

# The effect of preharvest 28.6% chitosan composite film sprays for controlling the soft rot on kiwifruit and its defence responses

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**Abstract:** This study evaluated the effects of preharvest plant 28.6% chitosan composite film (CTS-Fh) sprays on the postharvest quality and diseases in kiwifruit (*Actinidia deliciosa* cv. 'Guichang'), it was screened and prepared by mixing chitosan, calcium, dextrin, ferulic acid and auxiliaries. 28.6% CTS-Fh solutions at different concentrations were sprayed three times during the fruit growing season. The obtained results show that 28.6% CTS-Fh sprays remarkably promoted the improvement of the yield and quality of the kiwifruit, significantly ( $P < 0.05$ ) increased the Ca content and firmness, delayed the fruit ripening and softening, and enhanced the storability. Moreover, the kiwifruit soft rot was effectively controlled; the control efficiency was 61.68–88.79%. Additionally, the 28.6% CTS-Fh sprays significantly ( $P < 0.05$ ) increased the content and activity of some defence-related secondary metabolites and enzymes, and could also increase the cell wall compactness in the kiwifruit. These results suggest that 28.6% CTS-Fh might trigger several defence responses in the kiwifruit against pathogenic infections. The doses of 28.6% CTS-Fh 200–400 dilution times were recommended for the practical application with regards to the production of kiwifruit.

**Keywords:** kiwifruit soft rot; chitosan film; preharvest spray; storage quality

As an emerging fruit that the world recognised, the kiwifruit has a high nutritional value that exhibits a beneficial effect on a human's health (GUO et al. 2016). The harvested area and yield of the kiwifruit in the world has continuously increased in the 21<sup>st</sup> century. In China, the kiwifruit industry had rapidly developed, and the cultivation area and yield are ranked first in the world. However, postharvest

diseases of kiwifruit are a serious problem in storage, because of the invading pathogens, the kiwifruit is susceptible of becoming rot, which strongly limits the postharvest life and supply period of the kiwifruit (FATEMI et al. 2013). Kiwifruit soft rot is one of the main diseases to cause kiwifruit rot after the harvest, infection is mainly caused by *Botryosphaeria dothidea* and *Phomopsis* sp. usually results in

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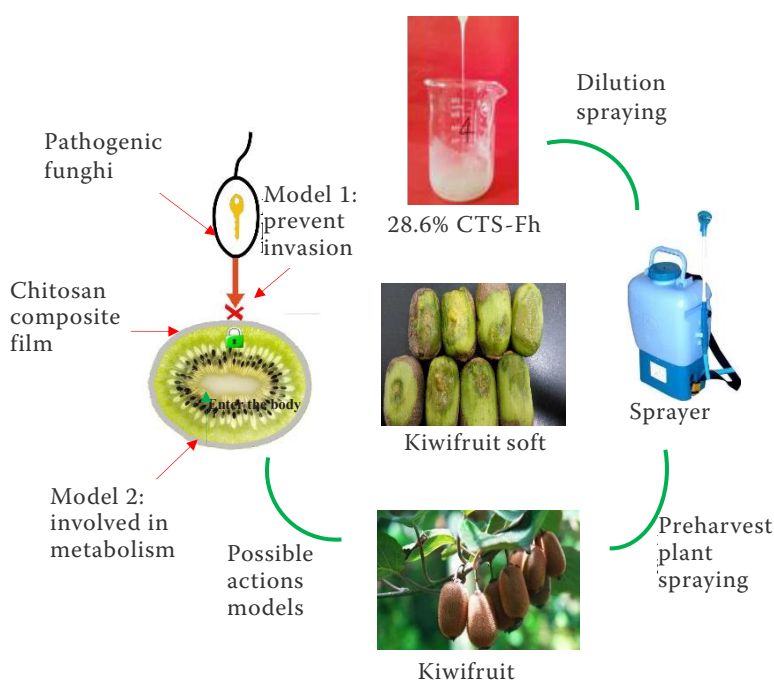


Fig. 1. The possible action mode of the preharvest 28.6% CTS-Fh sprays in the kiwifruit

serious postharvest losses of kiwifruit (LUONGO et al. 2011). The pathogens can enter the fruit tissues at the early growing stage, but stay latent in the tissues until the fruit ripens. Then, the invading pathogens begin to recover the capacity of the fruit, eventually causing disease symptoms on the fruit during storage (LUONGO et al. 2011; CHEN et al. 2015).

