ABSTRACT

Zn is an essential element for all life forms. In the current scenario, the entire world is at high risk of Zn deficiency, for which declining pristine Zn deposits in the soil are one of the major causes. Also, studies mentioned that the major Zn fraction in the soil is present in unavailable forms that can consequently lead to decreasing Zn levels in plants. A reduction in soil-Zn levels has detrimental effects on plant growth, microbial community structure, and soil fertility. Therefore, it is imperative to understand this issue in the "soil-plant-animal-human continuum" context as, like other essential nutrients, most of the Zn requirement derives from dietary food intake. The consumption of Zn-deficient food crops can also cause Zn deficiency in humans, as a majority of the population relies on staple crops to meet the Zn requirements, like other nutrients. Biofortification of Zn in food crops is one of the preferred approaches to increase dietary Zn intakes, for which the optimal levels of Zn in agricultural soils are desired. The gap between Zn demands for humans and its supply through the food chain is big enough to necessitate revolutionizing the agriculture industry with innovative solutions.

The present research aims to develop a nano-ZnO-based biofertilizer formulation through a green chemistry route for Zn-biofortification and improve overall growth and development in crop plants. The green synthesis of ZnO NPs can offer an eco-friendly approach as it minimizes toxic solvents. Recent years have witnessed significant research exploring low-cost biotemplates, such as agricultural residues, industrial wastes, weed biomass, etc., for synthesizing metal-based NPs (like ZnO NPs). The current work entails biogas slurry (BGS) in ZnO NPs synthesis (B-ZnO NPs), coupled with utilization as a low-cost substrate for mass multiplication of beneficial microbes and further development of nano-ZnO-based biofertilizer formulation.

The study began with the green synthesis of ZnO NPs at different pH (8 to 13) utilizing an aqueous extract of BGS (aq. BGS). In FTIR analysis, major peak assignments indicate the presence of phenols and other bioactive compounds bearing functional groups like alcohols, carboxylic acids, aromatics, etc., in an aq. BGS. The quantitative analysis of BGS extract revealed significant levels of phenols and flavonoids. The GC-MS chromatogram of BGS extract indicated 57 peaks, and the

identified compounds belong to fatty acids, esters, alcohols, aldehydes, terpenoids, ketones, and sterols. Overall, the FTIR and GC-MS results corroborated the existence of bioactive compounds in BGS extract that could act as a source of capping and stabilizing agents in ZnO NP synthesis. Further, the FTIR investigations indicated the presence of organic molecules on the surface of the B-ZnO NPs and suggested the capping potential of bioactive compounds present in aqueous BGS extract.

UV-Vis spectra displayed λ_{max} at 360-380 nm, possibly due to the strong SPR effect of B-ZnO NPs. XRD crystallite size of B-ZnO NPs showed a decreasing trend with an increase in the reaction medium's pH (up to 11). The PDI for B-ZnO NP-11 was lower than B-ZnO NP-8, indicating less agglomeration of NPs at higher pH. ZnO NPs obtained at greater pH displayed higher band gap values than those obtained at lower pH, which could be due to the decrease in NPs size with increased pH. The peak positions in XRD diffractograms for synthesized ZnO NPs were in close agreement with the JCPDS standard (JCPDS file no. 01-079-0206). The strong and sharp peaks were attributed to the high degree of crystallinity, and recorded "Bragg reflections" further confirmed the hexagonal wurtzite symmetry of ZnO NPs. FE-SEM analysis showed mixed morphologies of ZnO NPs, predominantly spheroid, oval, and hexagonal. TEM studies also favored higher pH for synthesizing small-sized ZnO NPs. At pH=8 and 11, B-ZnO NPs were comparatively smaller than C-ZnO NPs. These observations supported the potential role of aq. BGS in synthesizing stabilized ZnO NPs. Surface roughness was studied through AFM, and the average height distribution followed the trend B-ZnO NP-8 > CMC-ZnO NP-11 > C-ZnO NP-11 > B-ZnO NP-11. EDX mapping exhibited strong signals for elemental Zinc and Oxygen with wt% of Zn varied from ~73 to 80% in synthesized ZnO NPs. B-ZnO NPs synthesized at pH=11 were screened for further experimental studies based on synthesis conditions, structural attributes, and recovery.

