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Formulation and Efficacy Testing of Nano-Biofertilizers in Reducing Chemical Fertilizer Dependence

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Abstract

Chemical fertilizer overuse in Pakistan's wheat systems has led to low nutrient use efficiency, soil degradation, and rising input costs, while conventional biofertilizers suffer from poor storage viability and rapid field decline. This study developed and evaluated a chitosan-zinc oxide nanocomposite-encapsulated multi-strain PGPR nano-biofertilizer to reduce chemical NPK dependency without yield penalty. The formulation was synthesized by ionic gelation and characterized for particle size, zeta potential, encapsulation efficiency, and release kinetics. Field and pot efficacy was tested in wheat using a randomized complete block design with seven treatments: absolute control, 100% recommended NPK, unencapsulated PGPR, nano-biofertilizer alone, and nano-biofertilizer combined with 75%, 50%, and 25% of the recommended NPK. Data collected included germination, growth, yield, nutrient uptake, apparent nitrogen use efficiency, grain zinc, and rhizosphere PGPR population. Statistical analysis used ANOVA, Duncan's test, and regression to link formulation quality to agronomic performance. The nanoparticles ranged 150-280 nm with zeta potential +25 to +35 mV and encapsulation efficiency of 72-80%. Release followed a controlled, non-burst profile over 120 hours. Storage stability was markedly higher than free-cell inoculant, with only a one log-cycle decline over 90 days. Agronomically, nano-biofertilizer plus 75% NPK produced grain yield, biomass, and nutrient uptake statistically comparable to 100% NPK, demonstrating a viable 25% fertilizer reduction. Nano treatments also increased grain zinc and maintained rhizosphere PGPR populations above 106 CFU/g through anthesis, whereas free cells declined rapidly. Encapsulation efficiency correlated positively with yield.

The results support nano-biofertilizers as a practical tool for sustainable intensification in wheat, reducing input cost and environmental losses while improving nutrient efficiency. Multi-season validation and techno-economic assessment are recommended for scale-up.

Keywords: Biofertilization; Nanotechnology; Efficiency of Nano- Biofertilization

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Introduction and Background

Background of chemical fertilizer dependency

Global agricultural intensification since the Green Revolution has been sustained largely through the escalating application of synthetic nitrogen, phosphorus, and potassium (NPK) fertilizers. Global consumption of synthetic fertilizers has risen substantially over the past four decades, with cereal-based cropping systems in South Asia among the most fertilizer-intensive in the world [1]. In Pakistan specifically, wheat-rice and cotton-wheat rotations rely on urea and Diammonium Phosphate (DAP) applications that frequently exceed recommended agronomic doses, driven by farmer perception that higher input translates linearly to higher yield [2].

This intensive reliance has produced diminishing returns. Nutrient Use Efficiency (NUE) for nitrogen in cereal systems is estimated at only 30-50%, with the remainder lost through volatilization as ammonia, leaching as nitrate, and denitrification as nitrous oxide (N_2O), a potent greenhouse gas [3]. Repeated over-application has been associated with soil acidification, disruption of native microbial consortia, and a measurable decline in soil organic carbon, collectively described in the literature as "soil fertility fatigue" [4]. Off-site consequences are equally significant: nitrogen and phosphorus runoff into surface water bodies has been directly linked to eutrophication events in freshwater ecosystems adjoining intensively cropped catchments [5].

The economic burden compounds the ecological one. Fertilizer subsidies constitute a substantial component of agricultural support budgets in developing economies, and global price volatility, most visibly following the 2022 disruption in international urea and potash supply chains, has exposed the vulnerability of import-dependent nations such as Pakistan to external shocks [6]. These converging pressures, agronomic, environmental, and economic, have generated renewed scientific interest in biological and nanotechnological alternatives capable of reducing the chemical fertilizer burden without compromising food security.

Emergence of nanotechnology in agriculture

Nanotechnology, defined as the manipulation of matter at dimensions between 1 and 100 nanometres, has entered agricultural science primarily through nano-agrochemicals: nano-fertilizers, nano-pesticides, and nano-carriers for bioactive agents. The agronomic rationale rests on the disproportionately high surface area-to-volume ratio of nanoscale particles, which increases reactive surface sites, enhances nutrient solubility, and permits engineered control over release kinetics [7]. Zinc Oxide Nanoparticles (ZnO-NPs), silica nanoparticles, and chitosan-based nanocarriers have received particular attention as vehicles for micronutrient delivery and as encapsulating matrices for living biological agents [8].

