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Research article

Zinc-functionalized thymol nanoemulsion for promoting soybean yield

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ABSTRACT

Herein, we report zinc-functionalized thymol nanoemulsion (Zn-TNE) by sonication method and its characterization by DLS, HR-TEM, FEG-SEM-EDS, Cryo-FESEM, FTIR and AAS studies. Zn-TNE treated seeds bestowed better seedling vigor index and higher activities of seed stored food mobilizing enzymes (α-amylase and protease). Foliar application of Zn-TNE (0.01–0.06%, v/v) enhanced defense-antioxidant enzymes activities, balanced reactive oxygen species, induced higher content of chlorophyll-a, b and higher lignin deposition in soybean plants. In the field, Zn-TNE application (0.02–0.06%, v/v) significantly controlled bacterial pustule disease (PEDC value 28–79%) and increased grain yield up to 16.6% as compared with bulk thymol application and up to 50% from control. Disease control and higher yield in soybean could be explained by diverse bioactivities of Zn-TNE in maintaining cellular homeostasis of soybean plants. Study shows that Zn-TNE can further be maneuvered for slow delivery of other micronutrients for higher crop yield.

1. Introduction

The demand of agrochemicals has exactingly increased in recent years and is likely to be an impetus in the future to meet the required food production. Besides, hazardous environmental impacts of synthetic agrochemicals, agro-industries immeasurably emit carbon to the environment and lead to global warming. To address the growing concerns of agrochemical uses, efforts are needed towards sustainable and eco-friendly alternatives. In this line, plants are the potential resources of naturally derived biocompatible and biodegradable compounds which are safer for the ecosystem. Thymol (2-isopropyl-5-methylphenol), a phenolic component known for decades, found in *Thymus vulgaris* oil has excellent antimicrobial activity (Guarda et al., 2011). It contains a phenolic hydroxyl group and penetrates into microbial cell membrane leading to leakage of ions, cell contents and ultimately cell death (Chang et al., 2012). Conversely, thymol in its native form is ineffective due to its immiscibility in water (Pan et al., 2014). Therefore, in our previous study, we had prepared stable thymol nanoemulsion in which thymol nano-droplets were enclosed in plant based surfactant known as saponin. The developed nanoemulsion was thoroughly studied for its various physico-chemical properties to ensure its long term stability and consistent bioactivities (Kumari et al., 2018). Laboratory experiments further revealed that nano scale thymol exhibited significantly higher antimicrobial activity as compared with bulk thymol without imposing a toxic effect on plant growth. Concomitantly, we hypothesized that thymol nanoemulsion could be a promising carrier of micronutrient delivery in plants and can be introduced to field application to explore its disease control and plant growth promotry activities. Among the micronutrients, zinc deficiency is the most common problem rendering the plants susceptible to disease, causing repressed growth and yield (Duhan et al., 2017).

Zinc influences various biochemical and physiological functions in plants for optimum growth and is considered an important component of defense against diseases (Choudhary et al., 2017). Its defense activity coincides with balancing reactive oxygen species (ROS) by improving antioxidant enzyme activities during growth (Choudhary et al., 2019). Role of zinc in crop yield is imperative as it plays a crucial role in remobilization of photo-assimilates during reproductive and grain filling stages of the plant (Yu et al., 2015). Lower bioavailability of zinc to plants is due to its deficiency in soil and/or inability of plants to assimilate it from alkaline soil. In the current scenario, there is a need to develop a dual system for crop plants which could act as disease protective as well as yield promoting agent. With this notion and to expedite thymol from laboratory to field application, we herein report

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zinc-functionalized thymol nanoemulsion (Zn-TNE) and its field application to control disease and ultimately improve yield. In the present study, Zn-TNE was used to decipher the physiological and biochemical responses of plants in pot and field experiments.

2. Materials and methods

2.1. Plant material and chemicals

Thymol (Mol. Wt. 50,000) and *Quillaja* saponin (Mol. Wt. 56,000) were procured from Sigma-Aldrich, St. Louis, MO, USA. Dimethyl sulfoxide (DMSO), $ZnSO_4$ (Mol. Wt. 234.4), King's medium B and chemicals for enzyme assays were purchased from HiMedia, India. All chemicals and reagents were used as received. Deionized water was obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA, USA). Seeds of moderately susceptible soybean variety "JS-335" were purchased from certified seed supplier. Culture of *Xanthomonas axonopodis* pv. *glycines* was obtained from the Department of Plant Pathology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India.

2.2. Preparation of Zn-TNE

Nanoemulsion was prepared by ultrasonication method as described earlier (Saharan, 2010; Kumari et al., 2018). Thymol, *Quillaja* saponin and $ZnSO_4$ were used in the ratio of 30:5:2 (w/v) for nanoemulsion preparation. In brief, thymol and saponin were mixed in deionized water through probe (½ inch probe with 12.7 mm tip) sonication (Q500 sonicator, Qsonica, USA) for 50 min at 500 Watts, 20 kHz frequency, 5 s pulse on/off and 60% amplitude at room temperature (25 °C). After getting a semi-transparent suspension in midway of ultrasonication, $ZnSO_4$ was added for functionalization of nanoemulsion. The prepared nanoemulsion was stored at room temperature until further analyses and use.

2.3. Mean droplet diameter, polydispersity index (PDI) and zeta-potential measurements

Mean droplet diameter (z-averages), size distribution, polydispersity index (PDI) and zeta-potential of nanoemulsion were measured using dynamic light scattering on Zetasizer Nano ZS90 (Malvern, U.K.) at 25 °C at a scattering angle of 90°, in triplicates, before and after adding $ZnSO_4$ into nanoemulsion.

2.4. High resolution transmission electron microscopy (HR-TEM), cryogenic-field emission scanning electron microscopy (Cryo-FESEM) analyses

To reveal the internal structure, HR-TEM micrographs were acquired using a transmission electron microscope at an accelerating voltage of 300 kv (FEI- Tecnai G2, F30). For this, one drop of nanoemulsion was positioned on copper grid (200 mesh), stained with 2% phosphotungstic acid and kept for drying (Zhang et al., 2014). To examine the external architecture, Cryo-FESEM (JEOL-JSM-7600F) equipped with Cryo Unit Quorum (PP3000T, UK) was used. In brief, emulsion was freezed in liquid nitrogen at −196 °C and fractured. Further, samples were sublimed at −90 °C for 10 min and sputtered for 30 s at 10 mA and subjected to imaging at −140 °C.

2.5. Field emission gun-scanning electron microscopy-energy dispersive X-ray spectroscopy (FEG-SEM-EDS) and fourier transform infrared (FTIR) analyses

Elemental analysis of the nanoemulsion was carried out using FEG-SEM (JSM-7600F) equipped with EDS elementary analyzer (Oxford Instruments) sputter coating with gold. FTIR was used to determine the interactions of thymol with saponin and zinc. For bulk thymol analysis, KBr pellet method was adopted at the ratio of 1:99 of sample and KBr powder (Saharan et al., 2015). Attenuated total reflection (ATR) unit was used for the analysis of nanoemulsions (Wu et al., 2012). Measurement was carried out from 400 to 4000 cm^{-1} wave numbers with 1 cm^{-1} resolution using FTIR spectrophotometer (Alpha, Bruker, Germany) equipped with an ATR cell.

2.6. Release profile of thymol and zinc

The release kinetics of thymol was studied in deionized water at room temperature (25 °C) using dialysis membrane (Luo et al., 2011). Dialysis membrane-135 with a molecular weight cut-off of 10 kDa (HiMedia Laboratories, Mumbai, India) was loaded with 5 ml of Zn-TNE. The sealed membrane was placed in beaker containing 250 ml deionized water and kept on magnetic stirrer (Remi Laboratory Instruments, Mumbai, India) at 300 rpm. After 24, 48, 72, 96, 120, 144, 168 and 192 h, 5 ml sample from the beaker was withdrawn and 5 ml of fresh deionized water was added to maintain the volume. The samples were subjected for thymol quantification at 263 nm using spectrophotometer (UV–Vis spectrophotometer-118, Systronics, India). A standard curve of thymol was obtained using pure thymol in 0.01% DMSO (Gomes et al., 2011). Similarly, the release profile of Zn^{+2} was studied using the procedure explained above. Zn^{+2} was quantified using double beam atomic absorption spectrophotometer (AAS-4141 model, Electronics Corporation of India Ltd, India).

