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Chitosan nanoparticles improve yield, enzymatic activity, and bioactive compounds in tomato fruits
Nanopartículas de quitosán mejoran el rendimiento, actividad enzimática y compuestos bioactivos en frutos de tomate

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SUMMARY

Chitosan nanoparticles (NPsCS) are used as natural biostimulants in sustainable agriculture since they increase crop productivity and induce the synthesis of enzymatic and non-enzymatic antioxidants, protecting the plant from stress. The present study was developed to determine the effect of foliar application of NPsCS on yield, enzymatic activity, and content of bioactive compounds in tomato fruits. The trial was established completely randomized design with six escalating doses of NPsCS 0, 0.05, 0.1, 0.2, 0.4, and 0.8 mg mL^{-1}. The foliar spray of 0.2 mg mL^{-1} increases the fruits' yield, size, and firmness. High doses increase bioactive compounds and enzymatic activity. The foliar-applied NPsCS has excellent potential to be used as biostimulants to improve performance and obtain functional foods.

Index words: *antioxidant enzymes, biostimulant, Solanum lycopersicum* L.

RESUMEN

Las nanopartículas de quitosán (NPsCS) son utilizadas como bioestimulantes naturales en la agricultura sustentable, ya que incrementan la productividad de los cultivos e inducen la síntesis de antioxidantes enzimáticos y no enzimáticos, protegiendo a la planta del estrés. El presente estudio se desarrolló con el objetivo de determinar el efecto de la aplicación foliar de las NPsCS sobre el rendimiento, actividad enzimática y contenido de compuestos bioactivos en frutos de tomate. El ensayo se estableció en un diseño completamente al azar con seis dosis crecientes de NPsCS: 0, 0.05, 0.1, 0.2, 0.4 y 0.8 mg mL^{-1}. La aspersión foliar de 0.2 mg mL^{-1} aumentó el rendimiento, tamaño y firmeza de los frutos; en cambio dosis alta incrementan los compuestos bioactivos y la actividad enzimática. El uso de NPsCS aplicadas de forma foliar presentan un gran potencial para utilizarse como bioestimulantes para mejorar el rendimiento y obtener alimentos funcionales.

Palabras clave: *antioxidantes, bioestimulante, Solanum lycopersicum* L.

INTRODUCTION

The use of nanotechnology improves agricultural productivity by increasing the yield and nutritional quality of crops, as well as providing more excellent protection to the environment (Gondal and Tayyiba, 2022; Khairy *et al*., 2022). However, using metal nanoparticles can cause undesirable effects (Rizwan *et al*., 2017), as many of

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them are highly toxic and pollute the environment by harming the life of organisms (Vodyashkin, Kezimana, Vetcher and Stanishevskiy, 2022). Due to the above, there is a growing interest in ecologically friendly nanocomposites (Lira-Saldivar, Méndez, De los Santos and Vera, 2018).

In this regard, chitosan (poly β-(1,4)-N-acetyl-D-glucosamine, CS), a biopolymer, could be used for manufacturing nanocomposites with multiple advantages, including minimal toxicity and biodegradability (Chadha *et al*., 2022; Sangwan, Sharma, Wati and Mehta, 2023). NPsCS are very versatile and exhibit high stability and ease of preparation (Maluin and Hussein, 2020). These have multiple agricultural applications such as pesticides, herbicides, and insecticides and to obtain better quality food products with higher yields (Bandara, Du, Carson, Bradford and Kommalapati, 2020).

NPsCS can increase crop tolerance to biotic or abiotic stress (Wang *et al*., 2021). They are stimulants and potent inducers of antioxidant enzyme activity (Maluin and Hussein, 2020; Chandrasekaran, Kim, and Chun, 2020), decreasing the accumulation of reactive oxygen species (ROS) in plant cells, improving stress tolerance, growth, and yield of crops (Ishkeh *et al*., 2021); it also increases natural antioxidants in crops, which can generate benefits to human health (Ketnawa, Reginio, Thuengtung and Ogawa, 2022). On the other hand, tomato (*Solanum lycopersicum* L.) is the most produced and consumed vegetable worldwide and is considered a functional food because it is rich in fiber and contains a great variety of minerals, and bioactive compounds are beneficial to human health (Attia *et al*., 2021). These qualities make it an appropriate vegetable for biostimulation. Based on the above, this study aimed to determine the effect of foliar spraying with NPsCS on yield, enzyme activity, and bioactive compound content in tomato fruits.

