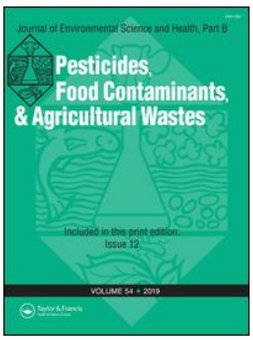

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Amphiphilic polymer based nanoformulations of mancozeb for management of early blight in tomato

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ABSTRACT

Controlled release (CR) nanoformulations of Mancozeb (Manganese-zinc double salt of *N, N*-bisdithiocarbamic acid), a protective fungicide, have been developed using poly (ethylene glycols) (PEGs) based functionalized amphiphilic copolymers and evaluated for the management of early blight in tomato. During the field experiment, it was observed that number of infected leaflets/plants were less in developed formulation treated plants as compared to commercial products. Number of infected leaflets per plant was 2.40–4.60 and the number of fruits per plant were 6.40–9.00 at 50 mg L^{-1}, whereas at 100 mg L^{-1}, the corresponding numbers were 2.10-4.10 and 6.30-9.10 respectively. These formulations can be used to optimize the release of Mancozeb to achieve disease control for the desired period depending upon the matrix of the polymer used. Importantly, sufficient amount of active ingredient remains available for a reasonable period of time after application leading to reduced number of applications of pesticide.

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Amphiphilic polymer; Nanoformulations; Mancozeb; Field bio-efficacy; Early blight of tomato

Introduction

There is a great surge in utilizing nanotechnological tools in crop protection.[1] Nano sized materials tend to show different behavior than their bulk form like gold, which is well known to be safe and chemically inert in bulk while at nano scale it becomes highly reactive and utilized in many fields.[2] Similarly, nano pesticides tend to show extraordinary pesticidal activities and are required in lesser quantities for effective pest management thereby, reducing pesticide load on the environment.[3–6]

Amphiphilic block copolymers with hydrophilic and hydrophobic segments have been investigated extensively because of their unique self-organization characteristics and wide range of potential applications, such as in drug delivery and separation technology systems. Amphiphilic polymers have very high solubilization power, low critical micelle concentration (CMC) and high stability.[7–9] Polymeric micelles composed of amphiphilic block copolymers demonstrate a series of attractive properties in drug and pesticide delivery systems, such as good biocompatibility and high stability *in vitro* and *in vivo* and can be successfully used for the encapsulation of various poorly soluble agents in aqueous solution.[10,11] Nano Sulphur was developed and compared for bio-efficacy with commercially available sulfur formulations *in vitro* for fungicidal efficacy at 1000 mg L^{-1} against *Erysiphe cichoracearum* in okra. It was observed that nano-sulfur was more effective than the commercial formulations of sulfur and required at lower doses for optimum control of powdery mildew disease in okra.[12] Integrated information from more than 3000 patents and 100 peer-reviewed publications and reports, has been reported.

Tomato is second most important remunerable solanaceous vegetable crop after potato, either for local consumption and exportation. It is native to South America and is widely cultivated in 140 countries of the world with an annual production of 16826000 metric tonnes.[13] High nutritive value and varied climatic adaptability made tomato cultivation more popular. Area under tomato in the India is about 8.65 lakh hectares and it is about 10.2% of the total cropped land under vegetables. Annual production of tomato in India is 1.68 MMT which is 11.5% of the total vegetable production and productivity of 19.5 metric tonnes per hectare. There has been a gradual increase in the area under tomato while the production has been fluctuating due to various diseases and insect pest damage. There are several diseases on tomato caused by fungi, bacteria, viruses, nematodes and abiotic factors.[14–16]

Among the fungal diseases in tomato, early blight caused by *Alternaria solani*, is the most threatening one which causes great reduction in the quantity and quality of fruit yield. It is an important disease of tropical and sub-tropical areas. It is now found in all continents of the world. The fungus causes disease in tomato, potato and eggplant. The causal organism is air borne and soil inhabiting causes disease on foliage (leaf blight), stem (collar rot) and fruit (fruit rot) and can result in severe damage during all stages of plant development disseminated by fungal spores.[17,18] It has increasingly become a limiting factor for successful cultivation of tomato and causes yield losses varying from 15 to