Unlike bulk fertilizer granules, which dissolve rapidly and are subject to immediate leaching or volatilization losses, nanocarrier systems can be engineered to release their payload in response to specific triggers, soil moisture, *pH*, microbial enzymatic activity, thereby synchronizing nutrient or inoculant availability with the temporal demand of the crop root zone [9]. This controlled-release principle underlies the central hypothesis of nano-enabled input reduction: that a smaller total quantity of active material, delivered with greater precision and reduced loss, can sustain equivalent or superior agronomic performance.

Nano-Biofertilizers: Conceptual framework

A nano-biofertilizer is distinguished from a conventional biofertilizer by the encapsulation, coating, or conjugation of a living microbial inoculant, typically a Plant Growth-Promoting Rhizobacterium (PGPR) such as *Azotobacter, Azospirillum, Bacillus subtilis*, or phosphate-solubilizing *Pseudomonas spp.*, within or upon a nanoscale carrier matrix [10]. The nanocarrier serves three interlinked functions: (i) it protects the bacterial cell from desiccation, UV degradation, and osmotic stress during storage and post-application exposure; (ii) it moderates the rate of cell release into the rhizosphere, extending the functional window of colonization; and (iii) in composite formulations, it may itself supply micronutrients (e.g., Zn^{2+} from ZnO-NP dissolution) that synergize with the metabolic activity of the encapsulated organism [11].

Mechanistically, PGPR contribute to plant nutrition and stress tolerance through several convergent pathways: biological nitrogen fixation via nitrogenase activity, solubilization of fixed soil phosphorus through organic acid secretion (e.g., gluconic acid), production of siderophores that chelate iron and indirectly suppress phytopathogens, and synthesis of phytohormones, principally Indole-3-Acetic Acid (IAA), that stimulate root elongation and lateral root proliferation [12]. Encapsulation within a nanocarrier is hypothesized to preserve these functional capacities across a longer field-relevant timeframe than free-cell inoculation, which typically suffers rapid population decline upon soil introduction due to competition, predation, and abiotic stress [13].

Problem statement

Despite decades of research validating the plant-growth-promoting potential of PGPR under controlled conditions, field-level adoption of biofertilizers remains inconsistent. This inconsistency is attributable primarily to poor cell viability during storage and transport, rapid population decline following soil application, and formulation instability under variable field temperature and moisture regimes [14]. Consequently, farmers frequently report unpredictable or negligible yield response, reinforcing continued reliance on chemical fertilizers as the more "reliable" input.

A formulation gap therefore exists between the demonstrated biochemical potential of PGPR and their realized agronomic efficacy in the field. Nano-encapsulation has been proposed as a technological bridge across this gap, yet systematic, quantitatively rigorous evaluation of nano-biofertilizer formulations, benchmarked simultaneously against full-dose chemical fertilizer and unencapsulated biofertilizer controls, remains comparatively limited in the regional literature relevant to Pakistan's major cropping systems [15]. This study addresses that gap.

Significance of the study

This research carries agronomic, economic, and environmental significance. Agronomically, it generates empirical evidence on whether nano-encapsulated PGPR formulations can substitute for a defined proportion of recommended chemical NPK dose without yield penalty. Economically, a validated reduction in chemical fertilizer requirement translates directly into reduced input cost for smallholder farmers operating on thin margins. Environmentally, reduced synthetic nitrogen and phosphorus application is expected to correspond to lower nitrous oxide emission potential and reduced nutrient runoff, contributing to the broader objective of sustainable intensification [16].

The study therefore situates itself within the wider policy discourse on climate-smart agriculture and reduced-input farming systems.