The *Actinidia deliciosa* cv. Guichang has a long storage period, good quality and high yield performance, and the planting area in Southwest China has reached 30,000 ha. According to the statistics, soft rot easily occurred in the Guichang kiwifruit due to *B. dothidea* and *Phomopsis* sp.; significant yield losses could be up to 30%, or even 50% in extreme cases. Currently, the approaches for suppressing the postharvest decay of the kiwifruit mainly rely on chemical fungicides (BARDAS et al. 2010). Although decay can be reduced significantly by synthetic fungicides, there are increasing concerns about fungicide residues and resistance and the harmful effects on the environment and man's health. Interestingly, alternative strategies for the management of the postharvest diseases have been created, such as the application of preharvest oligochitosan and chitosan (YAN et al. 2012).

Chitosan (CTS), a natural, non-toxic biopolymer, has good antibacterial, film-forming, antioxidant, renewable, nontoxic, biocompatible and biodegradable characteristics, obtained by the N-deacetylation of chitin (SHUKLA et al. 2012a,b, 2016; WILLIAM et al. 2016). Its antifungal activity has been documented

in several studies as a promising compound to effectively control phytopathogenic fungi both in the field and during the post-harvest condition (LIU et al. 2007; ZHANG et al. 2011; BOONLERTNIRUN et al. 2012; YAN et al. 2012). Importantly, it could activate defence responses in numerous plant species by inducing various defence-related compounds (LUONGO et al. 2011), and also as a fertilizer supplement to enhance plant growth (BOONLERTNIRUN et al. 2008; MONDAL et al. 2013). Calcium is a necessary element of the kiwifruit affecting the fruit development, quality and postharvest storability. Calcium pectates in the cell walls strengthen the plant tissues and protect the fruit from pathogenic bacteria infections (NESLIHAN et al. 2016). Ferulic acid is an abundant cinnamic acid derivative found in the plant world and has been utilised by microorganisms, also it has potential as a possible food preservative due to its good antibacterial performance (GARCIA et al. 2005). Dextrin is a natural film-forming substance of edible films, widely used in the storage and processing of horticultural products (LANDMAN; FOCK 2006).

Several studies reported the effects of chitosan composite coatings and calcium on the postharvest diseases and the quality of fruits (KOU et al. 2014; PETRICCIONE et al. 2015), nevertheless, no research work has been hitherto conducted into the effects of preharvest chitosan and calcium composite film spray on the decaying of kiwifruit during the storage. In this study, to investigate the potential role of pre-

harvest plants, 28.6% chitosan composite film (CTS-Fh) sprays were used during the fruit growth and development stage to discover the effect on the fruit's development, quality, storability, postharvest diseases and defence responses in the kiwifruit. 28.6% CTS-Fh was made by chitosan, calcium nutrition, antibacterial and film-forming substances and auxiliaries, and we preliminarily predicted the possible action modes of 28.6% CTS-Fh as shown in Fig. 1. The findings of this study will provide a scientific basis and new way for high-quality cultivation, organic control of soft rot and the green preservation of the kiwifruit.

## MATERIAL AND METHODS

**Study site and plant material.** The experiments were carried out on the kiwifruit cultivar 'Guichang' (*Actinidia deliciosa*), planted in 2002, in the kiwifruit garden in 2016 in the Xiuwen county of Guizhou province, China (26°49'02.2"N, 106°28'23.6"E, 1,354 m). A "T" type frame of the kiwifruit, with a spacing of 3.0 m × 3.0 m was used, the proportion of male:female vines was 1 : 8. The mean temperature in the kiwifruit garden was about 15–16°C, the rainfall was 1,293 mm, in loam soil. The soil (0–60 cm in depth) had a total organic matter content of 29.56 g/kg, a total nitrogen content of 1.42 g/kg, an alkali-hydrolysable nitrogen content of 98.47 mg/kg, an available phosphorus content of 4.40 mg/kg, an exchangeable calcium content of 17.84 cmol/kg. The pH value of the planting soil was 5.86.