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100%.[19] Tomato crop is damaged due to severe infection of *A. solani* every year in India. The disease severity was recorded up to 90% in Varanasi region.[20]

Mancozeb (ethylene bisdithiocarbamate salt) is widely used as a dithiocarbamate fungicide to protect fruit, nut and field crops from a range of fungal diseases.[21] The direct effect of Mancozeb upon core biochemical processes within the fungus results in inhibition of spore germination.[22] Mancozeb is marketed by the trade names Dithane M-45, Manzeb, Nemispot, and Manzane. It is used to control plant diseases in cereals, vegetables and fruits, including citrus, bananas, strawberries, pineapples, papaya, litchi etc. The problems with existing mancozeb formulations are higher doses of application for controlling the early blight of tomato and also have low solubility. Therefore, we prepared novel amphiphilic polymer based nanoformulations of mancozeb, which are required in low dose.

There are reports in literature of CR formulations based on commercially available polymers.[1] Also synthetic amphiphilic polymers for developing control release formulations of different bioactive molecules were shown to be promising for slow release and in our previous report also showed nanoformulation of mancozeb was more effective at *in-vitro* condition.[3,23–27] The present study is aimed to development and evaluation of nanoformulations of Mancozeb employing amphiphilic polymers under field conditions for controlling of early blight of tomato, which, to the best of our knowledge, is the first report of its kind.

Materials and methods

Materials

Mancozeb (technical grade) with purity of 99% w/w and commercial formulation 42% Suspension Concentrate (SC) were obtained from Insecticide (India) Limited, Rajasthan, India. Wet table Powder (WP), 75%, was obtained from Swal Corporation Limited, Mumbai, India. Double distilled water was used to prepare the reagents and solutions. Hydrochloric acid, Potato dextrose agar (PDA) was supplied by Himedia Laboratories Pvt. Limited, Mumbai, India etc. High pressure knapsack sprayer model HX-102 (Hymax Agro. India) with 16 L capacity and automatic agitation was used for spray application. Cultures of *A. solani* were obtained from the Department of Plant Pathology, PAU, Punjab, India. Tomato seedlings were obtained from the Seed Production Unit, ICAR- IARI, New Delhi, India.

Synthesis

The exceptional characteristics of poly(ethylene glycol) (PEG), including a wide-ranging solubility, lack of toxicity, noninterference with enzymatic activities and polypeptide conformations and ease of excretion from living organisms, make them perfect drug carrier. In this study, PEG-based amphiphilic copolymers **3a–3d** (dimethyl-5-hydroxyisophthalate and poly(ethylene glycols) of different molecular weights *viz.* 600, 1000, 1500 and 2000 were used for the encapsulation of Mancozeb and their functionalized products **6a–6d** & **7a-7d** were synthesized and characterized according to the method reported in literature.[27] The general method of synthesis of polymers is reproduced below (Scheme 1).

General method of coupling of bromohexadecane (5) with amphiphilic polymers (3a-3d)

Equimolar quantities of **3a-3d** (4.67, 7.07, 10.07 and 13.07 g) and **5** (1.832 g) were dissolved in dry acetone (10 mL) and to the resultant solution was added an equimolar amount of anhydrous potassium carbonate (0.828 g). The reaction mixture was refluxed at 60 °C and the progress of the reaction was monitored by TLC using ethyl acetate in petroleum ether (30%). After completion, potassium carbonate was removed by filtration and the solvent was removed under vacuum to give the products **7a-7d**.

