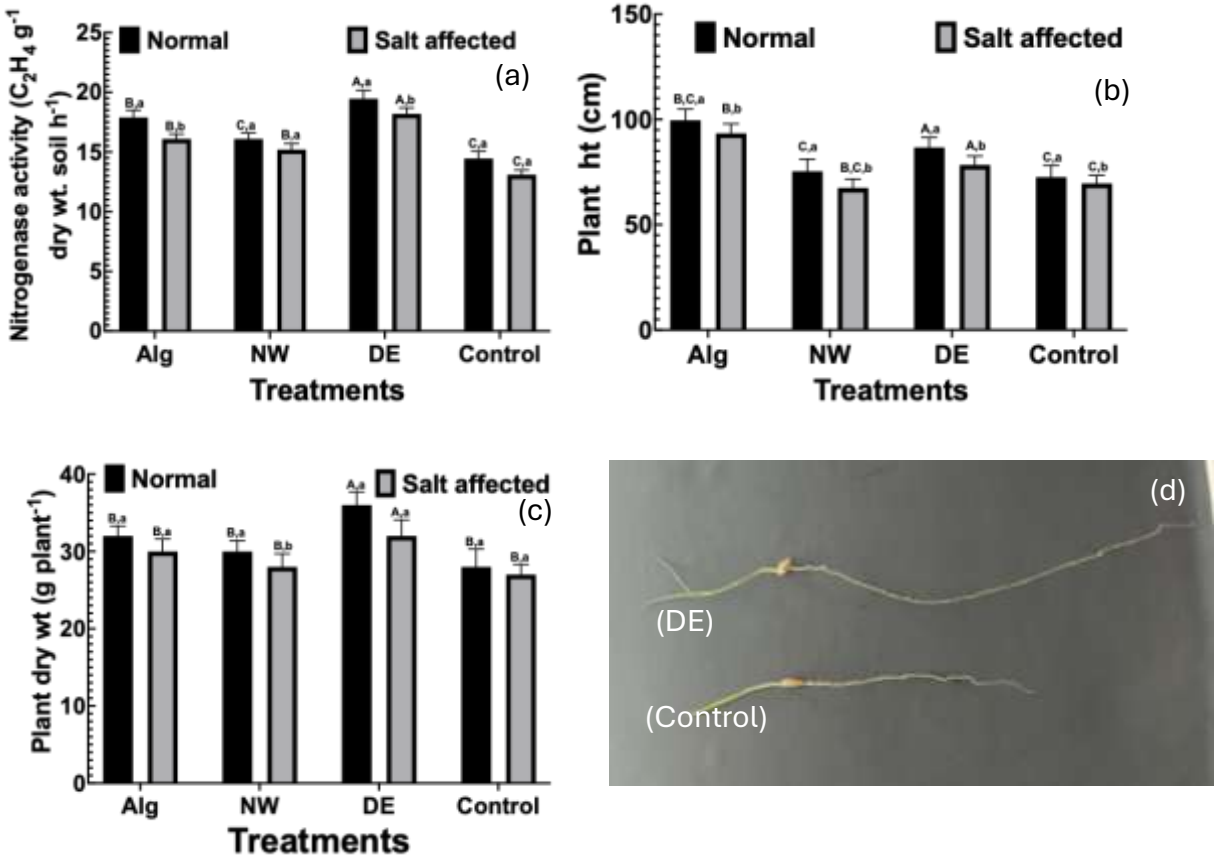
Fig. 4. *Azospirillum* colonization from alginate, non-woven biodegradable paper, and double-entrapped matrices under different normal and salt-affected conditions.

a) Population expressed as log Cfu g^{-1} b) Colonization of *A. brasilense in* rice c) Subsurface pellicle formation and colour change in N-free malic acid medium due to alkaline conditions d) Close view of the subsurface pellicle formation e) SEM view of *Azospirillum* under pellicle formation conditions f) Root length and g) root area observed under 45 DAS.

