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Research Article
Antiviral Activity of Curcumin Loaded Milk Proteins Nanoparticles on Potato Virus Y

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Abstract

Background and Objective: Potato is one of the world's leading vegetable crops. Potato viral diseases cause adversely effects on the agricultural sector. Recently there is a growing interest to control plant viruses using spices and herbs (including curcumin). Poor solubility of curcumin in water limited its applications. Therefore, the main objective of the present study was to evaluate the effect of antiviral activity of curcumin-milk proteins nanoparticles against potato virus Y (PVY). **Materials and Methods:** Curcumin-milk proteins nanoparticles were prepared via ionic gelation method. The antiviral activity of the resultant nanoparticles against PVY was evaluated at different concentrations (500, 1000 and 1500 mg/100 mL). Chlorophyll content as well as the activity of peroxidase (POX) and polyphenol oxidase (PPO) was examined. **Results:** Curcumin-milk proteins nanoparticles showed inhibitory effect on PVY in a concentration dependent manner. **Conclusion:** Curcumin-milk proteins nanoparticles displayed a successful tool to control the PVY under green house conditions.

Key words: Curcumin, milk proteins, nanoparticles, potato, potato virus Y

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the most cultivated crop worldwide[1]. In Egypt, potato has a significant position among all vegetable crops, where about 20% of the total area devoted for vegetable production is cultivated with potato[2]. Egypt is the 15th largest potato producer in the world, producing[3] 4,325,480,00 t in 2017. Therefore, infection of potato with numerous pathogens causes negative effects on the Egyptian quantitative and qualitative potato yield[4].

Plant viral diseases represent the second largest plant diseases after plant fungal diseases, leading to crop loss and great damage to agricultural industry[5]. Potato virus Y (PVY) is one of the most common and dangerous potato viruses that infecting potato worldwide leading to rejection of seed lots from certification programs[6,7]. Losses from this viral disease reach about 10-80% depending on the variety as well as its effects on the product quality[8]. Recently, biopesticides have gained more attention and exhibited great development potential. Many researches focused on developing and assessment of new biogenic plant antiviral materials including proteins, polysaccharides and small molecules such as alkaloids, flavonoids, phenols and essential oils from various sources[9].

Milk proteins, especially whey proteins, have showed a good potential as antibacterial and antiviral modifiers[10,11]. Lactoferrin, for instance, has been studied extensively for its antiviral properties[12] and its antiviral activity against potato virus X was reported by Taha *et al.*[13]. Also esterified whey proteins had antiviral activity against tomato curl viruses[14].

Chitosan is a nontoxic biodegradable polysaccharide that possesses various biological activities, including antibacterial, antiviral, antitumor and anticholesterol, therefore it is wide used in medicine and pharmacy[15]. Recently, researches have focused on the ability of chitosan to increase plant resistance to viruses[16,17]. It was reported its suppressive effect on many plant viruses[18,19].

Curcumin, is a natural compound derived from turmeric (*Curcuma longa*), widely used as spice and coloring agent in food[20] and displays many pharmacological activities[21]. Recently, curcumin extract was used to control alternaria solani in tomato[22]. Despite its bioactivities, its application is limited due to its poor solubility in water and limited absorption[23]. Therefore, many recent attempts were carried out to overcome these problems using different kinds of food-grade polymer materials[24-26]. It is worth noting that few studies have been carried out on curcumin to control virus populations. Therefore the aim of the present study was to develop and evaluate a new series of curcumin -milk proteins nanoparticles to control the PVY.

MATERIALS AND METHODS

Materials: Sodium caseinate (SC) was obtained from Friesland Campina DMV, Nederland. Whey protein concentrate (WPC 80%) was kindly attained from Onalaska Wisconsin, USA. Whey protein isolate (WPI), α-lactalbumin (α-La 97.46% protein) and β-lactoglobulin (β-lg 97.8% protein) were kindly provided by Davisco Foods International, USA. Chitosan (Cs) of low molecular weight of 100,000-300,000 and sodium tripolyphosphate (TPP) were purchased from Acros Organics, New Jersey, USA. Curcumin (Cur) (≥96.1% purity) was obtained from Kolorjet Chemicals Pvt Ltd., Mumbai, Maharashtra, India. Potato tubers cultivar Spunta was obtained from Brown Root project (Ministry of Agr. Egypt). Potato virus (PVY) was obtained from Virus and Phytoplasma Department, Plant Pathology Research Institute, Agricultural Research Center, Giza. ELISA Kits were purchased from LOEWE Biochemica, GmbH, DSMZ (Germany). All other chemicals were of analytical grade. This study was done between December, 2016 to April, 2018.