MATERIALS AND METHODS

Synthesis of Chitosan Nanoparticles

The NPsCS were synthesized at the Applied Chemistry Research Center in Saltillo, Coahuila, Mexico, by the ionic gelation method described by Kumaraswamy *et al*. (2018). The NPsCS have an average size of 111 ± 21 nm and a spherical shape, as previously reported by Ramírez-Rodríguez *et al*. (2021).

Growing Conditions and Plant Material

The research was carried out in a tunnel-type greenhouse belonging to the Horticulture Department of the Universidad Autónoma Agraria Antonio Narro, in Buenavista, Saltillo, Coahuila, Mexico, located at 25° 21′ N and 101° 01′ W and an altitude of 1790 meters of altitude. Seeds of tomato F1 Hybrid (MARIANA, SAKATA®) were germinated in agricultural foam plates. Twenty days after sowing, the seedlings were transplanted in 10 L black polyethylene bags with Peat moss and perlite 2:1 (*v*:*v*). The planting density was six plants per square meter. Steiner's (1984[1]) nutrient solution was used for crop nutrition, applied through a drip irrigation system with three daily irrigations. In the first week after transplanting, irrigation was done with a 25% nutrient solution, the second week with a 50% solution, the third week with a 75% solution, and the fourth week after transplanting with a 100% Steiner solution. The average temperature was 22.4 °C, while the average photosynthetic active radiation was 677 μmol m^{-2} s^{-1}, and the average relative humidity was 62%, monitored with a ThermoProTP359.

[1] Steiner, A. A. (1984). The universal nutrient solution. In *Proceedings 6th International Congress on Soilless Culture* (pp. 633-650). Wageningen, The Netherlands: ISOSC.

Treatments and Experimental Design

The NPsCS were prepared in deionized water with glycerin (reagent grade) and Bionex (Arysta®) as dispersing and coadjuvant agents, then sonicated for six minutes at an amplitude of 50% in an ultrasonic cleaner (Branson, 1510R-DTH), to obtain a concentration of 1 mg mL^{-1}. Subsequently, from this solution, solutions were prepared and applied by foliar sprays at the following concentrations: 0 (distilled water), 0.05, 0.1, 0.1, 0.2, 0.4, and 0.8 mg mL^{-1}, during the first hours of the morning (8:00 h), with four applications every 15 days during the crop cycle, starting 15 days after transplanting (DDT). The experiment was conducted in a completely randomized design with ten replicates per treatment, considering one plant as the experimental unit.

Sampling

At 100 DDT, ten fruits were harvested from each treatment and repetition, of uniform size and light red maturity, determining the physical quality of the fruit and the quantification of bioactive compounds.

Physical Quality of the Fruit

Tomato fruits were weighed on an analytical balance (Ohaus®, CS5000P). Fruit size was quantified by determining the polar and equatorial diameter using a digital vernier (ASK-500-196-30; Mintutoyo). Firmness was measured with an Extech penetrometer (FHT200). For this, the fruits were placed on a hard and fixed surface, recording the average of two measurements per fruit for each repetition and treatment.

Bioactive Compounds

Preparation of Extracts for Nutraceutical Quality

Two g of fresh pulp was mixed in 10 mL of ethanol in a screw-capped plastic tube, which was placed in a rotary shaker (ATR Inc., EU) for six h at five °C and 20 rpm. Subsequently, the tubes were centrifuged at 3000 rpm for 5 min, and the supernatant was extracted for analytical tests (Preciado-Rangel, Troyo, Valdez, García and Luna, 2020).