Encapsulation of mancozeb in nanospheres

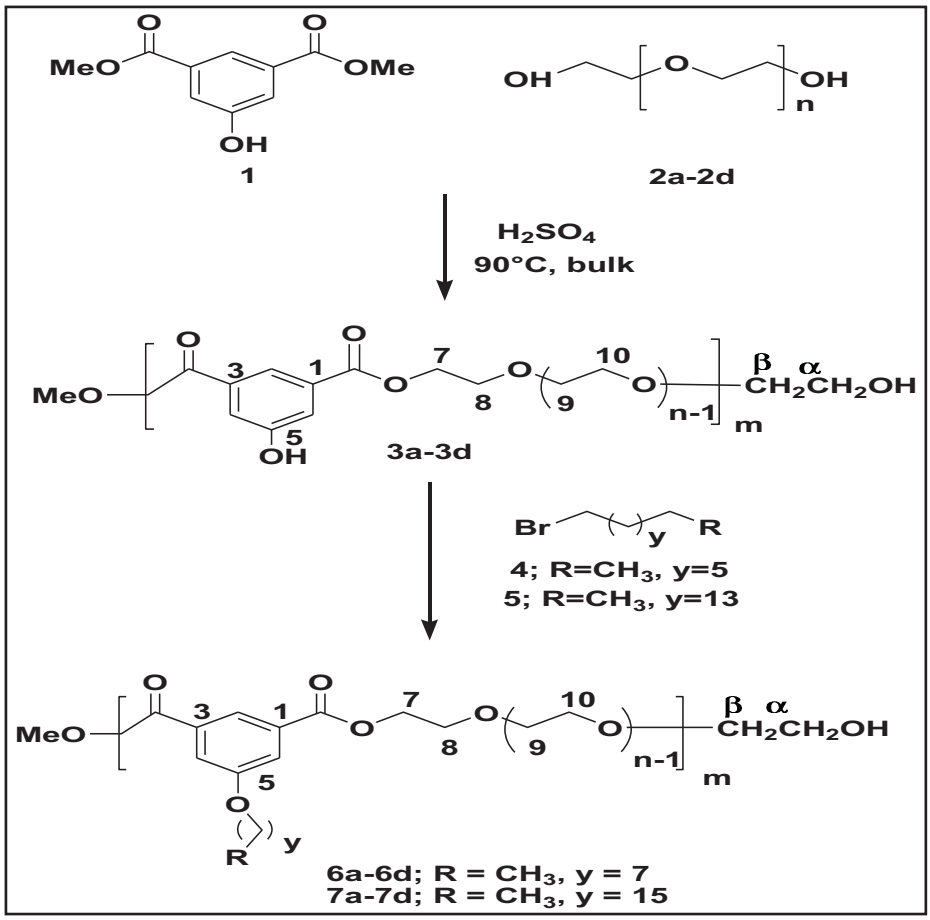
The solubility of the synthesized copolymers and Mancozeb was checked in different solvents. Dichloromethane was selected due to its high volatility. The amphiphilic polymers(1g) and Mancozeb (0.20 g, 99% purity) were dissolved in dichloromethane separately (in 1:5 a.i./polymer w/w ratio) and mixed together in round bottom flask at room temperature. In a typical procedure for encapsulation, the solution was stirred for 3 h. After removal of the solvent, the residue was dissolved in water and left on stirring for the formation of nanomicelles. In this step, Mancozeb gets encapsulated in the amphiphilic polymer and un-encapsulated/non-incorporated Mancozeb precipitates out of water. The non-incorporated pesticide was separated from the aqueous layer by filtration and encapsulation efficiency also reported in previous publication.[27] The filtrate was freeze dried and lyophilized to get the encapsulated material.[25]

Field bio-efficacy of mancozeb nanoformulations

Experimental details

The present investigation was carried out in the net house of Division of Plant Pathology, ICAR-Indian Agricultural Research Institute(IARI), New Delhi.

In general, it is believed that nano pesticides tend to show enhanced bio efficacy as compared to conventional formulations. Therefore, in order to evaluate for any increase in the bio-efficacy, the developed nanoformulations of Mancozeb were tested for their bio-efficacy in tomato fields against early blight of tomato. The efficacy of the nanoformulations and its commercial formulations 42SC and 75WP were evaluated on transplanted tomato at the farm of ICAR- IARI, New Delhi during the summer season 2016. Because of no clear-cut guidelines about nanoformulations, the entire experiment was conducted in controlled conditions. The experimental plot was sealed with two layers of high-density polyethylene sheets from 2 m below up to the bunds in order to eliminate any chances of leaching or percolation of nanoformulations. Soil of the experimental



Scheme 1. General method for the synthesis of amphiphilic copolymers **6a-6d** and **7a-7d**.

farm was alluvial (type ustochrepts; order inceptisol) in origin having sandy loam texture with 0.48% organic carbon and pH 7.4. During the experiment Urea, Single super phosphate and Muriate of Potash were applied 120, 60 and 40 Kg/ha respectively. Half dose of nitrogen and full dose of phosphorous and potassium were applied at the time of transplanting while the remaining nitrogen was top-dressed in two equal halves at 30 and 60 days after transplantation (DAT).