Methods

Preparation of chitosan/milk protein nanocomposite and curumin-loaded chitosan/milk proteins nanoparticles (NPs): Nanocomposites of milk proteins-chitosan were prepared by ionotropic gelation method according to Calvo *et al.*[27] with minor modification. Briefly, 1 mL of protein solution (10%) was added to 50 mL chitosan solution (0.2% prepared in 1% acetic acid) under mild stirring. Then, aqueous sodium tripolyphosphate solution (TPP) (0.5 %) was added drop wise to the mixture. The overall mixture was stirred for 30 min at 25°C. The resulting NPs were collected by centrifugation at 25,000 rpm for 30 min at 4°C and washed twice by distilled water and then freeze dried.

About 100 mg of curcumin was dissolved in absolute ethanol till the solution became clear, then added to the previously prepared fresh chitosan/protein nanosuspension and stirred for 30 min at 25°C, followed by centrifugation at 25,000 rpm for 30 min at 4°C. The resulted curcumin-loaded NPs were washed twice with distilled water and then freeze dried and kept at room temperature.

Characterizations of nanoparticles: Particle size, zeta potential, fourier-transform infrared spectroscopy (FTIR) spectra of tested materials also entrapment efficiency and the curcumin release percentage from the nanoparticles were measured as performed by Ali *et al.*[28].

Preparation of virus inocula: *Nicotiana* (N) *tabacum* cv. white barley was used as source plants of the virus infections.

Virus inocula were prepared by crushing infected leaves with distilled water (1:1 w/v) in a sterilized mortar. Sap extract was squeezed through 2 layers of muslin cloth and the solution was centrifuged at 5000 g for 10 min. (centrifuge 2K15, Sigma Chemical Co., Osterode, Germany). The clear supernatant was used as virus inoculum.

Antiviral activity: The green house experiments, isolation and detection of the virus were carried out at Virus and Phytoplasma Department, Plant Pathology Research Institute, Agricultural Research Center, Giza from August, 2017 to April, 2018. Potato tubers cultivar Spunta were planted in sterilized soil potting of 30 cm plastic pots (one tuber per pot) under usual and propitious conditions for potato plants. After one month of growth, plants were divided into 14 groups, each group containing 12 plants, 1 of them was healthy (negative control) and others (positive control and treated plants) were mechanically infected with the virus. Three concentrations (500, 1000 and 1500 mg/100 mL) of chitosan, curcumin, native proteins and curcumin loaded milk proteins-chitosan nanoparticles were examined for their antiviral activity. The infected plants were treated by spraying 20 mL of the tested solutions/plant after 7 and 14 days of infections. Two weeks later, leaf samples from each pot were collected and examined by DAS-ELISA and absorption was measured at 405 nm using ELX800 ELISA reader, Bio-TekInstruments, USA. The inhibition (%) was calculated by the formula according to Baranwal and Verma[29].

$$\text{Inhibition (\%)} = \frac{C - T}{C} \times 100$$

Where:

C = Number of healthy plants

T = Number of infected plants inoculated by virus mixed with tested solutions

Preparation of enzyme extract: Leaves of plants inoculated with PVY and sprayed with tested solutions were used to measure the oxidative enzymes activities. About 10 g of fresh plant leaves of each treatment were blended with 20 mL phosphate buffer solution (7.0 pH) and centrifuged at 4000 rpm for 20 min/4°C (centrifuge 2K15, Sigma Chemical Co., Osterode, Germany). Clear supernatants were used as crude enzymes to determine the activities of peroxidase and polyphenol oxidase according to Anand *et al.*[30].

Determination of peroxidase activity (POX): Peroxidase (POX) activity was assayed spectrophotometrically according to Kar and Mishra[31] by measuring the oxidation of pyrogallol to pyrogallin in the presence of H_2O_2. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at 25°C for 5min. then the reaction was stopped by adding 0.5 mL of 5% (v/v) H_2SO_4. The amount of pyrogallin formed was measured at 420 nm.

Determination of polyphenol oxidase activity (PPO): Polyphenol oxidase (PPO) activity was determined following the method of Mayer *et al.*[32]. The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µL of the enzyme extract. To start the reaction, 200 µL of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm.