3.4. Plant Growth under Normal and Salt Stress

Effects of different *Azospirillum*-based formulations were assessed on the nitrogenase activity, plant height, plant dry weight, leaf area, and grain yield (Figure 5). DE carrier system outperformed others by exhibiting nitrogenase activity of 19.4 C_2H_4 g^{-1} dry wt. soil h^{-1}, which was 9% higher than alginate, 21% higher than non-woven, and 35% higher than control under normal soil conditions. DE carrier system showed similar trends under salt affected conditions exhibiting a nitrogenase activity of 18.2 C_2H_4 g^{-1} dry wt. soil h^{-1}. This activity was 14% higher compared alginate, 26% higher than non-woven, and 38% higher compared to control. The DE carrier system of *Azospirillum* exhibited the highest increase in plant height of 100 cm under normal soil conditions, which was 15% higher than alginate based formulation, 33% higher than non-woven, and 37% higher than control. Similar trends were observed under salt affected conditions. In the DE carrier system, plant height was the highest with 93 cm, which was 18% higher than alginate, 39% higher than non-woven and 34% higher than control. For plant dry weight, DE carrier system recorded a plant dry weight of 36 g $plant^{-1}$, which was 13% higher than alginate, 20% higher than non-woven and 29% higher than control. Under salt affected conditions, a plant dry weight of 32 g $plant^{-1}$ was recorded in DE carrier system, and this was

7% higher than alginate, 14% higher than non-woven and 19% higher than control. The DE treatment exhibited a leaf area of 246 cm² under normal soil conditions which was 6% higher than alginate, 17% higher than non-woven, and 24% higher than the control. Under salt-affected conditions, a leaf area of 225 cm² was recorded in DE system, which was 5% higher than alginate, 10% higher than non-woven, and 25% higher, when compared to control. The DE carrier system recorded a grain yield of 5.4 g plant^{-1} under normal soil conditions, and this was 32% higher than alginate, 50% higher than non-woven, and 74% higher than the control. Under salt affected conditions, DE carrier system recorded a grain yield of 4.5 g plant^{-1}, and this was 25% higher compared to alginate, 41% higher than non-woven, and 67% higher compared to the control. Perrig et al., (2007) reported yield improvements ranging from 5-30% due to *Azospirillum* inoculation depending on the formulation efficiency and environmental conditions. Earlier findings by Abdel-Latef et al. (2020) and Khalid et al. (2017) supported a successful mitigation of salt stress by *Azospirillum* through improved physiological activities, osmolyte accumulation, and antioxidant enzyme activity.



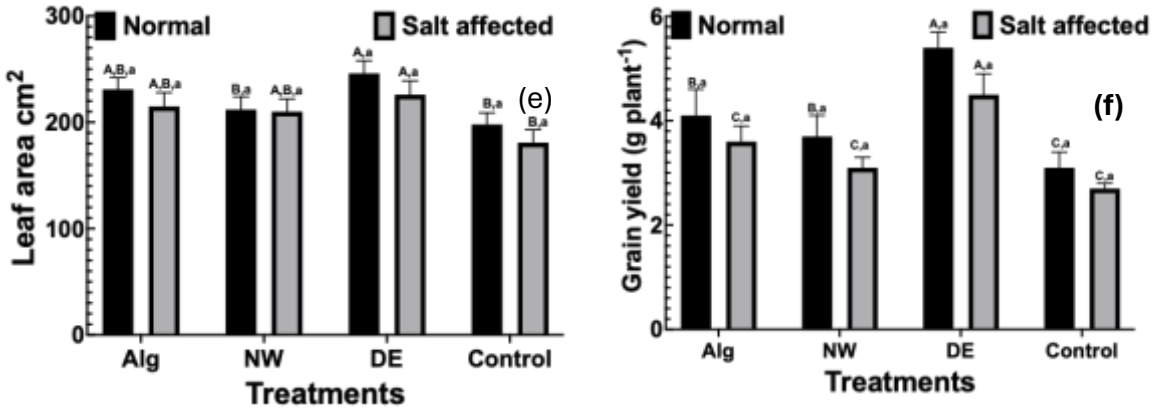


Figure 5. Effect of *Azospirillum* based alginate, non-woven biodegradable paper, and double-entrapped matrices under different normal, and salt-affected conditions on a) Nitrogenase activity, b) Plant height c) Plant dry wt. d) compared between control and double entrapped formulations e) plant leaf area and f) grain yield .