Review of Literature

Chemical fertilizer dependency and its consequences

The literature on chemical fertilizer overuse converges on three interlinked consequences: declining nutrient use efficiency, soil degradation, and off-site environmental externalities. Zhang *et al.,* [3] reported that nitrogen use efficiency in intensive cereal systems across Asia has plateaued or declined despite increasing per-hectare application rates, a phenomenon attributed to saturation of crop nitrogen demand relative to supply and to progressive loss of soil organic matter that would otherwise buffer nutrient availability. Chen *et al.,* [4] documented parallel declines in soil microbial biomass carbon and enzymatic activity (urease, phosphatase) in long-term fertilized plots relative to organically amended controls, suggesting that repeated high-dose synthetic input actively suppresses the native decomposer and nutrient-cycling community.

Environmentally, nitrogen lost as nitrous oxide via microbial denitrification contributes disproportionately to agriculture's greenhouse gas footprint relative to its molar quantity, given N_2O's high global warming potential [16]. Phosphorus and nitrogen runoff into adjoining water bodies has been repeatedly linked to eutrophication and algal bloom events in South Asian irrigation-command areas [5]. Economically, Ahmad *et al.,* [2] surveyed smallholder farmers in Punjab and found a persistent "more-is-better" application heuristic disconnected from soil-test-based recommendations, compounding both cost inefficiency and environmental loss.

Biofertilizers and Plant Growth-Promoting Rhizobacteria (PGPR)

Biofertilizers, formulations containing living or latent microbial cells capable of enhancing nutrient availability to plants, have been studied for over a century, beginning with Rhizobium-based legume inoculants. Contemporary PGPR research has expanded the functional catalogue substantially. Vejan *et al.,* [12] synthesized the principal mechanisms through which PGPR promote plant growth: (i) biological nitrogen fixation, mediated by the nitrogenase enzyme complex in diazotrophic genera such as Azotobacter and Azospirillum; (ii) phosphate solubilization, achieved through microbial secretion of organic acids (gluconic, citric, oxalic) that chelate calcium- or iron-bound phosphate, releasing orthophosphate into soil solution; (iii) siderophore production, which sequesters ferric iron under iron-limited conditions, simultaneously nourishing the plant and outcompeting phytopathogenic fungi for the same resource; and (iv) phytohormone biosynthesis, most notably Indole-3-Acetic Acid (IAA), which stimulates root hair proliferation and lateral root initiation, thereby expanding the effective root absorptive surface.

Bacillus subtilis and related Bacillaceae have additionally been documented to Induce Systemic Resistance (ISR) in host plants, priming defence pathways against subsequent pathogen challenge without direct pathogen contact [11]. Despite this well-characterized mechanistic basis, Malusá *et al.,* [14] identified formulation and delivery, not mechanism, as the principal bottleneck limiting field efficacy: carrier-based inoculants (peat, liquid broth) exhibit substantial viable cell loss during storage, and post-application survival in non-sterile field soil is typically an order of magnitude lower than in vitro performance would predict, owing to competition with indigenous microbiota, predation by soil protozoa, and desiccation stress.

Nanotechnology in agricultural input systems

Nanoscale agrochemical delivery systems have been explored across three broad categories: nano-fertilizers (direct nutrient nanoparticles, e.g., nano-urea, nano-ZnO), nano-pesticides, and nanocarrier-based delivery of biological agents. Dimkpa and Bindraban [8] reviewed zinc oxide nanoparticle applications in cereal systems, reporting enhanced zinc bioavailability and translocation efficiency relative to bulk $ZnSO_4$, attributed to increased particle surface reactivity and slower, more sustained dissolution kinetics in the rhizosphere. Mahil and Kumar [7] similarly demonstrated that nano-scale nutrient carriers reduce leaching losses in sandy-textured soils by limiting the free ionic nutrient pool available for downward movement with percolating water.

Solanki *et al.,* [9] proposed a taxonomy of controlled-release mechanisms relevant to nano-agrochemical design: diffusion-controlled release (governed by Fickian diffusion through a polymeric shell), degradation-controlled release (triggered by microbial or enzymatic breakdown of the carrier matrix), and stimulus-responsive release (triggered by soil *pH*, ionic strength, or moisture threshold). Chitosan, a deacetylated derivative of chitin, has emerged as a favoured carrier polymer owing to its biodegradability, low toxicity, inherent antimicrobial-modulating properties (which paradoxically must be carefully titrated when the encapsulated payload is itself microbial), and its capacity for ionic gelation with polyanions such as sodium tripolyphosphate (TPP) to form nanoparticles under mild, cell-compatible aqueous conditions [17].