Serological detection: Enzyme linked immune-sorbent assay (DAS-ELISA) was conducted to test the inhibitory effect of the tested samples according to the protocols described by Clark and Adams[33] using Microtitre plate wells coated with the antigen-specific coating-antibody (IgG). About 200 µL/well of (IgG) was diluted in coating buffer at 1:200 and incubated at 37°C for 4 h, according to the supplier's specifications then remove the reagent from the wells and wash the plates 4 times using the washing buffer (PBST). About 200 µL of the plant sample sap (prepared at a 1:20 dilution in sampling buffer) as well as negative and positive control. The plate was incubated at 4°C overnight. Plates rewashed 4 times as mentioned before. About 200 µL of antibody-AP-conjugate diluted (1:200) from original vial in conjugate buffer were added to each well and incubated at 37°C for 4 h. The plate was washed 4 times. About 200 µL of freshly prepared substrate (1 mg mL^{-1} 4-nitro phenyl phosphate) was dissolved in substrate buffer were added to each well and the plates were incubated at room temperature for 1-2 h. To stop the reaction, 50 µL of 3 M NaOH to each well were added. The alkaline phosphatase (AP) reacts with the substrate 4-nitrophenyl phosphate in an enzymatic reaction, resulting in yellow colored 4-nitrophenol as product. This color formed can be evaluated visually or measured in a spectrophotometer at 405 nm.

Total chlorophyll content: The SPAD-502 chlorophyll meter was used to determine the total chlorophyll in potato leaves according to Ali *et al.*[34]. The chlorophyll content was expressed as SPAD value[35].

Statistical analysis: A randomized complete block design with 2 factors (factor A: conc. and factor B: treatments) was

employed for analysis of chlorophyll content, peroxidase and polyphenol oxidase. The remind parameters used randomized complete block design with one factor with three replications for each parameter. The treatment means were compared by least significant difference (L.S.D.) test as given by Snedecor and Cochran[36] using Assistat program, while the standard deviation (SD) was calculated by using excel program.

RESULTS AND DISCUSSION

Characterizations of nanoparticles: The average particle sizes of milk proteins-chitosan nanocomposites ranged from 275.33-334.90 nm. The average particles sizes of casein, α-La and β-Lg nanoparticles (278.10, 290.83 and 274.80 nm, respectively) decreased as a results of loading curcumin while, it increased in case of WPI and WPC nanoparticles (462.80 and 439.90 nm, respectively). All nanoparticles either without or with curcumin are in nano scale. Zeta potential values ranged from -11.13 to +17.30. In case of curcumin loaded milk proteins-chitosan nanoparticles, the zeta potential ranged from -12.63 to +27.73. The FTIR results depict that the NPs were formed due to the interaction between the carboxyl group (-COO-) of protein and amino groups of chitosan. In addition, there was an interaction between chitosan protein NPs and curcumin, they gave the same spectra for curcumin chitosan protein NPs with slight shifting, which confirms that curcumin was loaded in the nanoparticles (data not shown).

The entrapment efficiency (EE %) of curcumin in the suspended protein nanoparticles ranged from 72.27-77.27% (data not shown).

The curcumin release (%) increased gradually in all treatments as the experiment time increased. It reached 96.33-97.83% after 48 h. which indicate that all the used milk proteins can be successfully used as carrier for curcumin (data not shown).

Inhibitory effect (%) of curcumin-loaded milk proteins nanoparticles: Data shown in Fig. 1 depict the external symptoms of PVY of infected plants including leaf crinkle and deformation while in treated plants, the symptoms disappeared to be almost the same as healthy plants. Data in Table 1 show the inhibitory effect (%) of the tested materials estimated by ELISA test. Generally, all the tested materials exhibited antiviral activity against PVY and this effect was concentrations dependent. The antiviral activity of curcumin was more pronounced than chitosan. The native WPC and β-lg showed higher antiviral effect compared to other milk proteins. All of the curcumin loaded nanoparticles form showed higher antiviral activity compared to the native form. It is also observable that the concentration 1500 mg/100 mL led to fully inhibition of the virus especially the nanoparticles of WPC, WPI and β-lg followed by α-la and casein. Also the inhibitory effect of the nanoparticles in most cases is higher than that of curcumin and chitosan alone. The antiviral effect of chitosan can be due to its ability to suppress viral infections by its effect on the plant. Chitosan enhances the plant resistance to infection and induce a broad spectrum of defense responses[37]. Also it was reported that chitosan inhibited the systemic propagation of viruses and viroids throughout the plant and enhancement of the host's hypersensitive response to infection[38]. Moreover, chitosan stimulates the plant immune response[39,40]. Although, there are many studies on the effect chitosan on plant virus diseases but there are no studies on the effect of curcumin on plant viruses. Many studies dealt with its effect on animal viruses or human viruses. Also it was reported that milk proteins may saturate or interact with the binding of viral DNA or RNA to inhibit viral replication process causing reducing infection of tobacco mosaic virus in pepper, tomato and tobacco[14].