3.5. Variabilities in *Azospirillum* Survivability, Transport, Release, and Effects in Plant Growth in Different Carrier Systems

Inter-relationships and variabilities of different carrier material properties were observed via Principal component analysis (PCA) showing that PC1 accounted for 97.1% of the variability and PC2 accounting for 2.9% of the variability (Figure 6.). DE carrier system was observed along the $D_e$ff, showing transport properties coupled with improved protection to the *Azospirillum* cells. On the other hand, non-woven carrier line-up with Weibull and k_1, and k_2, showing faster inactivation and a lower stability. Alginate associates with biomass vector, showing better maintenance of the microbial population compared to the non-woven carrier. PCA highlights the successful integration of survivability and transport phenomenon in the DE system compared to alginate and non-woven carriers. The separation of DE system from other indicate a perfect blend of both microbial protection and delivery mechanism. Mhlongo et al.

(2022) reported that PCA has been widely used to differentiation the PGPR treatments based on functional and metabolic traits.

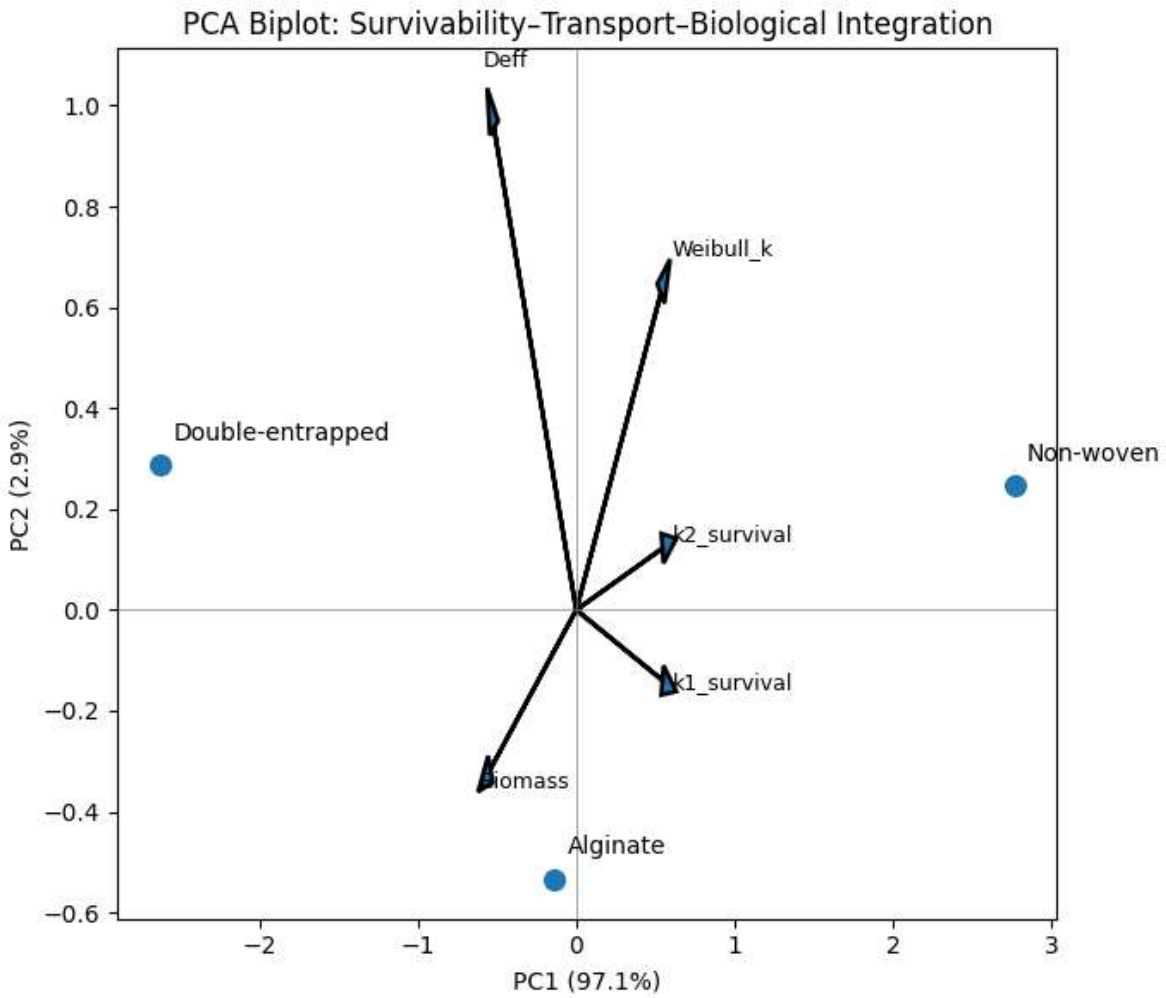


Fig. 6. Principal component analysis (PCA) of biplot integrating survivability, transport phenomenon, release kinetics, and biological parameters of different *Azospirillum* carrier systems. PC1 (97.1%) and PC2 (2.9%) are the PCA components that explain the total variance of the data set. Contribution and direction of the variables, $D_e$ff , k, k_1, k_2 and biomass are indicated by vectors.

Conclusion

Based on the analysis of various parameter such as survivability under storage conditions, transport phenomenon and release kinetics evaluation, and plant growth responses both under normal and salt affected conditions, it is observed that DE carrier system for *Azospirillum* performed better than alginate based carrier system by providing suitable microenvironment for *Azospirillum*. Diffusion-controlled protection has enabled the sustained release for *Azospirillum*, and this in turn enhances microbial persistence and plant growth performance, particularly under salt-stressed conditions.

Declaration of generative AI in scientific writing