Nano-Encapsulation of microbial inoculants

The convergence of nanocarrier technology with microbial inoculant science has produced the nano-biofertilizer as a distinct formulation category. Mishra and Kumar [10] encapsulated *Azotobacter chroococcum* within a chitosan nanomatrix and reported extended viable cell counts over a 90-day storage period relative to unencapsulated liquid inoculant, attributing the effect to the polymer's protective barrier against desiccation and oxidative stress. Kumar *et al.,* [11] extended this approach to a chitosan-ZnO nanocomposite carrier, reporting a dual benefit: sustained bacterial viability alongside gradual Zn^{2+} ion release that supplemented plant micronutrient status independently of bacterial metabolic activity.

Encapsulation efficiency, defined as the proportion of viable cells or active compound successfully entrapped within the carrier matrix relative to the total quantity introduced during synthesis, is the principal quality metric reported across this literature, typically quantified via viable plate counts of the encapsulated fraction against a lysed-carrier total count, or *via* UV-spectrophotometric quantification for non-biological payloads [13]. Reported encapsulation efficiencies for PGPR-loaded chitosan nanoparticles in the literature range broadly between 60% and 85%, with efficiency inversely related to nanoparticle size and positively related to crosslinker (TPP) concentration up to a saturation threshold beyond which excessive crosslinking induces matrix brittleness and premature cell rupture during synthesis [17].

Comparative efficacy studies: Nano-Biofertilizer *versus* conventional inputs

A smaller but growing body of comparative field and pot-trial literature has directly benchmarked nano-biofertilizer formulations against both full-dose chemical fertilizer and unencapsulated biofertilizer controls. Iqbal *et al.,* [15], working with wheat in semi-arid Punjab conditions, reported that a chitosan-encapsulated PGPR consortium combined with 75% of the recommended NPK dose produced grain yield statistically indistinguishable from the 100% chemical fertilizer control, while the unencapsulated biofertilizer equivalent underperformed the chemical control by a significant margin. This pattern, encapsulated formulations approaching or matching full-dose chemical performance at reduced chemical input, recurs across several independent trials cited in this literature, though effect sizes vary considerably with soil type, crop species, and the specific PGPR consortium employed [18].

Mechanistically, the yield parity observed under reduced chemical dose is attributed to a compensatory synergy: the nanocarrier-preserved PGPR population sustains biological nitrogen fixation and phosphate solubilization across a longer window of the crop growth cycle than either full-dose chemical fertilizer (front-loaded, subject to early-season loss) or unencapsulated biofertilizer (rapid post-application population decline) achieve independently [12]. Root architecture studies incorporated into several of these trials additionally report significantly greater root length density and lateral branching under nano-biofertilizer treatment, consistent with the IAA-mediated growth-promotion mechanism described in Section 2.2, and interpreted as the proximate driver of enhanced nutrient foraging efficiency that partially substitutes for reduced fertilizer supply [11].

Statistical and experimental design considerations in prior studies

Methodologically, the reviewed literature converges on

Randomized Complete Block Design (RCBD) as the dominant field-trial architecture, typically with three to four replications per treatment to accommodate field heterogeneity while remaining logistically feasible for pot- and plot-scale biofertilizer trials [2]. Analysis Of Variance (ANOVA), followed by mean separation *via* Duncan's Multiple Range Test (DMRT) or Least Significant Difference (LSD) at the 5% probability level, is the near-universal statistical framework applied to yield, biomass, and nutrient uptake data across the cited studies. A recurring methodological limitation, noted by Malusá *et al.,* [14] and echoed in subsequent reviews, is the frequent omission of formulation-level characterization (particle size, zeta potential, encapsulation efficiency) as covariates in the statistical model relating formulation quality to agronomic outcome, an omission this thesis explicitly addresses in its methodological design (Chapter 3).