Table 1: Antiviral activity of native milk proteins and curcumin loaded milk proteins-chitosan nanoparticles

	Infected			Healthy			Inhibition (%)		
	---------- (mg mL^{-1}) ----------			---------- (mg mL^{-1}) ----------			---------- (mg mL^{-1}) ----------		
Treatments	500	1000	1500	500	1000	1500	500	1000	1500
Control (-)	-	-	-	12	12	12	-	-	-
Control (+)	12	12	12	0	0	0	0.0	0.0	0.0
Chitosan	10	8	7	2	4	5	16.7	33.3	41.7
Curcumin	9	7	6	3	5	6	25.0	41.7	50.0
SC Native	11	9	7	1	3	5	8.33	25.0	41.7
Cur-Cs/SCNPs	5	4	3	7	8	9	58.3	66.7	75.0
α-La Native	10	8	6	2	4	6	16.6	33.3	50.0
Cur-Cs/α-LaNPs	6	3	2	6	9	10	50. 0	75.0	83.3
β-lg Native	9	7	5	3	5	7	25.0	41.7	58.3
Cur-Cs/ βlgNPs	5	4	0	7	8	12	58.3	66.7	100.0
WPI Native	8	7	6	4	5	6	33.3	41.7	50.0
Cur-Cs/WPINPs	3	2	0	9	10	12	75.0	83.3	100.0
WPC	8	6	4	4	6	8	33.3	50.0	66.7
Cur-Cs/WPINPs	6	2	0	6	10	12	50.0	83.3	100.0

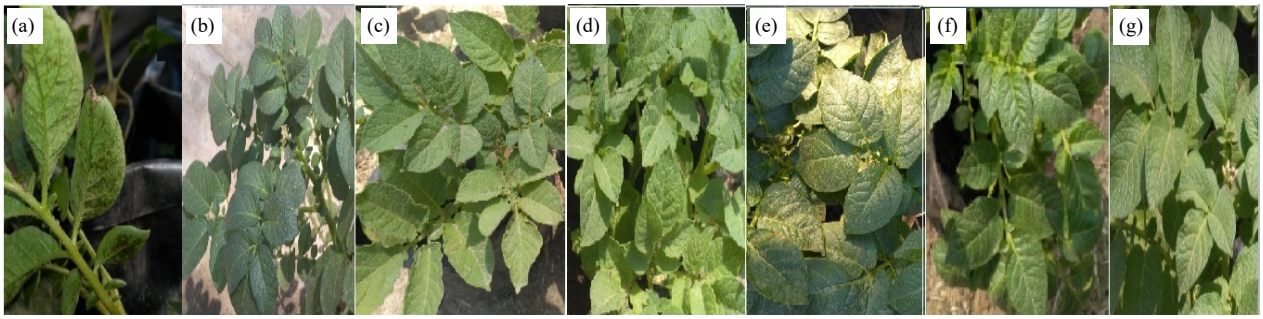

Fig. 1(a-g): Symptoms of PVY (a) Infected potato plants, (b) Healthy potato plants, (c) Cur-SCNPs, (d) Cur-α-LaNPs, (e) Cur-β-lgNPs, (f) Cur-WPINPs and (g) Cur-WPCNPs