2.7. Effect of Zn-TNE on α-amylase, protease enzymes activities and seedling growth in pot experiments

Seeds of soybean variety 'JS-335' were treated for 4 h with different concentrations *viz.* 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06% (v/v) of Zn-TNE along with control (water), bulk thymol (0.01%, w/v) and bulk saponin (0.01%, w/v) in aqueous suspension. Treated seeds were dried on creped seed germination paper (Axiva Sichem Biotech, Delhi) and sown in plastic plantation pots (100 × 100 mm, Polylab, India) filled with coco-soil and kept in laboratory conditions under 16/8 h dark and light at 27 ± 2 °C with regular watering. At different stages of germination (0, 1, 3, 5, 7 & 9 days after germination), α-amylase and protease activities were measured. Extract of α-amylase and protease were prepared in sodium acetate (100 mM, pH 4.7) and phosphate buffer (100 mM, pH 7.8) at 4 °C. Enzyme activities (μmol/min/g dry weight) were measured by DNS (3, 5-dinitrosalicylic acid) and FCR (Folin-Ciocalteu reagent) method as described earlier (Lowry et al., 1951; Bernfeld et al., 1955). Protein content (mg/g dry weight) in germinating seed was determined according to Lowry method using bovine serum albumin as standard (Lowry et al., 1951). At first trifoliate stage, foliar spray of Zn-TNE (until run-off i.e. 100 ml on each replication) was performed on seedlings. Chlorophyll *a* and *b* contents were quantified in first trifoliate leaf after 48 h of foliar spray. Various growth characters i.e. germination per cent, shoot - root length, root weight, hypocotyl length, fresh weight, dry weight and plant height were recorded after 2 weeks of sowing. Seedling vigor index (SVI) was calculated according to the formula (Abdul-Baki and Anderson, 1973):

$$\text{Seedling vigor index} = (\text{Germination \%}) \times (\text{Seedling length})$$

2.8. Effect of Zn-TNE on antioxidant-defense enzymes activities, ROS content and lignin deposition in pot experiment

Activities of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD), and defense enzymes *viz.* phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) were estimated in first trifoliate leaf after 0, 12, 24, 48, 72 and 96 h of foliar application of various treatments. For enzyme extraction, 0.2 g leaf samples were

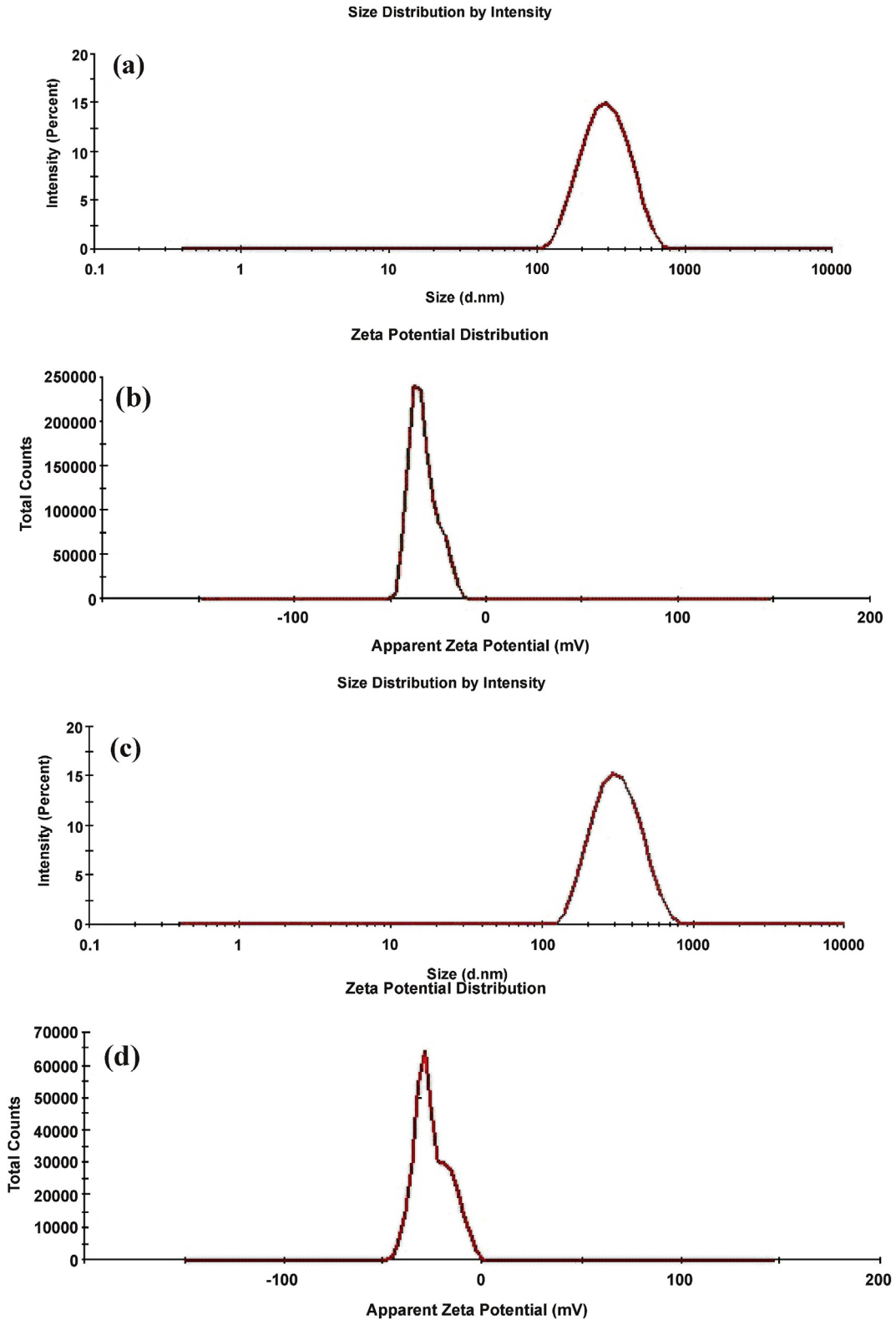

Fig. 1. DLS study (a) Z-average and (b) zeta-potential of thymol nanoemulsion and (c) Z-average and (d) zeta-potential of Zn-TNE.

homogenized in 5 ml of extraction buffer (phosphate buffer for SOD and PPO at pH 7.4 and 6.8, respectively; tris-HCl buffer at pH 7.5 for POD and borate buffer at pH 8.8 for PAL). The homogenates were centrifuged at 10, 000 × *g* for 20 min at 4 °C and supernatants were taken for enzymes assays. SOD (EC 1.15.1.1) activity was determined at 560 nm, as reduction of nitro-blue tetrazolium (NBT) as an indicator of superoxide anion production (Rao et al., 1996). POD (EC 1.11.1.7) activity was measured spectrophotometrically as described by Chance and Maehly (1955) by oxidation of guaiacol in the presence of hydrogen peroxide. PAL (EC 4.3.1.5) was estimated as described (Moerschbacher et al., 1988) where the deamination of L-phenylalanine to trans-cinnamic acid and ammonia was measured at 290 nm. PPO (EC