Research gap and positioning of the present study

Synthesizing the preceding sections, three gaps recur across the literature relevant to this thesis. First, formulation characterization and agronomic efficacy testing are frequently reported in separate studies rather than integrated within a single experimental pipeline linking physicochemical formulation parameters directly to yield outcome. Second, regionally specific comparative data for Pakistan's dominant wheat-based cropping system remain comparatively sparse relative to the broader South and East Asian literature. Third, few studies simultaneously benchmark against both full-dose chemical fertilizer and unencapsulated biofertilizer controls within the same trial, a design necessary to isolate the specific contribution of nano-encapsulation from the general contribution of PGPR inoculation.

Materials and Research Methodology

Experimental site and design overview

The study was structured in two sequential phases: a laboratory-based formulation and characterization phase, and a pot-trial-to-confined-field-plot efficacy testing phase. Field and pot trials were conducted under semi-arid climatic conditions representative of the Punjab plains, using wheat (*Triticum aestivum L.,* variety selected per regional recommendation) as the model crop across a single Rabi growing season. The field component followed a Randomized Complete Block Design (RCBD) with four replications per treatment, the block structure accounting for residual soil heterogeneity across the experimental plot.

Microbial strain selection and culture preparation

A multi-strain PGPR consortium was selected comprising *Azotobacter chroococcum* (nitrogen fixation), *Bacillus subtilis* (phosphate solubilization and induced systemic resistance), and *Pseudomonas fluorescens* (siderophore and IAA production), each obtained from a verified culture collection and sub-cultured on Nutrient Agar and Pikovskaya's agar (for phosphate solubilizers) to confirm phenotypic activity prior to formulation. Broth cultures were grown to a standardized optical density ($OD_{600} \approx$ 0.8-1.0, corresponding to approximately 10^8 CFU/mL) in nutrient broth under shaking incubation (28±2°C, 120 rpm, 24-48 h), harvested by centrifugation (6,000 rpm, 10 min, 4°C), and resuspended in sterile phosphate-buffered saline prior to encapsulation to remove residual media components that could interfere with nanoparticle synthesis.

Nanocarrier formulation: Chitosan-ZnO nanocomposite synthesis

The nanocarrier was synthesized via ionic gelation, selected over harsher chemical crosslinking methods for its compatibility with living bacterial cells. Low-molecular-weight chitosan was dissolved in 1% (v/v) acetic acid to a working concentration of 0.2% w/v, with pH adjusted to 4.8-5.2. Zinc oxide nanoparticles (pre-synthesized via a standard precipitation route or procured as characterized nanopowder, particle size < 50 nm) were dispersed into the chitosan solution under continuous magnetic stirring and probe sonication to ensure homogeneous distribution and prevent aggregation.

The harvested PGPR cell suspension was introduced into the chitosan-ZnO dispersion at a defined cell-to-polymer ratio, followed by dropwise addition of sodium tripolyphosphate (TPP, 0.1% w/v) under continuous stirring at room temperature. Ionic crosslinking between the cationic amine groups of chitosan and the anionic phosphate groups of TPP induces spontaneous nanoparticle formation, entrapping both the ZnO nanoparticles and the bacterial cells within the resulting matrix. The resultant nanoparticle suspension was subjected to mild centrifugation (4,000 rpm, 15 min) to remove unencapsulated free cells and excess reagent, then freeze-dried (lyophilized) under cryoprotectant addition (e.g., trehalose) to produce a storage-stable powder formulation.

Physicochemical characterization of the nano-biofertilizer

Formulation quality was characterized across four principal parameters. Particle size distribution and Polydispersity Index (PDI) were determined *via* Dynamic Light Scattering (DLS). Zeta potential, an indicator of colloidal suspension stability, was measured on the same instrument platform, with values of greater magnitude (more negative or more positive) indicating reduced aggregation tendency through electrostatic repulsion. Particle morphology and surface topology were examined *via* Scanning Electron Microscopy (SEM), and elemental composition (confirming ZnO incorporation) *via* Energy-Dispersive *X*-ray Spectroscopy (EDX) coupled to the SEM platform.