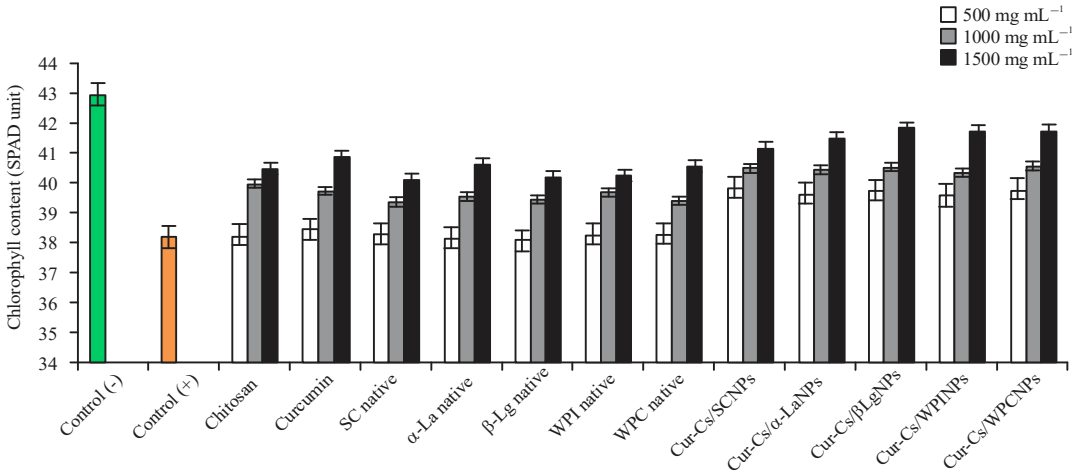

Fig. 2: Chlorophyll content (SPAD Unit) in potato plants infected by (PVY) as affected by native milk proteins and its curcumin nanoparticles

Chlorophyll content: Chlorophyll is a green molecule present in plant cells responsible for greenness of leaves and indicates the nitrogen status in the plant[41]. Leaf colour gives a good indication of chlorophyll content of leaves[42] and used as measure for chloroplast development, photosynthetic capacity, leaf nitrogen content or general plant health[43]. Chlorophyll content is affected by many factors such as plant genotype, nutrient, leaf thickness or biotic stresses like diseases. The SPAD 502 was designed to detect the chlorophyll content of leaves and used as an indirect indicator of plant N status[41].

As shown in Fig. 2 the negative control had high chlorophyll content (42.97), while the chlorophyll content in the positive control was reduced to 38.20. It is observable that both of chitosan and curcumin enhanced the chlorophyll content with non-significant differences and this effect is concentration dependent. Also nanoparticles had higher and significant differences as compared to native form. From these results, it can be concluded that by increasing the concentrations of the tested materials, the content of chlorophyll changed to be almost closed to the negative control readings, which proves the efficacy of tested materials to eliminate the undesirable effects of the virus. The obtained results declare that all curcumin/chitosan/milk proteins nanoparticles enhance the chlorophyll formation in infected plants which is in accordance with Zong *et al.*[44], who reported that chitosan increase the chlorophyll content in Rape in the presence of cadmium.

Oxidative peroxidase (POX) and polyphenol oxidase (PPO): Many defense enzymes are involved in defense reaction against plant pathogens including oxidative peroxidase (POX) and polyphenol oxidase (PPO), which catalyzes the formation of lignin and other oxidative phenols contributing to the formation of defense barriers for reinforcing the cell structure[45]. The antioxidant enzymes PPO and POX are the

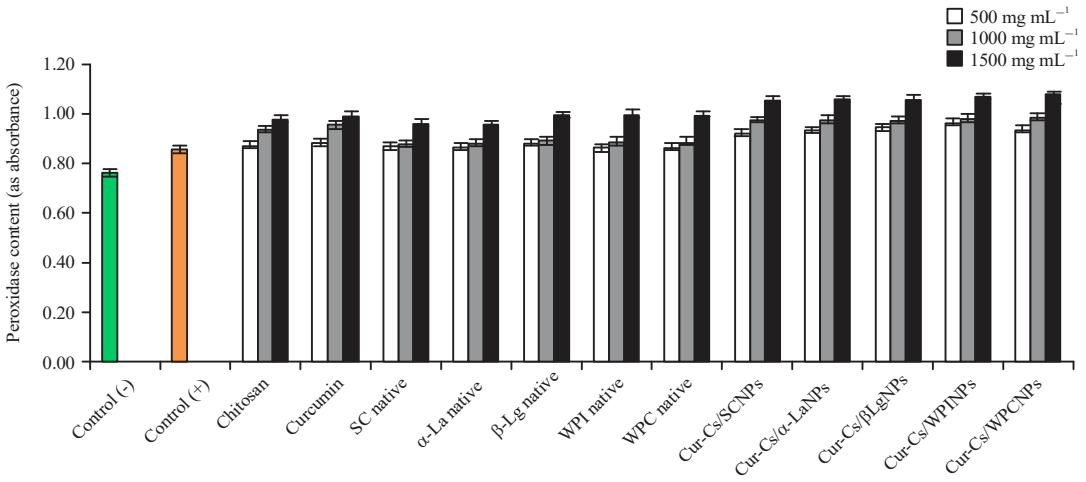

Fig. 3: Peroxidase content in potato plants infected by PVY as affected by native milk proteins and its curcumin nanoparticles