In order to compare the bio-efficacy of nanoformulations with the commercial formulations, the spraying of Mancozeb was done in different plots according to randomized block design (RBD) with three replications of each treatment, plot size was 3 m x 2.5 m, variety of the tomato plant was Pusa Rohini and spacing provided were 60 cm x 45 cm. Nanoformulations and commercial formulations were applied at 50 mg L^{-1} and 100 mg L^{-1} doses. Since the application of Mancozeb was performed as foliar spray, weather forecast was checked in order to avoid any chances of wash out of pesticide from canopy due to rain. Spraying operation was performed in the evening.

After 3 days of pesticides spraying, fungus (*A. solani*) were artificially inoculated.

Inoculation of tomato plants with pathogen (A. solani)

The fungus was on Potato dextrose agar (PDA) for sporulation for 7 days at 25 °C. Spore suspension was made in sterile water which was blackish in color and presence of spores was confirmed under microscope and quantified (10^4 spores/mL). Plants were uniformly sprayed with the prepared spore suspension and spray volume was used 100 mL per plant. After spraying, plants were covered with perforated plastic bags, whose internal surface was sprayed with water. Since temperature was on the lower side for infection, plants were kept in glasshouse and temperature was elevated. The symptoms appeared, within 2-3 days.

For spraying of pesticide formulations, high pressure Knapsack sprayer of 16 L capacity and automatic agitation was used. The average working pressure of sprayer was kept at 300 Kpa while the discharge rate was kept at 500 mL/min. Doses of developed formulations (50 and 100 mg L-1) were decided based on *in vitro* bioassay (ED_{50} values). Commercial formulation was applied at recommended dose. Blank (control) samples corresponded to tomato plants inoculated with *A. solani*, but not treated with fungicide.

Recording of observation

Data on the infected plants were recorded after one week of inoculation and at the time of harvesting. Observations were taken in terms of number of infected leaflet and number of

fruits per plant. Time from inoculation to harvesting was around 21 days.

Statistical analysis of data, obtained from pot experiment, was carried out using statistical software R Package.

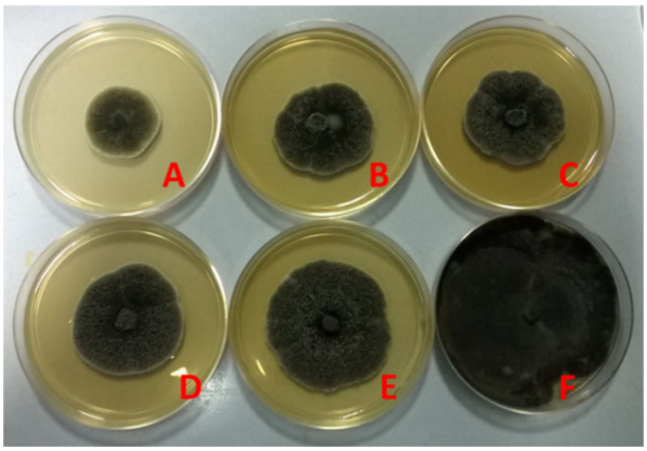


Figure 1. Fungicidal activity of developed formulations (**A2**) against *A. solani*. A = 25 mg L^{-1}, B = 12.5 mg L^{-1}, C = 6.25 mg L^{-1}, D = 3.125 mg L^{-1}, E = 1.562 mg L^{-1} and F = control

Results and discussion

We had reported the *in vitro* antifungal evaluation of developed formulations against *A. Solani* (Fig. 1). The results showed that the formulations, hexadecyl derivative of amphiphilic polymer with PEG-600 (A2), octyl derivative of amphiphilic polymer with PEG-1000 (A3), octyl derivative of amphiphilic polymer with PEG-1500 (A5), hexadecyl derivative of amphiphilic polymer with PEG-1500 (A6), octyl derivative of amphiphilic polymer with PEG-2000 (A7) and hexadecyl derivative of amphiphilic polymer with PEG-2000 (A8) (Composition of the nanoformulations are also

Table 1. Composition of the nanoformulations.