**Pathogenic fungi and chemicals.** The *B. dothidea* and *Phomopsis* sp. were provided by the Institute of Crop Protection (Guizhou University, Guiyang, China). The chitosan (deacetylation ≥ 90%, the molecular weight was 50KDa) was purchased from Huarun Bioengineering Co. Ltd. (Zhenzhou, China). The dextrin (purity ≥ 99.0%) and sodium benzoate (purity ≥ 99.0%) were purchased from Kermel Chemical Reagent Co. Ltd. (Tianjin, China). The ferulic acid (purity ≥ 99.0%) was purchased from Aladdin Industrial Co. Ltd. (Shanghai, China). The calcium nitrate (Ca-N, purity ≥ 99.5%) and glycerol (purity ≥ 99.5%) were purchased from Jinshan Chemical Reagent Co. Ltd. (Chongqing, China). The organosilicon (purity 100%) was purchased from Zhengan Agricultural Sci & Tech Co. Ltd. (Shijiazhuang, China). The 80% thiophanate-methyl wettable powder (Thiopsin-M WP) was purchased from Meibang Pesticide Co. Ltd. (Xian, China). The

potato dextrose agar (PDA) was obtained from Xiya Reagent Co. Ltd. (Chengdu, China).

**Preparation of the CTS-Fh.** The 28.6% CTS-Fh consists of 8.00% CTS + 10.00% dextrin + 1.00% FA + 5.00% Ca-N + 0.60% sodium benzoate + 3.00% glycerol + 1.00% organosilicon + 72.40% sterile water, the 24% CTS-Fh consists of 8.00% CTS + 10.00% dextrin + 1.00% FA + 5.00% Ca-N + 76.00% sterile water, the 19% CTS-Fh consists of 8.00% CTS + 10.00% dextrin + 1.00% FA + 81.00% sterile water, the 18% CTS-Fh consists of 8.00% CTS + 10.00% dextrin + 82.00% sterile water. They were made as follows: firstly, the dextrin was added into the sterile water and stirred and water-bathed at 85°C for 20 min; secondly, the other effective components were mixed with the dextrin solution in a glass beaker and stirred with an electric stirrer (JJ-1, Fuhua Instrument Co. Ltd., Jintan, China) for 12 hours. The CTS-Fh solution prepared in this way was subjected to ultrasonication (Scientz-08, Xinzhi Bio & Tech Co. Ltd., Ningbo, China) for 30 minutes.

**The impact of the CTS-Fh on the pathogenic fungi hyphal growth.** For determining the contact effects, 9 mL PDA was emptied into glass Petri dishes (90 mm in diameter) at a temperature of 40–45°C and when it solidified before 1 ml of the tested solution of CTS-Fh, it was evenly coated on the PDA plate. Then, a 5 mm diameter disc cut from the actively growing front of a 1-week old colony of the desired pathogenic fungus was then placed with the inoculum side down in the centre of each treatment plate, aseptically. The treated petri dishes were then incubated at 28°C till the fungal growth was almost complete in the control plates. All the experiments were done in quadruplicate for each treatment against *Botryosphaeria dothidea* and *Phomopsis* sp.. The diameters of the fungal growth were measured after 2, 3, 4, 5, 6 and 7 days. The formula for calculating the growth inhibition of the fungal hyphae is per the Equation: Inhibition rate (%) =  $[(D_c - D_t) / (D_c - 5)] \times 100$ , where  $D_c$  and  $D_t$  represent the mycelial growth diameter in the control and the CTS-Fh treated conditions, respectively.

The  $EC_{50}$  (effective dose for 50% inhibition) values were estimated statistically by a probit analysis with the probit package of SPSS 18.0 software.

**The field experiments.** On the 20<sup>th</sup> of April (Budding phase), the 20<sup>th</sup> of May (Fruit setting phase) and the 12<sup>th</sup> of August (Expanding final phase), 2015 to 2016, 28.6% CTS-Fh 100, 200, 400, 800 dilution times (dilut-t), Ca-N 0.5 g/l, 80% Thiopsin-M WP 500 dilut-t and irrigation water (control) were

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sprayed onto the kiwifruit plants, respectively. A total of seven treatments, twenty-one plots were arranged in a randomised block design with three replicates. Each plot consisted of six trees, only the interior four trees were used for the measurements. About 1.50 l of each treatment solution was sprayed on each tree, each time.

**The plant sampling and analysis.** The kiwifruit samples of 100 fruits were randomly collected and divided into 2 groups from each plot on the 23<sup>rd</sup> of September, and stored at room temperature ( $25 \pm 1^\circ\text{C}$ ). The fruits of the first group were used for determining the fruit quality parameters, the resistance to the related substances, the respiratory intensity, the firmness and so on. The fruits of other group were used to investigate the weight loss and the incidence of soft rot.