Total Phenolic Compounds

Total phenol content was measured by the Folin-Ciocalteau method (Singleton, Orthofer, and Lamuela, 1999). 300 µL of the extract was mixed, and 1080 mL of distilled water and 120 µL of Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis MO, USA) were added, vortexing for 10 s. After 10 min, 0.9 mL of sodium carbonate (7.5% w/v) was added, vortexing for 10 s. The solution was allowed to stand at room temperature for 10 min. The solution was allowed to stand at room temperature for 30 min, and then its absorbance was read at 765 nm in a UV-vis spectrophotometer (VE-5100uv-VELAB). The phenol content was calculated by a standard curve using gallic acid as a standard (Sigma, St. Louis, Missouri, USA). Results were reported as mg gallic acid equivalent per 100 g fresh weight (mg equiv AG-100 g^{-1} fresh weight (FW).

Total Flavonoids

The colorimetric method determined the total flavonoid content (Zhishen *et al*., 1999). For this purpose, 250 µL of the ethanolic extract was mixed with 1.25 mL of distilled water in a test tube, and then 75 µL of 5% $NaNO_2$ solution was added.

After 5 min, 150 µL of 10% $AlCl_3$ + H_2O solution was added and allowed to stand for another 6 min; then, a volume of 500 µL of 1 M NaOH plus an additional 275 µL of distilled water was added. All components were mixed by stirring in a vortex. The absorbance was measured immediately at 510 nm using a UV-vis spectrophotometer (VE-5100uv-VELAB). Results were expressed as mg quercetin equivalents per 100 g^{-1} fresh weight (mg equiv Q-100 g^{-1} FW).

Antioxidant Capacity

The antioxidant capacity was determined with the in vitro DPPH+ method (Brand-Williams, Cuvelier and Berset, 1995). To determine antioxidant capacity, 50 µL of sample and 1950 µL of $DPPH^+$ solution was mixed, and after 30 min of reaction, the absorbance of the mixture was read at 517 nm in a UV spectrophotometer (Genesys 10). The standard curve was fitted with Trolox (Aldrich, St. Louis, Missouri, USA). The results were reported as antioxidant capacity (µM equiv Trolox-100 g^{-1} FW).

Vitamin C

Vitamin C concentration was determined by the method of Klein and Perry (1982). For extraction, 10 mg of sample and 1 mL of H_3PO_4 (0.36 M) were added and centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 5000 rpm for 10 min at four °C. Subsequently, 200 µL of the supernatant and 1 mL of 2.6-dichlorophenolindophenol (2.6 D) (0.09 M) were homogenized for subsequent reading in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 515 nm. The results were expressed as mg g^{-1} dry weight (DW).

Lycopene

With 100 mg of lyophilized sample and 2 mL hexane, an extract was obtained and mixed in a vortex for 30 sec, then sonicated for 5 min and centrifuged at four °C for 10 min at 10 000 rpm. The supernatant was filtered and quantified at 472 nm. The concentration was obtained using the calibration curve previously plotted with the lycopene standard (Bunghez, Raduly, Doncea, Aksahin and Ion, 2011). A curve was performed with lycopene standard Sigma-Aldrich brand 98% purity. The results were expressed in milligrams per kilogram.

Preparation of Extracts for Total Protein and Enzyme Activity

The enzymatic extract was obtained using the methodology of Ramos *et al*. (2010), which refers to the placement of 200 mg of lyophilized plant tissue (LABCONCO 2.5 freezone lyophilizer) in a 2 mL tube, with the addition of 20 mg of polyvinylpyrrolidone and 1.5 mL of phosphate buffer pH 7-7.2 (0.1 M), and then centrifuged at 12000 rpm for 10 min at four °C in a microcentrifuge (Labnet Int. Inc., PrismTM C2500-R). The supernatant was collected and filtered on a PVDF membrane with a pore size of 0.45 microns.

Total Protein

Total protein (TP) concentration was determined according to the Bradford colorimetric technique (Cheng, Wei, Sun, Tian and Zheng, 2016). The results were expressed in mg g^{-1} DW.

Superoxide Dismutase

The superoxide dismutase (SOD) assay (EC 1.15.1.1) was performed using the commercial kit 19160 SOD (Sigma-Aldrich). Samples and blanks were assayed, then incubated at 37 °C for 20 min. Finally, it was read at an absorbance of 450 nanometers.