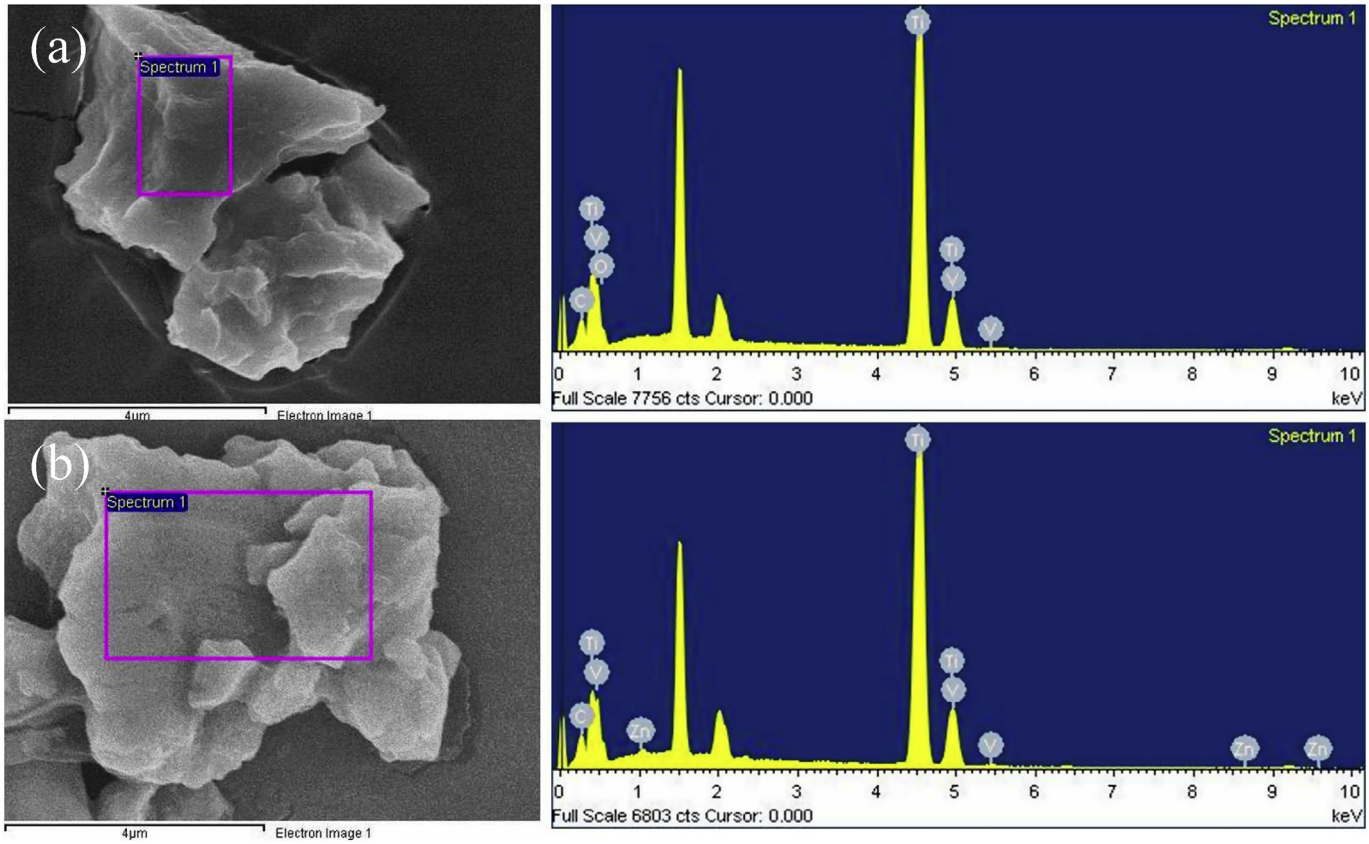

Fig. 2. FEG-SEM-EDS elemental analysis of (a) thymol nanoemulsion and (b) Zn -TNE.

1.10.3.1) was assayed according to Taneja and Sachar (1974) and activity was expressed as change in absorbance at 490 nm. The activities of all enzymes were expressed as µmol/min/g tissue. To estimate ROS contents, leaf samples were collected at 48 h after foliar application of Zn-TNE. Superoxide radicals were measured at 540 nm by monitoring the nitrite formation from hydroxylamine hydrochloride as described (Elstner and Heupel, 1976). H_2O_2 content was determined as change in color due to formation of chromic acetate at 570 nm (Daudi et al., 2012). Lignin deposition in control and treated plants were studied by staining the cross sections of stem and leaf for 3 min in 20% phloroglucinol prepared in HCl as described (Jensen, 1962). The stained sections were observed under light microscope (Olympus SZ 51, Japan).

2.9. Effect of Zn-TNE on disease assessment and crop yield in field

Field experiment was conducted in the year 2016–17 (July to October) at research farm of Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, India, (24.58° latitude, 73.70° longitude) on a standard clay type soil in randomized block design (RBD) with three replication. The field soil had pH 8.2, EC. 0.56 dSm^{-1} and zinc content 1.75 mg/kg. In each replication, seeds were grown in a plot (size 3.6 m^2, where row to row and plant to plant spaces were 0.3 m and 0.1 m, respectively) having three rows (each 3 m). Three best performing concentrations of Zn-TNE (0.02, 0.04 and 0.06%, v/v) optimized in pot experiments were selected for seed treatment as well as foliar application in field. The test field was maintained as per standard agronomic and plant protection management. First foliar spray (until run-off i.e. 1000 ml on each replication) of Zn-TNE was applied after 25 days of sowing (first trifoliate leaf stage) using a Knapsack battery sprayer (YS-095-2, Yes Instrument). Artificial inoculation with *X. axonopodis* pv. *glycines* was carried out after 35 days of sowing as per the standard method (Kim et al., 2011). In brief, for the preparation of inoculum, the bacterial strain was cultured on King's medium B plates at 29 ± 1 °C for 48 h. The bacterial culture was diluted with 10 mM $MgCl_2$ to obtain 1×10^8 CFU/ml at an optical density of 0.5 at 600 nm. Soybean plants were inoculated by spraying the bacterial suspension on to leaf surface using an atomizer. For bacterial pustule disease assessment, leaves from each replicate were selected for observation. Disease severity (DS) was evaluated on a scale of 0–5 (leaves with no visible symptoms = 0; a few individual lesions = 1; many individual lesions = 2; small patches of coalesced lesions = 3; medium sized patches of coalesced lesions = 4; and large patches of coalesced lesions = 5) (Odubanwo et al., 2013). Further, DS and percentage efficacy of disease control (PEDC) were calculated by using formula described elsewhere (Chester, 1959; Wheeler, 1969).

DS = Sum of all individual disease rating × 100/Total number of plant assessed × maximum rating

PEDC = DS in control − DS in treatment × 100/DS in control

Number of nodules/plant and weight/nodule were recorded after 45 days of sowing. Various growth characters namely plant height, root length, root weight, number of pods/plant, number of branches/plant, stem diameter, number of seeds/pod and 100 seed weight were recorded at the end of physiological maturity (95 days).

3. Statistical analysis

Statistical analysis of the data was performed with JMP software version 12. The significant differences among treatment groups were determined using the Turkey Kramer HSD at $p = 0.05$. All experiments were performed in three replications (triplicates) and each replication consisted of minimum three (for laboratory experiments) and ten samples (for field experiments) from randomly selected plants.

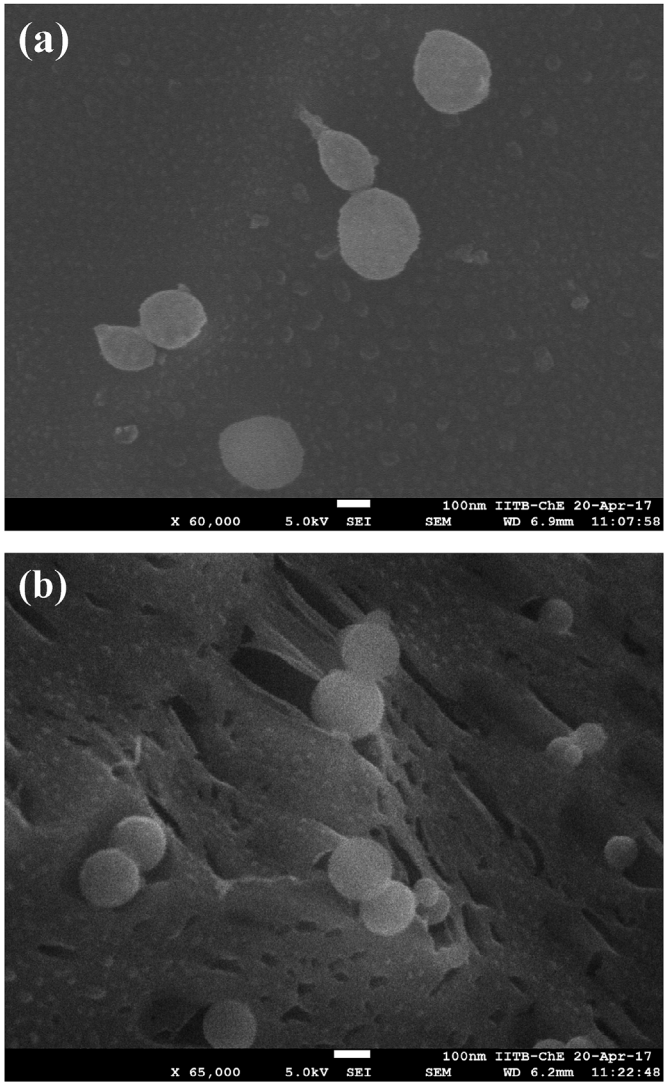

Fig. 3. Cryo-FESEM images of (a) thymol nanoemulsions at 60kx and (b) Zn-TNE at 65kx magnification.