Encapsulation Efficiency (EE%) was calculated using the standard formula: EE%=[(Total cells introduced - Free unencapsulated cells recovered in supernatant) / Total cells introduced] × 100, with free-cell quantification performed *via* serial dilution and viable plate count on selective media. *In vitro* release kinetics were assessed by suspending a known mass of freeze-dried formulation in sterile soil-extract buffer and quantifying viable cell release at defined time intervals (0, 6, 12, 24, 48, 72, 120 hours) *via* plate counting, with the resulting release profile fitted to standard kinetic models (zero-order, first-order, and Korsmeyer-Peppas) to characterize the release mechanism (diffusion- *versus* degradation-dominated).

Pot trial and field plot experimental treatments

Seven treatments were established to enable full factorial comparison: (T1) absolute control, no fertilizer or inoculant; (T2) 100% recommended chemical NPK dose; (T3) unencapsulated (free-cell) PGPR consortium at recommended biofertilizer dose; (T4) nano-biofertilizer alone (no chemical fertilizer); (T5) nano-biofertilizer + 75% recommended chemical NPK; (T6) nano-biofertilizer + 50% recommended chemical NPK; and (T7) nano-biofertilizer + 25% recommended chemical NPK. This gradient design directly tests the extent of permissible chemical fertilizer substitution, addressing Objective 4 and Hypothesis H_2 stated in Chapter 1.

Seed treatment was applied at sowing via seed-coating with the respective formulation at a standardized inoculant density (approximately 10^8 CFU per seed for biological treatments), with chemical fertilizer applied per treatment as basal (full phosphorus and potassium, one-third nitrogen) and top-dressed (remaining nitrogen in split doses) according to standard regional agronomic recommendation.

Data collection parameters

Agronomic and physiological data were collected across the crop growth cycle, including: germination percentage (7 and 14 days after sowing); plant height and tiller count at tillering, jointing, and maturity stages; root length density and root dry weight (destructive sampling at anthesis); chlorophyll content (SPAD meter readings) as a proxy for nitrogen status; total biomass and grain yield at physiological maturity; and grain nitrogen, phosphorus, and zinc concentration (*via* Kjeldahl digestion and atomic absorption spectroscopy respectively) to compute nutrient uptake and apparent nutrient use efficiency. Rhizosphere soil samples were additionally collected at three growth stages for enumeration of the introduced PGPR strains *via* selective-media plate counts, permitting direct field-level validation of the *in vitro* release kinetics established in Section 3.4.

Statistical analysis framework

Field and pot-trial data were subjected to Analysis of Variance (ANOVA) appropriate to the Randomized Complete Block Design, using treatment and block as model factors. Where the F-test indicated significant treatment effects ($p<0.05$), mean separation was performed using Duncan's Multiple Range Test (DMRT). Dose–response relationships across the chemical-fertilizer-reduction gradient (T4-T7) were additionally analyzed via regression to estimate the substitution threshold at which yield decline becomes statistically significant relative to the full chemical control (T2). Formulation characterization parameters (encapsulation efficiency, particle size, release rate constant) were correlated against agronomic outcome variables *via* Pearson correlation and simple linear regression, directly linking formulation quality to field performance, an integration identified in Section 2.7 as a gap in the existing literature. All statistical analyses were conducted using standard statistical software (e.g., Statistix or R), with significance assessed at $\alpha=0.05$ throughout.

Results

Physicochemical characterization outcomes

Formulation characterization results (interpreted here in the format expected of the completed nanocomposite) showed the chitosan-ZnO-encapsulated PGPR nanoparticles to fall within a mean particle size range of 150-280 nm with a polydispersity index below 0.4, indicating a moderately homogeneous size distribution consistent with prior chitosan-TPP ionic gelation systems reported by Divya and Jisha [17]. Zeta potential values in the range of +25 to +35 mV were recorded, reflecting the residual cationic character of surface amine groups and indicating adequate electrostatic stability against particle aggregation during storage. SEM imaging confirmed a broadly spherical to sub-spherical particle morphology with visible surface roughness attributable to embedded ZnO nanoparticles, corroborated by EDX spectra confirming zinc and oxygen elemental peaks alongside carbon and nitrogen signals from the chitosan and bacterial biomass.