During the preparation of this Ms., the authors utilized generative artificial intelligence (AI) technology, specifically ChatGPT and Gemini for language polishing and all the responses generated where checked with human interferences.

Authorship contribution statement

A.B– Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. M.M. J: Writing – original draft, Resources, Investigation, Formal analysis, Data curation. R.N: Writing –Methodology, Data curation. R.T: Methodology, Formal analysis, Data curation. S.N: Methodology, Investigation, Formal analysis. D.I.W: Writing – review & editing, Validation, Supervision, Conceptualization. T.S: Writing – review & editing, Writing.

Declaration of competing interest

Authors declare no competing interests.

Funding Declaration in the manuscript

There is no funding for this work.

Reference:

1. Abdel Latef, A.A.H., et al., "PGPR-mediated salt stress tolerance," *Plant Physiology and Biochemistry*, 147, 45–53 (2020).
2. Albert, I. and Mafart, P., "Application of Weibull model in food microbiology," *International Journal of Food Microbiology*, 100, 197–211 (2005).
3. Avsaroglu, M.D., Buzrul, S., Alpas, H. and Bozoglu, F., "Use of Weibull model for microbial inactivation," *International Journal of Food Microbiology*, 110, 181–186 (2006).
4. Balla, A., Silini, A., Cherif-Silini, H., et al., "Encapsulation technologies for microbial inoculants," *Agronomy*, 12, 179 (2022).
5. Bashan, Y. and de-Bashan, L.E., "How the plant growth-promoting bacterium Azospirillum promotes plant growth—a critical assessment," *Advances in Agronomy*, 108, 77–136 (2010).
6. Bashan, Y. and Holguin, G., "Interactions of Azospirillum with plants," *Canadian Journal of Microbiology*, 41, 844–852 (1995).
7. Bashan, Y., de-Bashan, L.E., Prabhu, S.R. and Hernandez, J.P., "Advances in plant growth-promoting bacterial inoculant technology," *Plant and Soil*, 378, 1–33 (2014).
8. Bashan, Y., Hernandez, J.P., Leyva, L.A. and Bacilio, M., "Alginate microbeads as inoculant carriers for plant growth-promoting bacteria," *Biology and Fertility of Soils*, 35, 359–368 (2002).
9. Cerf, O., "Tailing of survival curves of bacterial spores," *Journal of Applied Bacteriology*, 42, 1–19 (1977).