	Polymers	
Formulations	PEG block	Alkyl chain
A1	PEG-600	Octyl
A2	PEG-600	Hexadecyl
A3	PEG-1000	Octyl
A4	PEG-1000	Hexadecyl
A5	PEG-1500	Octyl
A6	PEG-1500	Hexadecyl
A7	PEG-2000	Octyl
A8	PEG-2000	Hexadecyl

Figure 2. Infected and nanoformulation treated tomato plants.

Table 2. Number of infected leaflets per plant in field study at 50 mg L^{-1} and 100 mg L^{-1}.

Treatments	Number of infected leaflets at 50 mg L^{-1}	Number of infected leaflets at 100 mg L^{-1}
A1	$4.70^{bc} \pm 0.141$	$4.00^{bc} \pm 0.141$
A2	$2.40^{f} \pm 0.283$	$2.10^{f} \pm 0.283$
A3	$4.60^{bc} \pm 0.283$	$4.10^{bc} \pm 0.283$
A4	$3.60^{de} \pm 0.283$	$3.60^{de} \pm 0.283$
A5	$2.70^{f} \pm 0.141$	$2.40^{f} \pm 0.141$
A6	$3.00^{ef} \pm 0.424$	$2.60^{ef} \pm 0.282$
A7	$3.60^{de} \pm 0.566$	$3.50^{de} \pm 0.566$
A8	$3.90^{cd} \pm 0.424$	$3.50^{cd} \pm 0.424$
SC	$6.00^{b} \pm 0.565$	$5.50^{b} \pm 0.565$
WP	$6.10^{b} \pm 0.424$	$5.70^{b} \pm 0.424$
Blank (Control)	$10.20^{a} \pm 0.282$	$10.30^{a} \pm 0.283$

Note. Means with the same letter are not significantly different.

Table 3. Number of fruits per plant in field study at 50 mg L^{-1} and 100 mg L^{-1}.

Treatments	Number of fruits per plant at 50 mg L^{-1}	Number of fruits per plant at 100 mg L^{-1}
A1	$6.40^{de} \pm 0.289$	$6.70^{de} \pm 0.289$
A2	$9.00^{a} \pm 0.848$	$9.10^{a} \pm 0.848$
A3	$6.00^{e} \pm 0.283$	$6.30^{e} \pm 0.141$
A4	$6.40^{de} \pm 0.565$	$6.30^{de} \pm 0.565$
A5	$8.00^{b} \pm 0.283$	$8.20^{b} \pm 0.283$
A6	$7.00^{cd} \pm 0.283$	$7.50^{cd} \pm 0.283$
A7	$6.90^{cd} \pm 0.424$	$7.10^{cd} \pm 0.424$
A8	$7.30^{bc} \pm 0.141$	$7.40^{bc} \pm 0.141$
SC	$5.70^{e} \pm 0.141$	$6.00^{e} \pm 0.141$
WP	$5.60^{e} \pm 0.283$	$5.80^{e} \pm 0.283$
Blank (Control)	$2.00^{f} \pm 0.141$	$2.00^{f} \pm 0.283$

Note. Means with the same letter are not significantly different

given in Table 1) were found to be the most active against *A. solani* with Effective Dose for 50% mortality (ED_{50}) values 1.72, 1.31, 1.87, 1.67, 1.45 and 2.08 mg L^{-1} respectively, which were at par when compared with the commercial formulations.[27] However, owing to their slow release nature, we evaluated these products, along with the commercial formulations, in pot culture and field experiment to check their performance *vis-a-vis* commercial products.

Antifungal bioassay against early blight of tomato

Assessment of early blight symptom

The symptoms appeared on leaves, stems and fruit after inoculation of pathogen. The development of disease was first developed on the lower and older foliage of the plant canopy and then progressed upward, especially during the period of fruit development. Initial symptoms were small, dark-colored, circular lesions, often surrounded by chlorotic (yellow) tissue. As the lesions expand, concentric rings or ridges became evident within them. The disease was more damaging on the foliage (where it caused lesions and premature defoliation) than on stems and fruit. Lesions on stems were oval or elongated, brown and sunken with concentric rings. These lesions eventually girdled and killed the affected stems. Fruits infected at maturity was seen to develop sunken lesions, dark-colored circular spots with concentric rings.