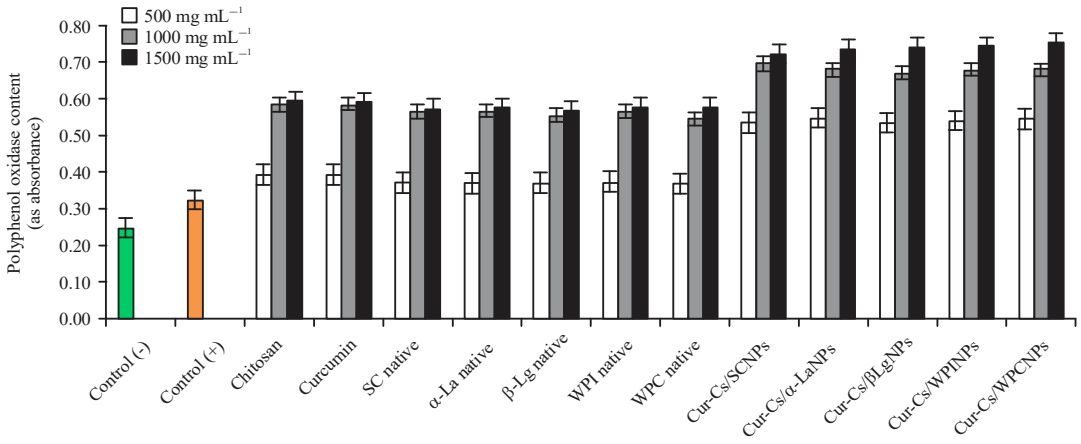

Fig. 4: Polyphenol oxidase content in potato plants infected by PVY as affected by native milk proteins and its curcumin nanoparticles

indication for detoxifying ROS synthesized during stress response[46,47]. Plant can synthesize and accumulate phenolic compounds in response to stress[48]. The antioxidant activity of phenolic compounds can play an important role in neutralizing ROS[48,49]. In agriculture, chitosan is widely used to mimic plant diseases. It has been used as a bio-fungicide, bactericide and virucide, which stimulate the plant defense system against the pathogen and induces the immune system of plants[50,51].

Data in Fig. 3 and 4 show POX and PPO activities (as absorbance) in healthy and infected plants. It was 0.760 and 0.250 for healthy plants and 0.850 and 0.320 for infected plants. An increase in enzymatic activity of both enzymes as a result of PVY infection was observed, compared with healthy plants. Non-significant differences were observed between the effect of both of chitosan and curcumin on the oxidative enzymes. It is worth to be mentioned that the values of peroxidase enzyme (POX) and the polyphenol oxidase (PPO) were increased significantly by increasing the concentration of both of native form or the curcumin loaded nanoparticles. It is also observable that all curcumin nanoparticles had significant effect on the determined enzymes as compared to chitosan, curcumin and the native milk proteins.

Chirkov *et al.*[52] reported that treatment of potato plants infected with potato virus X (PVX) with chitosan showed resistance to PVX virus. Also, tomato plants treated with chitosan showed resistance to tomato mosaic virus and improved vegetative growth[53]. Similarly, chitosan in formulation with plant growth promoting rhizobacteria (PGRP) conferred resistance to leaf curl virus in tomato plant[54]. Also, chitosan was found effective against squash mosaic virus (SMV)[55,56]. Furthermore, Sharif *et al.*[51] suggested that this effect

might be due to some peculiar properties of the host plant which initiate the antiviral reactions after chitosan treatment. Chandra *et al.*[57] demonstrated that chitosan nanoparticles produce significantly higher defense response in plants compared to natural chitosan and increased activity of the defense enzymes. Although many researches were conducted on the effect of chitosan or chitosan nanoparticle on the oxidative enzymes in plants, no available data were obtained about effect of curcumin or the tested milk proteins on plant viruses.

CONCLUSION

The obtained results revealed that all curcumin-milk proteins nanoparticles at the level of 1500 mg mL^{-1} especially of WPC, WPI and β-Lg followed by α-La and lastly casein displayed a successful tool to control the potato viral infection (PVY) under greenhouse conditions.

SIGNIFICANT STATEMENT

This study elucidate the significance of application of nanotechnology in controlling plant virus diseases using promising natural components (milk proteins, chitosan and curcumin). These results will consider as a pioneer data which will help other researchers to continue in this new field.

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