10. Chang, C.P., Lee, J.H. and Park, H.J., Mechanical stability of polymer-coated alginate systems, *Carbohydrate Polymers*, 50, 331–336 (2002).
11. Cochran, W.G., Mosteller, F. and Tukey, J.W., “Estimation of bacterial populations by the most probable number method,” *Biometrics*, 6, 105–116 (1950).
12. Córdoba, A., et al., “Diffusion properties of alginate matrices,” *Carbohydrate Polymers*, 97, 426–434 (2013).
13. Crank, J., *The Mathematics of Diffusion*, 2nd Ed., Oxford University Press, London, UK (1975).
14. Dias, D.R., “Mass transfer in immobilized microbial systems,” *Biochemical Engineering Journal*, 137, 346–356 (2018).
15. Döbereiner, J., “Forage grasses and grain crops,” in *Methods for Evaluating Biological Nitrogen Fixation*, F.J. Bergersen (Ed.), John Wiley & Sons, New York, pp. 535–555 (1980).
16. Döbereiner, J., Marriel, I.E. and Nery, M., “Ecological distribution of Spirillum lipoferum,” *Canadian Journal of Microbiology*, 22, 1464–1473 (1976).
17. Driessen, T., et al., “Diffusion–reaction modeling in porous systems,” *Chemical Engineering Science*, 227, 115–126 (2020).
18. Flores, M., et al., “Alginate encapsulation and microbial release,” *Journal of Applied Microbiology*, 114, 109–120 (2013).
19. Fogler, H.S., *Elements of Chemical Reaction Engineering*, 5th Ed., Prentice Hall, USA (2016).
20. Gombotz, W.R., and Wee, S.F., “Protein release from alginate matrices,” *Advanced Drug Delivery Reviews*, 31, 267–285 (1998).
21. Gaumann, T., Huber, A. and Vogel, H., “Multilayer polymer coatings for enhanced matrix stability,” *Journal of Applied Polymer Science*, 78, 345–352 (2000).

22. Hardy, R.W.F., Holsten, R.D., Jackson, E.K. and Burns, R.C., “The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation,” *Plant Physiology*, 43, 1185–1207 (1968).

23. He, X., et al., “Modified alginate carriers for slow release,” *Colloids and Surfaces B*, 136, 121–128 (2015).

24. Herrmann, L. and Lesueur, D., “Challenges of PGPR inoculant formulation,” *Applied Microbiology and Biotechnology*, 97, 8859–8873 (2013).

25. Hiemstra, H., Oxygen diffusion and microbial survival in immobilized matrices, *Biotechnology and Bioengineering*, 25, 1851–1865 (1983).

26. Hill, A.V., “The diffusion of oxygen and lactic acid through tissues,” *Proceedings of the Royal Society B*, 104, 39–96 (1928).

27. Hungria, M., Nogueira, M.A. and Araujo, R.S., “Inoculation of cereals with *Azospirillum*: sustainability and yield gains,” *Biology and Fertility of Soils*, 58, 1–17 (2022).

28. Joe, M.M., Chauhan, P.S., Bradeeba, K. and Sa, T., “Visualization of Azospirillum colonization and stress adaptation in rice roots,” *Rhizosphere*, 21, 100456 (2022).

29. Joe, M.M., Devaraj, S., Benson, A. and Sa, T., “Survival and efficacy of alginate-encapsulated *Azospirillum* in rice,” *World Journal of Microbiology and Biotechnology*, 28, 347–356 (2012).

30. Joe, M.M., Devaraj, S., Benson, A., Sa, T., and Dawwam, G.E., “Rice root colonization and plant growth promotion by Azospirillum brasilense,” *Journal of Basic Microbiology*, 53, 1–10 (2013).

31. Khalid, M., Bilal, M., Hassani, D., Iqbal, H.M.N., Wang, H. and Huang, D., “Mitigation of salt stress in plants by Azospirillum brasilense,” *Botanical Studies*, 58, 5 (2017).

32. Kim, K.Y., Jordan, D. and Krishnan, H.B., “Expression of genes from Azospirillum brasilense and colonization behavior in rice roots,” *FEMS Microbiology Letters*, 245, 117–123 (2005).

33. Kloepper, J.W. and Schroth, M.N., “Plant growth-promoting rhizobacteria on radishes,” *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, 2, 879–882 (1978).

34. Kong, H.J. and Mooney, D.J., “Polymer-based biomaterials and alginate hydrogels,” *Nature Reviews Materials*, 2, 1–13 (2003).