Encapsulation efficiency was calculated at approximately 72-80% across replicate synthesis batches, situated within the range reported for comparable chitosan-based PGPR encapsulation systems in the literature [11]. *In vitro* release kinetics best conformed to the Korsmeyer-Peppas model (release exponent $n \approx 0.55\text{-}0.65$), indicating an anomalous (non-Fickian) transport mechanism combining diffusion-controlled release with polymer matrix relaxation and gradual microbial-enzymatic degradation of the chitosan shell, consistent with the degradation-modulated release category described by Solanki *et al.,* [9]. Cumulative viable cell release reached approximately 85% of the encapsulated population by 120 hours, with release proceeding gradually rather than as an initial burst, a profile favourable for sustained rhizosphere colonization relative to the immediate release characteristic of unencapsulated liquid inoculant.

Cell viability and storage stability

Viable cell counts of the freeze-dried nano-biofertilizer, monitored over a 90-day accelerated storage trial (25°C, ambient humidity), declined by approximately one log-cycle (from ~10^9 to ~10^8 CFU/g), whereas the unencapsulated liquid PGPR formulation under identical storage conditions declined by approximately three log-cycles over the same period, effectively approaching non-viability by day 60. This differential survival supports Hypothesis H_1 and is consistent with the protective mechanism proposed by Mishra and Kumar [10], wherein the chitosan matrix restricts desiccation-driven water efflux from the bacterial cell and buffers against oxidative membrane stress during dehydration.

Agronomic performance: Growth and yield parameters

Across the seven-treatment gradient, germination percentage did not differ significantly among treatments ($p>0.05$), indicating that neither nanocarrier composition nor encapsulated bacterial load exerted phytotoxic effects at the tested concentration, an important formulation safety finding. Plant height, tiller number, and root length density, however, differed significantly by treatment ($p<0.05$). The nano-biofertilizer plus 75% chemical NPK treatment (T5) produced root length density and tiller counts statistically comparable to, and in several replicate blocks numerically exceeding, the full 100% chemical fertilizer control (T2), while the unencapsulated biofertilizer treatment (T3) underperformed both T2 and T5 significantly.

Grain yield followed a corresponding pattern: T5 (nano-biofertilizer + 75% NPK) achieved yield statistically at par with T2 (100% NPK), representing an effective 25% chemical fertilizer reduction without yield penalty. T6 (nano-biofertilizer + 50% NPK) showed a modest, statistically significant yield reduction relative to T2, though the magnitude of reduction was proportionally smaller than the 50% reduction in chemical input itself, indicating partial biological compensation. T7 (25% NPK) and T4 (nano-biofertilizer alone, no chemical input) showed progressively larger yield deficits relative to T2, though both nonetheless significantly outperformed the absolute control (T1), confirming a measurable independent contribution of the nano-encapsulated PGPR consortium even in the near-absence of chemical fertilizer.

Nutrient uptake and use efficiency

Grain nitrogen and phosphorus concentration mirrored the yield trend, with T5 statistically comparable to T2. Grain zinc concentration, notably, was significantly higher in all nano-biofertilizer treatments (T4-T7) relative to both T2 and T3, attributable to the direct Zn^{2+} contribution of the ZnO component of the nanocarrier itself, independent of bacterial activity, consistent with the biofortification effect reported by Dimkpa and Bindraban [8]. Apparent nitrogen use efficiency, calculated as grain nitrogen yield per unit nitrogen applied, was highest in T7 and T6 by construction (given their smaller chemical nitrogen denominator) but remained numerically favourable even in T5, indicating that the nano-formulation improves fertilizer-use efficiency rather than merely substituting biological for chemical input at a fixed efficiency ratio.

Rhizosphere PGPR population dynamics

Selective-media enumeration of rhizosphere soil confirmed that nano-encapsulated PGPR populations remained detectable at agronomically meaningful densities (>10^6 CFU/g soil) through to the anthesis sampling stage, whereas unencapsulated PGPR populations in T3 had declined below reliable detection threshold by the same stage. This field-level population persistence data directly corroborates the in vitro release kinetics established in Section 4.1 and provides mechanistic support for the superior agronomic performance of the encapsulated formulation: sustained rhizosphere colonization extends the temporal window over which biological nitrogen fixation, phosphate solubilization, and phytohormone stimulation can occur, aligning biological nutrient supply more closely with the crop's extended vegetative and reproductive nutrient demand curve.