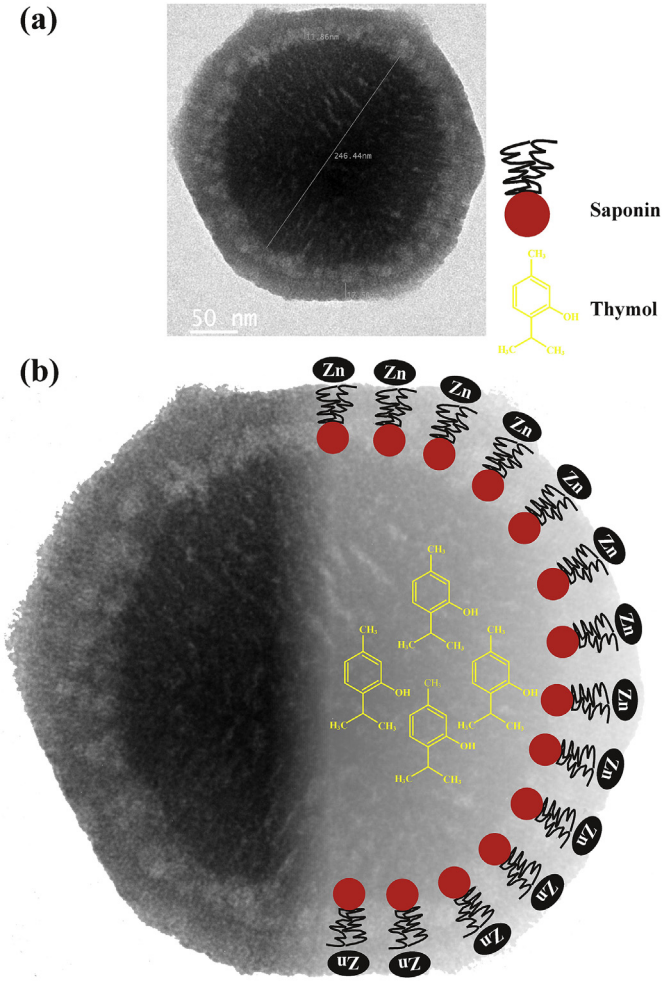

Fig. 4. HR-TEM image of (a) Zn-TNE and (b) hypothetical model of Zn-TNE exhibiting layers of zinc, saponin and thymol.

4. Results and discussion

4.1. Characterization of Zn-TNE

To confirm the interactions of zinc with nanoscale thymol formulation, the formulations prepared with and without zinc were studied by dynamic light scattering. Mean droplet diameter (z-average) 276.2 nm, polydispersity index (PDI) value 0.15 and zeta-potential −31 mV at pH 5.7 (Fig. 1a and b) were found in formulation without zinc. Formulation with zinc (Zn-TNE) showed z-average droplet size 292.5 nm, which was higher as compared with former formulation (Fig. 1c and d). The PDI values (0.14) remained almost same in both the formulations while zeta-potential substantially decreased (−25 mV) in Zn-TNE as compared with formulation without zinc (−31 mV). Kumari et al. (2018) concluded that negative zeta-potential of thymol nanoemulsion was due to –COO^- group of glucuronic acid of saponin at nano-droplet interface. Irrefutably, -COO^- groups interacted with Zn^{+2} on the interface of nano-droplets that led to decrease in zeta-potential (from −31 to −25 mV) of Zn-TNE (Fig. 1b, d). Owing to layering of Zn^{+2} on the surface of nano-droplets, the hydrodynamic diameter of Zn-TNE increased as compared with formulation with thymol only (Fig. 1c). Presence and absence of zinc in nanoemulsion was confirmed in elemental analysis by FEG-SEM-EDS study (Fig. 2a and b). Nanoemulsion without zinc did not show any peak for zinc while Zn-TNE exhibited specific peak for it. Moreover, two additional peaks representing titanium (Ti) and vanadium (V) traces which released from probe tip during sonication appeared in EDS micrographs (Fig. 2). Cryo-SEM micrograph of Zn-TNE revealed that droplets were precisely spherical, rigid and smooth due to the coating of zinc, whereas thymol nanodroplets were irregularly spherical, droopy and rough (Fig. 3a and b). HR-TEM study clearly differentiated various layers of Zn (outermost), saponin (middle) and thymol (centre) in Zn-TNE (Fig. 4a and b). FTIR spectroscopy was used to determine the interactions among various components of nanoemulsion. Nanoemulsion showed noticeable peak at 1341 cm^{-1} giving clue of zinc interaction with carboxylate group of saponin. No noticeable peak was observed in formulation without zinc, however a widened peak representing the hydrophilic interaction was observed at 3331 cm^{-1} (Fig. 5a and b).

4.2. Release kinetics of thymol and zinc

A rapid release (64%) of thymol was observed in first 24 h (Fig. 6). It is predicted that this is the unbound form of thymol present in the suspension which rapidly crossed the dialysis membrane. After 24 h, thymol release was quite slow (80% up to 192 h) as these molecules were covered with saponin. A similar release pattern was also followed by Zn^{+2} (57% up to 24 h and 79% up to 192 h) (Fig. 6).

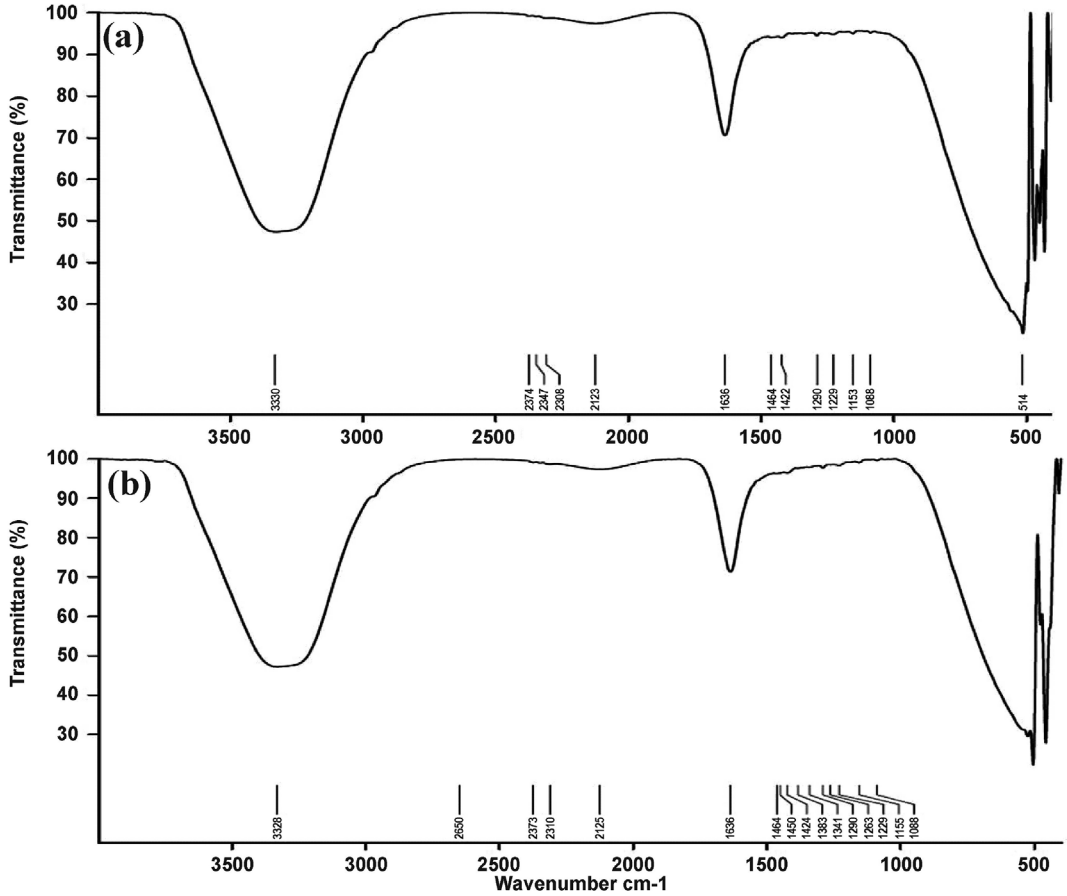

Fig. 5. FTIR spectra (a) thymol nanoemulsions and (b) Zn-TNE.

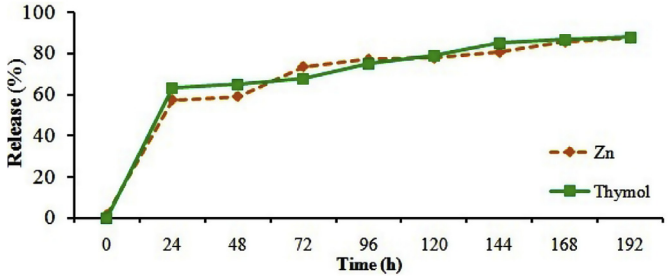

Fig. 6. Kinetics of thymol and Zn release from Zn-TNE.