Glutatión Peroxidase

Glutathione peroxidase (GPX) activity (EC 1.11.1.9) was quantified following the methodology of Flohé and Günzler (1984). 200 µL of the extract, 400 µL of GSH (0.1 mM), and 200 µL of Na_2HPO_4 (0.067 M) were homogenized and placed in a water bath at 25 °C for 5 min. Then, 200 µL of H_2O_2 (1.3 mM) was added to react for 10 min. The reaction was stopped by the addition of 1 mL of trichloroacetic acid (1%) and then centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 3000 rpm for 10 min at four °C. To determine GPX activity, 480 µL of the supernatant, 2.2 mL of Na_2HPO_4 (0.32 M), and 320 µL of DTNB (1 mM) were homogenized and subsequently read in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 412 nm. Results were expressed as U g^{-1} PT, where U corresponds to mM reduced glutathione equivalents per milliliter per minute.

Statistical Analysis

Bartlett's test was run on the data obtained to test the homogeneity of variance, and the normality of the data was tested with the Kolmogorov-Smirnov and Shapiro-Wilk W tests. Subsequently, an analysis of variance was performed, and where a difference was detected between treatments, Fisher's test ($P \leq 0.05$) was used for the separation of means using the statistical program InfoStat version 2020 (Di Rienzo *et al*., 2020).

RESULTS AND DISCUSSION

Fruit Quality

Foliar spraying of NPsCS positively affected tomato fruits' yield, weight, size, and firmness (Table 1). With the 0.4 and 0.8 mg mL^{-1} doses of NPsCS, the highest values were obtained in yield and fresh fruit weight concerning the control, with an increase of 25.9 and 42%, respectively. Fruit size was affected by the dose of 0.8 mg

Table 1. Average values of yield (R), fruit weight (PF), polar diameter (DP), equatorial diameter (DE), and firmness of tomato fruits (F) by foliar application of NPsCS.

NPsCS	R	PF	DP	DE	F
mg mL^{-1}	g $planta^{-1}$	g	cm		N
0	4198.2±71.79 b*	88.50±36.89 b	67.56±11.94 bc	47.40±7.32 b	56.60±17.80 b
0.05	4221.1±150.7 b	86.84±38.56 b	68.39±13.32 abc	46.69±7.52 b	50.89±10.02 b
0.1	3942.6±108.97 c	88.83±43.86 b	62.25±15.95 c	50.98±11.33 ab	73.75±22.38 a
0.2	4187.8±119.93 b	91.69±30.27 b	68.86±8.32 abc	48.24±5.64 ab	73.5±11.32 a
0.4	4367.4±72.70 a	125.69±25.39 a	73.42±7.82 ab	54.26±4.58 a	73.5±10.29 a
0.8	4437.2±54.53 a	111.43±22.96 ab	77.93±8.19 a	45.87±6.00 ab	77.5±11.21 a

*Mean values with different literals are significantly different (LSD $P \leq 0.05$).

mL^{-1} NPsCS, the polar diameter of the fruit increased by 25.1%, and the equatorial diameter with 0.4 mg mL^{-1} NPsCS increased by 14.4%, both concerning the control. Tomato fruit firmness increased with 0.8 mg mL^{-1} NPsCS, increasing 36.9% concerning the control treatment. Chitosan has a high affinity towards plant cell membranes, resulting in enhanced reactivity in the plant system (Maluin and Hussein, 2020), allowing the penetration of NPs that increase cellular metabolic activity (Lin *et al.*, 2009), thus managing to promote crop yield (Siskani, Seghatoleslami and Moosavi, 2015). NPsCS can stimulate plant growth by containing small amounts of nutrients such as C, O, N, and P, in addition to positively stimulating cell division and elongation, enzyme activation, and protein synthesis, leading to increased crop quality and productivity (Chakraborty *et al*., 2020; Prajapati *et al*., 2022). Parvin *et al*. (2019) reported that chitosan application increases fruit weight and size and, consequently, yield in *Solanum lycopersicum* L. The benefits of chitosan use are very diverse and range from a biostimulant effect by increasing the synthesis of growth regulators (Akhtar *et al*., 2022; Malerba and Cerana, 2016; Stasińska and Hawrylak, 2022); to improving nitrogen metabolism and as a consequence plant productivity Mondal *et al*. (2012) however, there is no single dose, as the plant response depends on the concentration, species and developmental stage of the plant (Balusamy *et al*., 2022; Hoang *et al*., 2022; Malerba and Cerana, 2016; Parvin *et al*., 2019), so further research is needed.