Evaluation of formulations

As evidenced from our previous work on *in vitro* evaluation of developed formulations, which showed that developed formulations offer slow release of Mancozeb, experiment was conducted for evaluation of persistency or efficacy for longer duration, against early blight of tomato.

Field efficacy against early blight of tomato

The formulations were further tested in field experiment against the disease at 50 mg L^{-1} and 100 mg L^{-1} doses at the rate of 600 L/ha. The number of infected leaflets and number of fruits were recorded seven days after spraying and at the time of harvesting. Results of the study showed that formulations could effectively control the early blight infection on tomato as compared to commercial formulations and untreated control (Fig. 2). Formulations A2, A5 and A6 were more effective as there was a reduction in number of infected leaves with significant difference ($P < 0.05$) (Table 2). Formulation A2 was the most effective as number of fruits were significantly higher than other treatments (Tables 2 and 3).

Based on the field performance of nanoformulations (at 50 mg L^{-1}), a relationship was observed as:

Number of fruits per plant= 9.936-0.768 x (number of infected leaflets per plant) with R^2=0.806, indicated that use of these formulations could increase about 75% fruit yield over the control (non-spray application).[28]

In our previous report, we have found that the Mancozeb nano formulations showed excellent *in vitro* antifungal activity against *A. solani* and ED_{50} values varied from 1.31 to 2.79 mg L^{-1}, which was at par when compared with the commercial formulations and the developed formulations offer slow release of Mancozeb and period of optimum availability (POA) was up to 35 days[27] which may have longer duration of efficacy as compared to commercial formulations. Therefore, developed nano formulations were

further evaluated in pot culture against the fungal disease, early blight in tomato. Formulation of the chemical offered dose reduction and was found to increase efficacy for a longer period as nano formulations were found to be more active than commercial formulations. Efficacy of the developed formulations for a longer duration has been due to slow release of the formulation. In the present study through field observation, it was observed that a single application of 60 g a.i./ha was effective in comparison to its commercial formulation where 650 g a.i./ha is recommended. Therefore, there has been almost 10 times reduction of chemical use and cost. Previous reports on controlled release of nano formulations for acephate against mustard aphids,[10] carbofuran and imidacloprid against potato aphid[3] supported the fact that slow release of Mancozeb is imparted through encapsulation by polymers.

The variation of the Mancozeb release from matrix at different time is due to rate of degradation of matrix at different time. The fast rate of degradation observed during 25–60 days could be due to complete release of Mancozeb from the developed nanoformulations.[27]

The rate of release of Mancozeb in water was found to be faster in commercial formulations (75WP and 42SC) than the developed formulations as it showed maximum release within 15 days. This could be due to large entrapping of the chemical inside the developed formulations over commercial one and also reported nanoformulations were more effective than commercial formulations.[27] In case of developed formulations, the micelles, formed by the aggregation of amphiphilic polymers, entrap the active ingredient and protect it from environmental and microbial degradation making active ingredient release optimum and for a longer duration than the commercial formulation. Similar results have been reported for carbofuran, β-cyfluthrin, imidacloprid, thiram and carbendazim.[11,24–26,29]

Nanomaterial based pesticide formulations have been reported to have better environmental stability, controlled release, targeted activity and physical stability. These materials can potentially protect the active ingredients from premature degradation such as volatilization, photolysis, rapid evaporation, etc.[1]

The slow releasing properties of the developed formulations might be helpful as effective means to prevent any losses from drainage or run off. Such protection mechanisms reduce the exposure of pesticides to the environment and retain their activity for a longer period of time. The requirement of pesticides for repeated and indiscriminate use can also be reduced.[11]

Conclusion

We have found that A2 formulation was superior among all the developed nano formulations of Mancozeb for management of early blight in tomato. Encapsulation of Mancozeb in nano micelles was achieved and explored for targeted delivery of Mancozeb for efficient pest control. These formulations can be used to optimize the release of Mancozeb to achieve disease control for the desired period depending upon the matrix of the polymer used. Also, the developed CR formulations may help in reducing the doses of Mancozeb application owing to its slow release nature. A single application of the formulation can be manipulated for disease control during the whole growth span of the crop. Noticeably, maximum amount of the active ingredient remains available for a reasonable period of time after application. Nano material-based pesticides with their controlled release behaviour have better pest control efficacy and can reduce environmental risk and thereby can provide an eco-friendly way for pest management.

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