4.3. α-Amylase and protease activities; and growth of soybean in pot experiment

Activities of α-amylase and protease and content of total protein was measured in germinating seeds at 0, 1, 3, 5, 7 and 9 days of germination (Fig. 7). At 0 day, negligible activities of α-amylase and protease were observed in all the treatments. On 5th day, α-amylase activity was maximum (2.22, 2.39, 3.49, 3.20, 3.17 and 3.45 μmol/min/g dw) in Zn-TNE (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06%) treated seedlings as compared with bulk thymol. In all concentrations of Zn-TNE, α-amylase activity increased by 1.8–2.4 folds as compared with bulk thymol (Fig. 7a). Similarly, protease activity was also affected by Zn-TNE (Fig. 7b). Maximum protease activity was recorded during 7th to 9th days in all the treatments of Zn-TNE. Total protein content was found maximum at 7th day and increased by 1.05–2.38 folds in Zn-TNE as compared with bulk thymol (Fig. 7c). After 7th day, protein content started to decline and protease activity started to increase. These data suggest that Zn-TNE significantly induced seed stored food (starch and protein) mobilization by enhancing the activities of α-amylase and protease in germinating seeds.

To evaluate the effect of Zn-TNE on growth characteristics of soybean, per cent germination, shoot-root length, root weight, hypocotyl length, fresh-dry weight, plant height, seedling vigor index and content of chlorophyll *a* and *b* were recorded (Table 1). Statistical analyses showed that Zn-TNE significantly enhanced the growth of soybean plants in pot experiments as compared with control, bulk thymol and bulk saponin treatments. Higher values of per cent germination, hypocotyl length, dry weight and seedling vigor index were recorded in 0.02–0.05% Zn-TNE treated plants as compared with control and bulk thymol (Table 1). However at 0.06% concentration, various growth parameters remained unaffected as compared with other treatments. A significant increase in chlorophyll *a* (9.65–20.53 mg/g) and b contents (0.71–1.57 mg/g) was recorded in 0.01–0.06% Zn-TNE treated plants. Contrarily, chlorophyll *a* and *b* contents were found minimum (7.88 and 0.64 mg/g) in bulk thymol treatment (Table 1).

4.4. Antioxidant-defense enzymes activities, ROS contents and lignin deposition in pot experiment

Zn-TNE increased the activities of antioxidant-defense enzymes (SOD, POD, PAL and PPO). SOD activity in Zn-TNE treated plant leaves showed 21.0–62.4% increase at 48 h as compared with bulk thymol treatment and 1.3–29.5% increase as compared with control (Fig. 8a). Similarly, at 48 h, 71.6–176.9% and 68.4–171.7% higher POD activity was recorded in 0.01–0.06% Zn-TNE treated plants as compared with control and bulk thymol treatment (Fig. 8b). Likewise, Zn-TNE treatment increased PAL activity throughout the experiment and maximum activity was found at 72 h which was 2.4–22.8% higher as compared with bulk thymol and 42.07–70.31% higher as compared with control

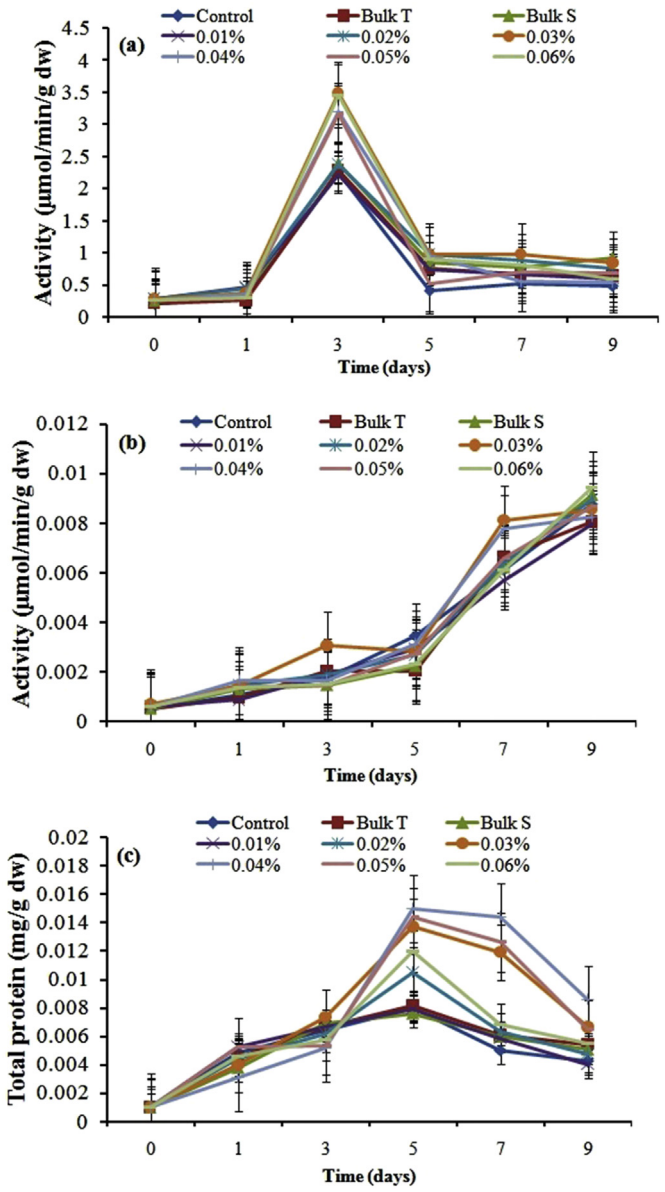

Fig. 7. Effect of Zn-TNE on (a) α-amylase (b) protease and (c) protein content in germinating soybean seeds. Each value is the mean of triplicates.

(Fig. 8c). PPO activity was also enhanced by 8.2–29.1% in Zn-TNE treatment as compared with bulk thymol at 24 h. As compared with control, PPO activity in 0.01–0.06% Zn-TNE treatment increased from 26.9 to 85.9% at 24 h (Fig. 8d). Concentration of O_2^- (superoxide) was slightly higher in Zn-TNE treatments at day 2 and remained constant in proceeding days (Fig. 9a). H_2O_2 content was higher in Zn-TNE treated leaves by 41.67–91.67% on 1st day, 30.77–64.84% on 2nd day and 2.4–207.1% on 3rd day as compared with bulk thymol treatment (Fig. 9b). Lignin deposition was also higher in leaves and stems of Zn-TNE (0.06%, v/v) treated plants as compared with control (Fig. 10a, b, c and d).

4.5. Growth parameters of soybean in field conditions

Seeds of soybean variety 'JS-335' treated with Zn-TNE for 4 h were sown in field. Three concentrations *viz.* 0.02, 0.04 and 0.06% (v/v) of nanoemulsion were selected from pot experiments to test under field conditions. Foliar application of Zn-TNE was made after 25 days of sowing while artificial inoculation with *X. axonopodis* pv. *glycines* was

Table 1
Effect of Zn-TNE on growth parameters of soybean in pot conditions.