Furthermore, the study implicates tripartite interaction between synthesized ZnO NPs, beneficial microbes, and plants to develop nano-ZnO-based biofertilizer formulation. Firstly, the BGS and Neem DOC combination (60:40, w/w) was optimized for cultivation and mass multiplication of *T. harzianum* (MTCC 801) (TH), *A. vinelandii* (MTCC 124) (AV), and *P. fluorescens* (MTCC 9768) (PF). The combination (v/v) of BGS (60%) and Neem DOC (40%) aqueous extracts promoted seedling growth in *R. sativus*. Next, interaction studies of B-ZnO NP-11 with TH, AV, and PF were

performed, and no inhibitory activity was recorded against any tested microbial culture up to 50 ppm concentrations. The results were comparable with other tested ZnO NPs (B-ZnO NP-8, C-ZnO NP-8, and C-ZnONP-11) up to 50 ppm treatments. The bacterial kinetics studies demonstrated the growth-promoting effects of B-ZnO NPs at lower concentrations.

The bipartite interaction of ZnO NPs and *R. sativus* seeds showed increased seedling growth at lower doses. The B-ZnO NPs up to 50 ppm treatment significantly improved germination indices. Overall, observing seedling length, phytobiomass content, and vigour indices, the growth-promoting effects of B-ZnO NP-11 were more pronounced than those of B-ZnO NP-8. The RL/PL and tolerance index (TI) indicated inhibitory effects of nano/bulk ZnO on seedling growth at higher doses, more evident at 500-1000 ppm. TI greater than one was obtained for germination indices at 50 ppm ZnO NPs and 200 ppm bulk ZnO treatments. The results suggested that the *R. sativus* seedling significantly tolerates higher doses of bulk-ZnO than nano-ZnO. However, the growth-promoting effects on *R. sativus* seedlings (SL, FWB, VI-1, and VI-2) were observed at lower doses (up to 50 ppm) and comparatively higher in the case of nano-ZnO (more prominent in B-ZnO NP-11) than its bulk form. Seed germination studies are imperative to establish the effective concentration of ZnO NPs that can vary with the plant type, NPs' treatment mode, and their structural properties (like size, morphology, etc.). Such investigations could further limit the NP's exposure and reduce the nanotoxicity risks.

Finally, the microbial cultures cultivated on an optimized combination of BGS and Neem DOCs (organic/bio-fertilizer mix, abbreviated as OBM) and different doses of ZnO NPs were combined to develop the nano-biofertilizer formulations and tested on wheat and spinach. The pot and micro-plot studies on wheat showed the highest biomass production, grain yield, and Zn contents in grains and straw in the case of T_3 formulation (B-ZnO NP-11 @50 mg/kg of soil + OBM). The mass balance studies on pre- and post-harvest soil showed higher Zn uptakes in nanoform. An analysis of the T_3 wheat harvest further confirmed the higher bioavailability of nano-Zn. Comparing the T_3 harvest with the positive control (T_7) demonstrated synergistic effects of B-ZnO NPs and microbial consortia on wheat growth. The pot experiments on spinach showed growth-prompting effects of B-ZnO NPs at lower doses (10-25 mg/kg of soil). The treatment formulation comprised of B-ZnO NP-11 (25 mg/kg of soil)

exhibited significant improvement in the FWB of spinach, and higher MSI values indicated the potential role of B-ZnO NPs in drought resistance. However, further studies are required to get mechanistic insights into the role of ZnO NPs in alleviating abiotic stress. Overall, the present study's findings exhibited encouraging results, and the tripartite interaction of beneficial microbes/B-ZnO NPs/plants has been effectively materialized into nano-ZnO-based biofertilizer formulations to ameliorate soil health and plant productivity via sustainable routes.