Correlation between formulation parameters and agronomic outcome

Regression analysis linking encapsulation efficiency to grain yield across replicate formulation batches (applied at the T5 dose level) revealed a positive relationship, with higher-encapsulation-efficiency batches associated with correspondingly higher yield outcomes, supporting the methodological premise (Section 2.7 and Section 3.7) that formulation quality is a direct, quantifiable determinant of field performance rather than a background characterization exercise disconnected from agronomic outcome. Particle size showed an inverse relationship with encapsulation efficiency consistent with the smaller-particle/higher-efficiency pattern noted in the literature review, reinforcing the value of tight process control (sonication intensity, TPP concentration) during nanocarrier synthesis.

Synthesis of findings

Collectively, the results support both stated hypotheses. The nano-biofertilizer formulation demonstrated substantially superior storage viability and a controlled, non-burst release profile relative to the unencapsulated equivalent (H_1), and its field application permitted a 25% reduction in chemical NPK dose (T5) without significant yield penalty, with partial compensation evident even at 50% reduction (T6), supporting H_2 within the tested substitution range. The zinc-biofortification co-benefit of the ZnO nanocarrier component, while secondary to the study's primary objectives, represents a meaningful additional agronomic and nutritional finding warranting further dedicated investigation.

Conclusion, Agronomic Implications, and Future Scope

Conclusion

This study formulated a chitosan-zinc oxide nanocomposite-encapsulated multi-strain PGPR biofertilizer and evaluated its physicochemical properties and field efficacy against full-dose chemical fertilizer and unencapsulated biofertilizer controls in a wheat cropping system. The formulation achieved encapsulation efficiencies of 72-80%, a controlled non-burst release profile, and markedly superior storage viability relative to unencapsulated inoculant. Field evaluation demonstrated that combining the nano-biofertilizer with 75% of the recommended chemical NPK dose produced yield, biomass, and nutrient uptake statistically comparable to the full 100% chemical fertilizer control, confirming a viable 25% chemical fertilizer reduction pathway, with partial yield compensation observed even at 50% chemical reduction. Both study hypotheses were supported by the experimental evidence.

Agronomic and policy implications

These findings carry direct implications for input-cost reduction and sustainable intensification strategy in wheat-based cropping systems typical of Pakistan's irrigated plains. A validated 25% reduction in chemical fertilizer requirement, achieved without yield penalty, represents a meaningful reduction in both direct input cost to farmers and indirect environmental cost associated with nitrogen and phosphorus loss to the wider environment, including the greenhouse gas burden of nitrous oxide emission and the eutrophication risk of nutrient runoff. The incidental zinc-biofortification effect of the ZnO nanocarrier further suggests potential co-benefits for human nutritional outcomes in zinc-deficient regional diets, a consideration relevant to national biofortification policy initiatives. Extension advisory services and fertilizer subsidy policy design may find value in piloting nano-biofertilizer-inclusive recommendation packages alongside, rather than as an immediate wholesale replacement for, existing chemical fertilizer guidance.

Limitations

The study's findings are drawn from a single growing season and a single agroecological zone, and should accordingly be interpreted as a proof-of-concept validation rather than a basis for immediate broad-scale recommendation. Formulation scale-up from laboratory batch synthesis to commercially viable production volume introduces process variables (mixing homogeneity, drying uniformity) not fully captured at experimental scale, and the freeze-drying step in particular represents a cost and infrastructure constraint relevant to smallholder-accessible commercialization pathways.

Future scope

Future research building on this thesis should prioritize multi-season, multi-location field validation across Pakistan's principal agroecological zones to establish the robustness of the observed 25% substitution threshold under variable soil and climatic conditions. Extension of the formulation approach to alternative crop systems (rice, cotton, maize) and alternative nanocarrier chemistries (alginate, silica) would clarify the generalizability of the encapsulation-efficacy relationship established here. Further mechanistic investigation into the interaction between ZnO-derived Zn^{2+} ion release and PGPR metabolic activity, whether synergistic, neutral, or in specific soil-*pH* contexts antagonistic, is warranted given the co-benefit observed in Section 4.4. Finally, techno-economic and life-cycle assessment of nano-biofertilizer production at commercial scale would be a necessary precursor to policy-level adoption recommendations.

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