Treatment (%)	Germination (%)	Shoot length (cm)	Root length (cm)	Root weight (g)	Hypocotyl length (cm)	Fresh weight (g)	Dry weight (g)	Plant height (cm)	SVI	Chl-a (mg/g)	Chl-b (mg/g)
Control	26.6 ± 1.6^{b}	23.6 ± 0.48^{b}	9.4 ± 0.06^{ab}	0.11 ± 0.013^{c}	9.6 ± 0.30^{abc}	0.83 ± 0.01^{ab}	0.08 ± 0.005^{ab}	33.8 ± 0.11^{ab}	818.3 ± 63.0^{b}	6.8 ± 0.02^{i}	0.5 ± 0.006^{i}
Bulk S (0.01)	30.0 ± 2.9^{ab}	23.2 ± 0.13^{b}	9.5 ± 0.26^{ab}	0.15 ± 0.001^{ab}	8.6 ± 0.33^{c}	0.92 ± 0.01^{a}	0.09 ± 0.004^{ab}	33.2 ± 0.37^{b}	999.6 ± 10.6^{b}	15.0 ± 0.04^{d}	1.0 ± 0.005^{d}
Bulk T (0.01)	41.6 ± 6.0^{ab}	23.2 ± 0.53^{b}	9.7 ± 0.33^{ab}	0.13 ± 0.006^{abc}	9.5 ± 0.06^{abc}	0.78 ± 0.06^{b}	0.07 ± 0.007^{b}	33.7 ± 1.09^{b}	1367.4 ± 21.8^{ab}	7.8 ± 0.03^{h}	0.6 ± 0.000^{h}
Zn-TNE											
0.01	35.0 ± 2.9^{ab}	23.1 ± 0.06^{b}	9.6 ± 0.13^{ab}	0.12 ± 0.005^{bc}	8.9 ± 0.17^{bc}	0.76 ± 0.01^{b}	0.08 ± 0.003^{ab}	33.5 ± 0.52^{b}	1159.0 ± 92.8^{ab}	9.6 ± 0.11^{g}	0.7 ± 0.008^{g}
0.02	53.3 ± 9.3^{a}	23.7 ± 0.35^{b}	10.1 ± 0.59^{ab}	0.16 ± 0.006^{a}	9.8 ± 0.24^{ab}	0.85 ± 0.00^{ab}	0.10 ± 0.005^{a}	34.5 ± 1.12^{ab}	1840.6 ± 32.7^{a}	19.7 ± 0.05^{b}	1.3 ± 0.021^{b}
0.03	41.6 ± 1.6^{ab}	26.4 ± 0.74^{a}	10.4 ± 0.29^{a}	0.16 ± 0.002^{a}	9.8 ± 0.13^{ab}	0.94 ± 0.00^{a}	0.10 ± 0.002^{a}	37.0 ± 0.65^{a}	1545.0 ± 73.5^{ab}	20.5 ± 0.06^{a}	1.5 ± 0.003^{a}
0.04	45.0 ± 5.7^{ab}	23.0 ± 0.29^{b}	10.0 ± 0.11^{ab}	0.14 ± 0.005^{abc}	10.0 ± 0.17^{a}	0.88 ± 0.01^{ab}	0.10 ± 0.002^{a}	33.1 ± 0.40^{b}	1323.0 ± 79.4^{ab}	18.1 ± 0.10^{c}	1.2 ± 0.006^{c}
0.05	40.0 ± 2.9^{ab}	22.7 ± 0.53^{bc}	9.8 ± 0.37^{ab}	0.12 ± 0.007^{abc}	9.6 ± 0.13^{abc}	0.87 ± 0.00^{ab}	0.08 ± 0.001^{ab}	32.9 ± 0.29^{b}	1316.3 ± 88.0^{ab}	14.3 ± 0.06^{e}	1.0 ± 0.008^{e}
0.06	38.3 ± 6.6^{ab}	20.8 ± 0.43^{c}	8.6 ± 0.50^{b}	0.12 ± 0.009^{bc}	9.5 ± 0.13^{abc}	0.81 ± 0.03^{ab}	0.08 ± 0.003^{ab}	28.8 ± 0.70^{c}	1094.6 ± 16.8^{ab}	12.4 ± 0.02^{f}	0.8 ± 0.000^{f}

Various growth parameters were recorded after 15 days of sowing. Each value is mean of triplicates and each replicate consisted of 5 plants. Mean ± SE followed by same letter is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD. Bulk T (bulk thymol) dissolved in 0.1% dimethyl sulfoxide (DMSO) and Bulk S (bulk saponin, 0.01%, w/v) mixed in water.

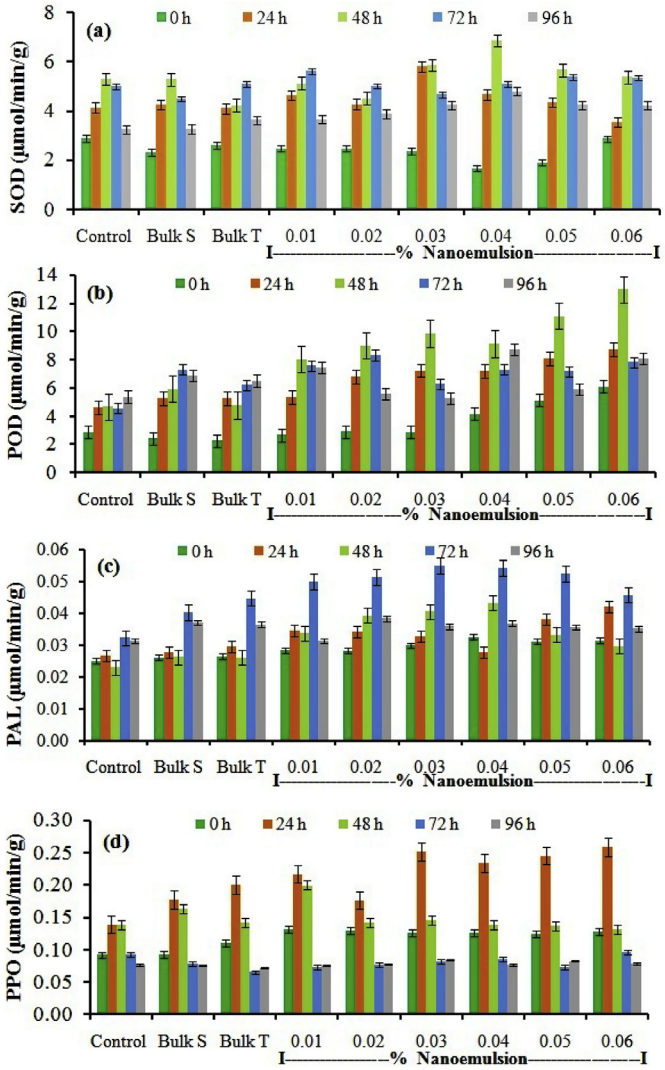

Fig. 8. Effect of Zn-TNE on (a) SOD (b) POD (c) PAL (d) PPO activities in soybean leaves after 0, 12, 24, 48, 72 and 96 h of foliar spray. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Control with water, Bulk T (bulk thymol, 0.01%) dissolved in 0.1% dimethyl sulfoxide (DMSO) and Bulk S (bulk saponin, 0.01%, w/v) mixed in water.

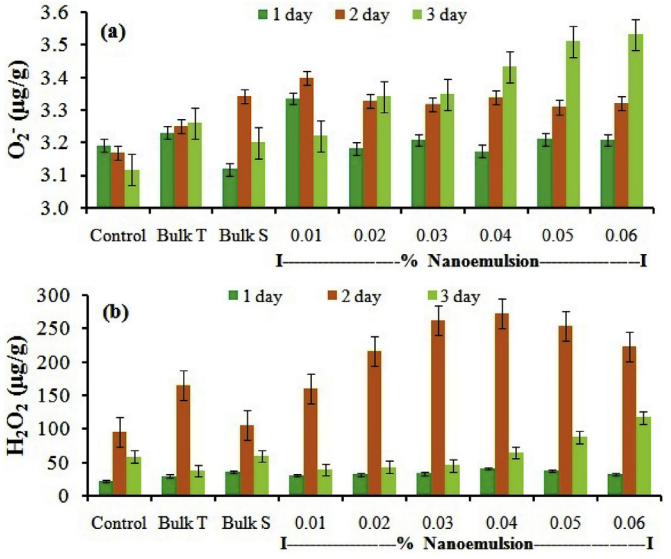

Fig. 9. Effect of Zn-TNE on (a) O_2^{-1} and (b) H_2O_2 content in soybean leaves after 1, 2 and 3 days of foliar spray. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Control with water, Bulk T (bulk thymol, 0.01%, w/v) dissolved in 0.1% dimethyl sulfoxide (DMSO) and Bulk S (bulk saponin, 0.01%, w/v) mixed in water.