35. Liu, Y., et al., “Microbial survival in non-woven matrices,” *Journal of Industrial Microbiology and Biotechnology*, 44, 789–798 (2017).

36. Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Sundaram, S.P., & Sa, T. (2006). Nodulation and plant-growth promotion by methylotrophic bacteria isolated from tropical legumes. *Microbiological Research*, 164, 114–120

37. Malusá, E., Vassilev, N., Freitas, H. and Glick, B.R., “PGPR commercialization challenges,” *Applied Microbiology and Biotechnology*, 96, 1031–1042 (2012).

38. Membré, J.M. and Leguérinel, I., “Modeling microbial behavior under environmental variability,” *Food Microbiology*, 115, 104243 (2024).

39. Mhlongo, M.I., Piater, L.A. and Dubery, I.A., “Metabolomic profiling of PGPR using multivariate analysis,” *Metabolites*, 12, 763 (2022).

40. Panigrahi, S., et al., “Biphasic microbial survival modeling,” *Food Research International*, 140, 110049 (2021).

41. Patra, P. and Klumpp, S., “Population dynamics under stress conditions,” *Physical Biology*, 10, 045001 (2013).

42. Peleg, M. and Cole, M.B., “Reinterpretation of microbial survival curves using Weibull model,” *Critical Reviews in Food Science and Nutrition*, 38, 353–380 (1998).

43. Peleg, M., “Modeling microbial populations with non-linear survival curves,” *Critical Reviews in Food Science and Nutrition*, 39, 353–380 (1999).

44. Perrig, D., Boiero, M.L., Masciarelli, O.A., Penna, C., Ruiz, O.A., Cassán, F.D. and Luna, M.V., “Plant-growth-promoting compounds produced by *Azospirillum brasilense*,” *Applied Microbiology and Biotechnology*, 75, 1143–1150 (2007).

45. Phaiboun, A., Zhang, Y., Park, B. and Kim, M., “Survival kinetics of bacteria under stress,” *Scientific Reports*, 5, 18229 (2015).

46. Piccoli, P., Bottini, R. and Cassán, F., “Gibberellin production by *Azospirillum* spp.,” *Journal of Plant Growth Regulation*, 18, 19–23 (1999).

47. Serrano-Aroca, Á., et al., “Limitations of alginate hydrogels,” *International Journal of Biological Macromolecules*, 105, 113–123 (2017).

48. Tan, Y., et al., “Non-woven materials for microbial immobilization,” *Materials Science and Engineering C*, 108, 110403 (2020).

49. Thuler, D.S., Floh, E.I.S., Handro, W. and Barbosa, H.R., “Plant growth regulators produced by Azospirillum,” *Genetics and Molecular Biology*, 26, 205–210 (2003).

50. van Veen, J.A., van Overbeek, L.S. and van Elsas, J.D., “Fate and activity of microorganisms introduced into soil,” *Microbiology and Molecular Biology Reviews*, 61, 121–135 (1997).

51. Viswambharan, S. and Praveen, K.H., “Chemical Fertiliser (NPK) and Its Importance in Agriculture – A Comprehensive Overview,” *International Journal for Multidisciplinary Research*, 6(3) (2024).

52. Weibull, W., “A statistical distribution function of wide applicability,” *Journal of Applied Mechanics*, 18, 293–297 (1951).

53. Wu, S.C., Cao, Z.H., Li, Z.G., Cheung, K.C. and Wong, M.H., “Effects of bioencapsulation on oxygen diffusion and bacterial survival,” *Bioresource Technology*, 102, 105–112 (2012)

54. Xiu, X.D., Lin, S.X.Q. and Ozkan, N., “Mass transfer and diffusion limitations in immobilized systems,” *Chemical Engineering Science*, 58, 99–107 (2003).

55. Young, C.C., Rekha, P.D., Lai, W.A. and Arun, A.B., “Encapsulation of PGPR in alginate beads,” *Biotechnology and Bioengineering*, 95, 76–83 (2006).

56. Zhang, Y., et al., “Microbial immobilization in polymer matrices,” *Bioresource Technology*, 289, 121–129 (2019).