Bioactive Compounds

Foliar spraying of NPsCS increased bioactive compounds (phenolics and flavonoids) and antioxidant capacity in tomato fruits (Table 2). The dose of 0.8 mg mL^{-1} of NPsCS increased antioxidant capacity by 81.3%, phenols by 6.6%, and flavonoids by 38.5% concerning the control treatment. Jahani, Behnamian, Dezhsetan, Karimirad and Chamani (2023) reported an increase in antioxidant capacity in *Solanum lycopersicum* L with the application of NPsCS. Similar responses are obtained with CS in *Raphanus sativus* L (Supapvanich, Anan and Chimsonthorn, 2019). Chandra *et al*. (2015) reported increased phenols and flavonoids in *Camellia sinensis*. Other investigations have reported increased content of these bioactive compounds due to NPsCS application in *Oryza sativa* L. (Divya, Thampi, Vijayan, Varghese and Jisha, 2020), *Rosa damascena* Mill. (Ali, Issa, Al-Yasi, Hessini and Hassan, 2022), in *Triticum aestivum* L. (Hajihashemi and Kazemi, 2022). The vitamin C content was affected by the NPsCS (Table 2). With the dose of 0.4 mg mL^{-1}, the highest accumulation was achieved concerning the control. Similar results are reported by El Amerany *et al*. (2022), indicating that the application of CS increased the levels of natural antioxidants such as vitamin C, phytic acid, pantothenic acid, lycopene, and flavonoids. Vitamin C

Table 2. Effect of NPsCS foliar spray on bioactive compounds in tomato fruits.

NPsCS	Total phenolic	Total flavonoids	Antioxidant capacity	Vitamin C	Lycopene
mg mL^{-1}	mg equiv GA·100 g^{-1} FW	mg equiv Q·100 g^{-1} FW	µmeq TROLOX 100 g^{-1} PF	- - - - - - - - - mg kg^{-1} - - - - - - - - -	
0	21.04±4.53 ab*	24.19±17.14 b	30.45±4.41 b	4982.5±39.48 ab	47.72±10.93 c
0.05	22.4±6.62 a	31.71±4.13 ab	30.78±6.48 b	4823.33±178.98 b	117.19±11.22 a
0.1	20.22±11.67 b	34.71±7.39 ab	50.50±14.74 a	4872.3±137.9 ab	79.72±30.07 bc
0.2	22.21±13.81 a	30.41±9.56 ab	55.06±13.63 a	5030.00±60.83 a	80.11±17.42 bc
0.4	21.45±7.12 ab	30.41±9.17 ab	45.12±6.18 ab	5030.00±123.56 a	88.54±10.53 ab
0.8	22.39±10.91 a	40.45±6.12 a	55.23±8.83 a	5003.33±75.72 ab	113.60±36.53 a

*Mean values with different literals are significantly different (LSD $P \leq 0.05$).