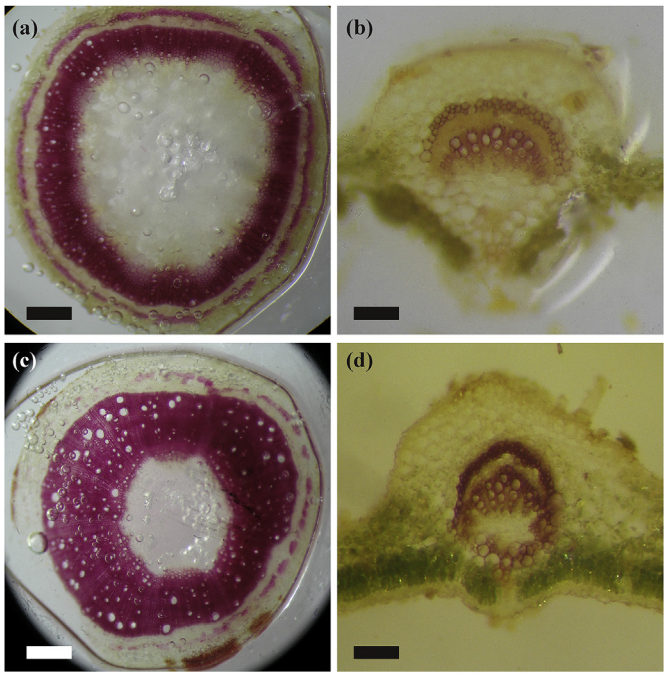

Fig. 10. Effect of Zn-TNE on lignin deposition in (a) stem (b) leaf sections of control plant and (c) stem and (d) leaf sections of Zn-TNE (0.06%, v/v) treated plants after 30 days of foliar spray. Bar = 0.03 mm.

carried out after 35 days of sowing. Growth data were recorded at the end of physiological maturity (95 days). Treatments with Zn-TNE (0.02, 0.04 and 0.06%) significantly affected growth parameters of soybean (Fig. 11a, b and c). Maximum plant height was recorded at 0.04% concentration and minimum in control-2 (with water treatment and inoculation). Root length and root weight was maximum in 0.06% and minimum in control-2 and control-1 (without water and without inoculation). Number of pods/plant, number of branches/plant, stem diameter, number of seeds/pod, number of nodules/plant, weight/nodule, 100 seed weight and grain yield was observed significantly higher in 0.06% as compared with other treatments (Table 2).

4.6. Occurrence of bacterial pustule disease in field conditions

DS and PEDC data were recorded after 15 days of inoculation using 1 to 5 standard disease rating scale. Control-2 plants (with water treatment and inoculation) exhibited 68.2% DS while all the plants treated with Zn-TNE (0.02–0.06%) expressed DS in the range of 48.9–13.8%. In bulk thymol treated plants, 62.2% DS was recorded. Treatment with Zn-TNE exhibited higher disease control (28.15–79.42%) as compared with other treatments. Maximum PEDC (79.4%) was found at 0.06% Zn-TNE (Table 3).

Zinc plays wider roles in plants through amending plant metabolism and contributes greatly to combat abiotic and biotic stresses. In fact, zinc acts as a key structural and catalytic component of various enzymes of electron transfer and redox reactions and is imperative for growth (Rajasekaran and Santra, 2015). It is possibly involved in various enzymes of food remobilization during grain filling stage which is very decisive for higher yield during biotic and abiotic stresses (Yu et al., 2015). Therefore, its deficiency in crop plants drastically reduces the yield and situation becomes more critical in crops growing in zinc deficit and/or alkaline soil. Various zinc based fertilizers/

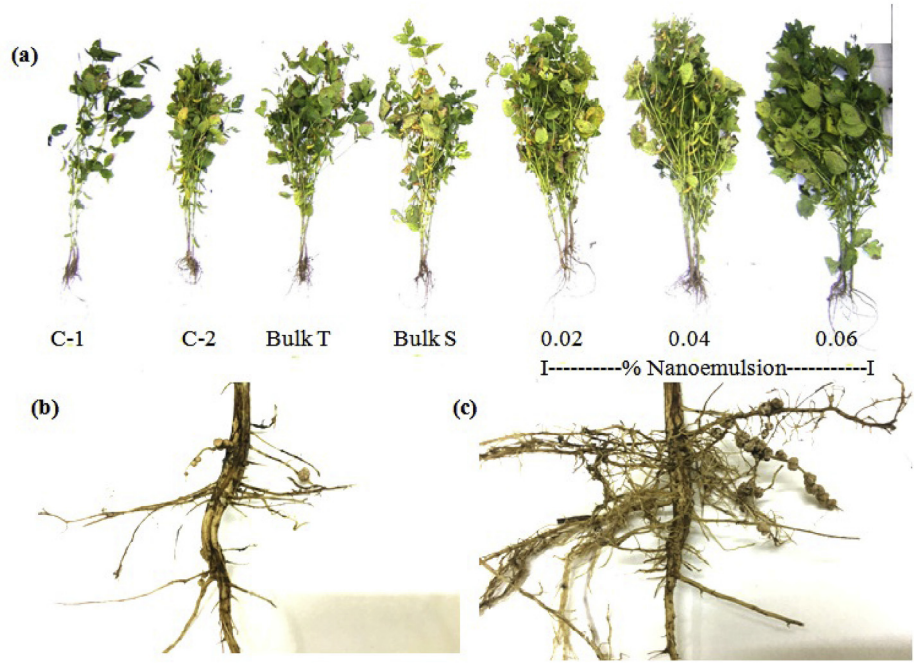

Fig. 11. Effect of Zn-TNE on (a) plant growth of soybean (b) nodules in control and (c) nodules in 0.06% Zn-TNE. Concentration of Zn-TNE ranging from 0.02 to 0.06%, v/v exhibited visual differences in plant growth.

agrochemicals have been developed and are being used for higher yield. However, the efficacy of soil amended with fertilizers/agrochemicals is poor as it may drift off from the target site. Additionally, other biological and physical factors are also responsible for lesser efficacy of such soils (Correa and Salgado, 2011). Moreover, uncontrolled and/or sudden exposure of zinc to plant cells also causes toxicity and residual effects (Gupta and Kalra, 2006). In recent years, numerous research groups have developed various types of zinc based nanomaterials for use in crops (Raliya et al., 2017). Technology translation of metal based nanomaterials has not been easy going as expected, due to the contradictory reports regarding toxicity of metal nano-materials to ecosystem and their fate in food crops (Maurer-Jones et al., 2013). Recent studies have serious reservations to nanomaterials particularly toxicity of zero valent metal nano-particulates in food-stuffs due to genotoxicity reports in human cell lines (Cox et al., 2016; Yang et al., 2018). This situation becomes more complicated as nano-agri-inputs get more attention for replacement of conventional agrochemicals. From an agriculture perspective, unknown interplay of nanomaterials with living organisms and their fate in soil, water and air needs to be examined for safe use (Zuverza-Mena et al., 2017). To overcome the toxicity issue mainly of metal based micronutrients and their higher use efficiency, biocompatible and biodegradable nano-carriers can be used for controlled delivery to crops (Kumaraswamy et al., 2018). In recent years, bioactive materials of plants and animals origin are being exploited through nanotechnology for their up-scale use. In current study, we have exploited plant based thymol and saponin as nanoemulsion which is safer as these compounds are biodegradable. Based on aforesaid, we elusively anticipate that thymol nanoemulsion could further be improved for more promising applications in crops, mainly towards growth promotry/yield aspects. Physico-chemical properties of thymol nanoemulsion especially the availability of $–COO^-$ group could further be a docking site for functionalization of nanoemulsion for use as a carrier for micronutrient delivery. In this nexus, we have developed plant based biocompatible, biodegradable and dual functional Zn-TNE not only for controlling bacterial disease but also for strengthening the defense and antioxidant systems of plants for yield improvement. Zn-TNE showed potential physico-chemical characteristics by DLS, HR-TEM, FEG-SEM-EDS, Cryo-FESEM, FTIR and AAS studies as compared with our previously reported thymol nanoemulsion without zinc (Kumari et al., 2018). Increased hydrodynamic diameter and decreased zeta-potential confirmed the binding of zinc on nanodroplets of thymol nanoemulsion. Cryo-SEM revealed smooth, hardy surface texture of Zn-TNE nano-droplets as compared with nano-droplets without zinc (Fig. 3). The observation can be attributed to the presence of zinc on Zn-TNE nano-droplets. FTIR micrograph showed the appearance of a new peak at 1341 cm^{-1}due to the interaction of $–COO^-$ group with zinc (Tang et al., 2017). Presence of zinc could also be confirmed in FEG-SEM-EDS analysis. Zinc, saponin and thymol layer in Zn-TNE was further clearly outlined by HR-TEM study (Fig. 4).

Seed treatment with Zn-TNE significantly improved seedling growth in soybean and biochemical analysis in germinating seeds suggested that Zn-TNE gravely induced α-amylase and protease activities for better mobilization of starch and protein to developing embryo (Fig. 7a, b and c). The study on Zn-TNE was further expended in pot and field experiments to ascertain in-depth study of plant innate immunity and subsequent plant growth/yield. Application of Zn-TNE considerably up-regulated the SOD, POD, PAL and PPO activities as compared with bulk thymol and control. Balance of ROS is vital for fecund plant metabolism and sustained growth under disease condition. Enhanced activity of SOD rapidly dismutates the toxic O_2^- into less toxic H_2O_2 and up-scaled POD activity can scavenge H_2O_2 into H_2O. Balanced content of H_2O_2 is essential for optimum growth since it acts as a signaling molecule for various metabolic reactions. H_2O_2 also activates adjoining plant cells to commence systemic acquired resistance (Das and Roychoudhury, 2014). Plant defense enzymes namely POD, PAL and PPO participate in the biosynthesis of suberin, melanin and lignin. Lignin is a hard and hydrophobic molecule of cell wall, so its higher deposition in stem and leaf (Fig. 10 a, b,c and d) imparts resistance against pathogenic microbes (Miedes et al., 2014). To arrive at conceivable conclusion, Zn-TNE was tested in field conditions for disease control and yield in soybean. Higher value of PEDC (79.4%) indicates a considerable control of bacterial pustule disease at 0.01–0.06% (v/v) concentrations of Zn-TNE (Table 3). Thymol is well known for its antimicrobial activity due to the presence of hydroxyl group and its nanoform is even more effective in inhibiting bacterial growth and subsequent disease control as evident in *in vitro* study (Kumari et al., 2018).

Table 2
Effect of Zn-TNE on plant growth in field conditions.

Treatment (%)	Plant height (cm)	Root length (cm)	Root weight (g)	No. of pods/plant	No. of branches/plant	Stem diameter (cm)	No. of seeds/pod	No. of nodules/plant	Weight/nodule (mg)	100 seed weight (g)	Grain yield (kg/plot)
Control-1	64.40 ± 0.91^{ab}	24.93 ± 0.81^{bc}	1.90 ± 0.21^{c}	34.60 ± 6.00^{d}	12.80 ± 0.57^{b}	1.88 ± 0.13^{c}	2.40 ± 0.02^{d}	7.86 ± 0.76^{c}	26.33 ± 0.71^{b}	7.59 ± 0.17^{a}	0.54 ± 0.06^{bc}
Control-2	62.86 ± 2.77^{ab}	22.93 ± 1.04^{c}	2.05 ± 0.22^{bc}	37.20 ± 4.12^{d}	13.33 ± 0.40^{b}	1.96 ± 0.06^{bc}	2.41 ± 0.01^{d}	8.80 ± 0.52^{bc}	28.00 ± 0.94^{ab}	7.68 ± 0.55^{a}	0.58 ± 0.12^{bc}
Bulk S(0.01)	55.86 ± 3.11^{b}	26.13 ± 1.64^{abc}	2.27 ± 0.12^{bc}	43.46 ± 2.99^{cd}	12.73 ± 0.46^{b}	1.94 ± 0.05^{bc}	2.46 ± 0.06^{cd}	9.66 ± 0.75^{abc}	28.91 ± 1.47^{ab}	7.61 ± 0.58^{a}	0.51 ± 0.06^{c}
Bulk T(0.01)	64.66 ± 2.37^{ab}	31.06 ± 3.27^{abc}	2.90 ± 0.49^{bc}	53.80 ± 2.19^{cd}	12.80 ± 0.75^{b}	2.28 ± 0.19^{abc}	2.55 ± 0.07^{bcd}	9.80 ± 0.20^{abc}	28.33 ± 0.79^{ab}	7.92 ± 0.80^{a}	0.90 ± 0.15^{abc}
$ZnSO_4$(0.01)	63.76 ± 0.77^{ab}	33.03 ± 1.09^{ab}	2.91 ± 0.66^{bc}	70.10 ± 1.26^{bc}	13.13 ± 0.20^{b}	2.19 ± 0.09^{abc}	2.59 ± 0.04^{abc}	9.90 ± 0.11^{abc}	29.90 ± 1.56^{ab}	8.18 ± 0.27^{a}	0.88 ± 0.13^{abc}
Zn-TNE											
0.02	64.86 ± 1.13^{ab}	34.93 ± 1.79^{ab}	3.68 ± 0.32^{abc}	72.00 ± 2.66^{bc}	13.80 ± 0.41^{b}	2.52 ± 0.04^{ab}	2.61 ± 0.01^{abc}	12.06 ± 0.33^{a}	29.08 ± 1.41^{ab}	8.58 ± 0.43^{a}	0.96 ± 0.21^{ab}
0.04	69.20 ± 1.33^{a}	35.33 ± 0.56^{ab}	4.60 ± 0.29^{ab}	88.40 ± 8.23^{ab}	16.40 ± 1.22^{ab}	2.65 ± 0.08^{a}	2.66 ± 0.01^{ab}	11.33 ± 0.78^{ab}	33.25 ± 2.51^{a}	8.99 ± 0.19^{a}	0.97 ± 0.18^{ab}
0.06	68.20 ± 1.51^{a}	36.86 ± 4.15^{a}	5.74 ± 1.21^{a}	116.66 ± 12.47^{a}	18.40 ± 1.55^{a}	2.89 ± 0.20^{a}	2.75 ± 0.02^{a}	11.53 ± 0.35^{ab}	33.08 ± 0.06^{a}	9.68 ± 0.29^{a}	1.08 ± 0.15^{a}

Each value is mean of triplicate and each replicate consisted of 3 plants. Mean ± SE followed by same letter in column of each treatment are not significant different at $p = 0.05$ as determined by Tukey–Kramer HSD. Control-1 (without water treatment and without inoculation), Control-2 (with water treatment and inoculation), Bulk S (bulk saponin, 0.01%) dissolved in water, Bulk T (bulk thymol, 0.01%) dissolved in 1% DMSO (Dimethyl sulphoxide).

Table 3
Effect of Zn-TNE on bacterial pustule disease in field conditions.

Treatment (%)	Bacterial pustule	
	DS (%)[A]	PEDC (%)[A]
Control-1	00.00 ± 0.00^{d}	00.0 ± 0.00^{c}
Control-2	68.22 ± 2.47^{a}	00.0 ± 0.00^{c}
Bulk S (0.01)	62.44 ± 3.44^{ab}	8.95 ± 4.51^{c}
Bulk T (0.01)	62.22 ± 1.73^{ab}	12.07 ± 3.07^{c}
$ZnSO_4$ (0.01)	59.13 ± 1.78^{abc}	15.6 ± 2.43^{c}
Zn-TNE		
0.02	48.88 ± 3.63^{bc}	28.15 ± 8.35^{bc}
0.04	33.33 ± 4.37^{c}	50.98 ± 7.73^{ab}
0.06	13.77 ± 5.12^{d}	79.42 ± 8.15^{a}

Disease data were recorded after visual appearance of symptoms using 0 to 5 standard disease rating scale. [A]Each value is mean of triplicates and each replicate consisted of 3 plants. Mean ± SE followed by same letter is not significantly different at $p = 0.05$ as determined by Tukey–Kramer HSD. Bulk S (bulk saponin, 0.01%) dissolved in water and Bulk T (bulk thymol, 0.01%) dissolved in 1% DMSO (Dimethyl Sulphoxide). DS (disease severity). PEDC (per cent efficacy of disease control) was calculated as compared with control-2 (with water treatment and inoculation). Control-1 (without water treatment and inoculation).

5. Conclusion

In conclusion, the remarkable control of bacterial pustule disease of soybean in field conditions can be realized by two facts. Firstly, thymol component of nanoformulation wield excellent bactericidal activity and secondly, its zinc component galvanizes the plant innate immunity, sustains oxidative homeostasis and overcomes the severity of pathogenic infection. Further, notably higher values of plant height, root length, root weight, number of pods/plant, number of branches/plant, stem diameter, number of seeds/pod, number of nodules/plant, weight/nodule, 100 seed weight and grain yield/plot were also recorded in Zn-TNE treatments. Application of Zn-TNE (0.06%, v/v) increased 100 seed weight by 18% as compared with bulk thymol and 21% with control-1. At same dose, Zn-TNE improved grain yield (kg/plot) by 16.6% as compared with bulk thymol and 50% with control-1. Altogether, Zn-TNE proved effective in controlling disease and enhancing growth and yield of soybean crop. To summarize, the developed Zn-TNE could be an alternative to synthetic agrochemicals and an eco-friendly approach for sustainable agriculture coupled with protection of biosphere.

Author's contribution

V.S. designed the study. S.K., R.C.C., R.V.K., and D.B conducted the experiments. P.B., R.R., A.P. and V.S. provided the overall supervision of the project. V.S. wrote the manuscript, all the authors read and revised the manuscript, and all authors approved the final version of the manuscript for submission.

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Declaration of competing interest

None.

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