is important because it is a powerful antioxidant by trapping reactive oxygen species (ROS) and reversing or minimizing oxidative damage (Meena *et al*., 2020). It also plays an essential role in photosynthesis as an enzymatic cofactor (including the synthesis of ethylene, gibberellins, flavonoids, and anthocyanins) (Kawashima *et al*., 2015). Therefore, higher vitamin C accumulation will improve fruit quality (Paciolla *et al*., 2019). Lycopene synthesis was affected by NPsCS, with the dose of 0.05 mg mL^{-1} increased by 145% concerning the control (Table 2). Lycopene is a ROS-deactivating antioxidant (Imran *et al*., 2020). Other studies found that foliar application of CS increases lycopene content in *Solanum lycopersicum* L. (Parvin *et al*., 2019), so an increase of lycopene would also be expected due to foliar application of NPsCS. The results obtained by foliar application of NPsCS in the rise of bioactive compounds can be attributed to the fact that CS and NPsCS, in addition to having a biostimulant action, also have an elicitor activity that generates reactive oxygen species (ROS), which stimulate the biosynthesis of bioactive compounds such as enzymatic and non-enzymatic antioxidants (Ishkeh *et al*., 2021; Singh, 2016; Stasińska and Hawrylak, 2022; Malerba and Cerana, 2016; Wang *et al*., 2021). The above shows that NPsCS can be used in agriculture as a natural biostimulant because it favors the biosynthesis of bioactive compounds reflecting higher fruit quality (Paciolla *et al*., 2019).

Enzymatic Activity

Proteins were positively affected by the foliar application of NPsCS (Table 3); with the 0.05 mg mL^{-1} dose of NPsCS, the highest concentration of proteins was obtained. Previous studies have indicated that NPsCS application increases total amino acids (Balusamy *et al*., 2022) and proteins Hajihashemi and Kazemi (2022). This increase in protein content may be due to the N content in NPsCS, which plays an important role in protein synthesis (Behboudi *et al*., 2018), or to the stimulation of the plant defensive system conducive to stimulation of ROS and accumulation of proteins such as chitinase and activation of peroxidase, SOD and CAT enzymes (Bandara *et al*., 2020; Chun and Chandrasekaran, 2019; Li *et al*., 2015).

The highest dose of NPsCS (0.8 mg mL^{-1}) produced the most increased SOD activity in tomato fruits (Table 3), with no modification in GPX enzyme activity. The stress caused by nanoparticles can produce ROS (Katiyar, Hemantaranjan and Singh, 2015), which modifies the activity of antioxidant enzymes, some transcription factors, and proteins involved in the stress response (López-Vargas *et al*., 2018). The elevated SOD activity caused by NPsCS could be responsible for ROS balance, degeneration, and scavenging to protect the plant from oxidative stress (Chun and Chandrasekaran,

Table 3. Effect of foliar application of NPsCS on the total protein content and enzymatic activity of SOD and GPX in tomato fruits.

NPsCS	Enzyme activity (U)		
	Total protein	SOD	GPX
mg mL^{-1}	g kg^{-1}		
0	2.46±0.61 b*	1.48±0.10 ab	1.51±0.80 a
0.05	4.59±0.52 a	0.85±0.02 b	1.78±0.17 a
0.1	3.81±0.71ab	1.57±0.41ab	1.52±0.17a
0.2	3.44±0.41ab	1.05±0.33 ab	1.59±0.38 a
0.4	4.04±0.42 a	1.89±0.56ab	1.50±0.71 a
0.8	3.56±0.38 ab	2.34±0.04 a	1.60±0.20 a

*Mean values with different literals are significantly different (LSD $P \leq 0.05$).

2019). Faizan *et al*. (2021) report an increase in SOD activity in *Solanum lycopersicum* L. with the application of NPsCS. By increasing SOD activity, the accumulated H_2O_2 also decreased, maintaining GPX activity at a basal level (Hajihashemi and Kazemi, 2022), which could explain why there are no differences in GPX enzyme activity in tomato fruits treated with NPsCS.

CONCLUSIONS

Foliar spraying of NPsCS increased yield, bioactive compounds, and enzyme activity in tomato fruits. The medium dose (0.2 mg mL^{-1}) optimized yield, fruit size, and weight, whereas the high dose (0.8 mg mL^{-1}) increased the biosynthesis of bioactive compounds and enzyme activity in tomato fruits. The foliar application of NPsCS can be used as a natural biostimulant to increase yield and obtain functional foods.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

The data sets used or analyzed from this study are available from the author by correspondence upon reasonable request.

COMPETING OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Conceptualization and research: S.C.R.R. and H.O.O. Validation and formal analysis: S.G.M. Writing, revision, editing, and visualization: S.C.R.R. and P.P.R. Supervision: P.P.R. Acquisition of funds: H.O.O.

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