**The fruit quality parameters.** The longitudinal, transverse and lateral diameter of 20 fruits of each replicate were measured with a digital calliper, the fruit shape index and single fruit volume were calculated according to the above measurement results. The single fruit weight was measured with an electronic analytical balance. The soluble solid content was measured with a digital refractometer (PAL-1, Yishida Sunshine Tech Co. Ltd., Beijing, China), the total soluble sugar was analysed by the Anthrone colorimetric method. The dry matter was evaluated by the difference between weighed samples before and after 24 h in a stove at  $1,000^\circ\text{C}$ , while the titratable acidity was determined by titration with 0.01 mol/l NaOH. The chlorophyll content was analysed by an acetone–ethanol (v/v, 2 : 1) extraction with the UV-VIS (T6, Purkinje General Instrument Co. Ltd., Beijing, China) method, the soluble protein content was determined by the Coomassie Brilliant Blue method. For the vitamin C analysis, 5.00 g of flesh from the equatorial position of the fruit was homogenised under an ice bath and placed into a 50 ml centrifuge tube with 20 ml of 0.1% oxalate. The samples were centrifuged at 10,000 g for 20 min at  $3^\circ\text{C}$  with a Sorvall (Biofuge Stratos, Thermo scientific Co. Ltd., USA). Each 10  $\mu\text{l}$  supernatant sample was regenerated through 0.20  $\mu\text{l}$  cellulose membrane filters. The vitamin C assay was performed using a Waters HPLC system (1260, Agilent, USA), a ZORBAX Eclipse XDB-C18 analytical column (4.6 cm  $\times$  250 mm  $\times$  5  $\mu\text{m}$ , Agilent, USA), an infinity VL two pump, and an absorbance detector at 265 nm. The mobile phase was 100% MeOH: 0.1 M  $\text{KH}_2\text{PO}_4$  1 : 9 (v/v), and the flow rate was 1.0 ml/min.

**The fresh-keeping parameters.** The respiration intensity was analysed by the alkali absorption

method, the firmness was measured with a fruit sclerometer (GY-4, Aidebao Instrument Co. Ltd., Leqing, China). The weight loss and softening rate were determined at time 0 and after 5, 10, 15, 20 and 25 days, expressed to two percentage points using the Equation:

Weight loss (%) =  $(W_0 - W_1)/W_0 \times 100$ , where:  $W_0$  and  $W_1$  – the initial and final sample weights, respectively.

The calcium contents were analysed as described by SIMSEK and AYKUT (2007), with slight modifications. For each sample,  $1.00 \pm 0.005$  g of flesh from the equatorial position of the fruit was homogenised and placed into a vessel and covered with 10 ml of 69% nitric acid ( $\text{HNO}_3$ ) and 2 ml of 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). And transferred to a microwave digestion system (MDS-2002AT, Sineo microwave chemical Tech Co. Ltd., Shanghai, China) at  $175^\circ\text{C}$  for digesting. The digested samples were diluted to 25 ml with ultrapure water and extracted with Whatman no. 5B filter paper. The extracts were analysed using the atomic absorption spectrometry (AAS) (AA800, Perkin Elmer Co. Ltd., USA).

**The disease control parameters.** Investigation on the incidence of soft rot, according to the Equation: Cumulative incidence (%) = number of diseased fruit/total number of fruit  $\times$  100, Disease index =  $\sum(\text{disease scale value} \times \text{number of the fruit within each scale}) / (\text{total number of fruit} \times \text{the highest scale}) \times 100$  and Control effect (%) = (Disease index of control – Disease index of treatment)/Disease index of control  $\times$  100 to calculate disease incidence, disease index and control effect. The grading standard of the disease degree: 0 – no disease; 1 – cumulative diameter less than 1 cm; 2 – about 1–2 cm; 3 – about 2–3 cm; 4 – about 3–4 cm; 5 – about 4–5 cm; 6 – more than 5 cm.

Total phenolic content was determined according to the Folin-Ciocalteu procedure (YANG et al. 1999), and the data were expressed as gallic acid equivalents in mg/g FW. The total flavonoid content was determined using the colorimetric method described previously by DEWANTO et al. (2002), and the data were expressed as rutin equivalents in mg/g FW. The malondialdehyde (MDA) content was measured using the thiobarbituric acid (TBA) method described by DING et al. (2007), and expressed as  $\mu\text{mol/g}$  FW. The catalase (CAT) activity was determined according to MILOSEVIC and SLUSARENKO (1996) and expressed as  $0.01 \Delta\text{OD}_{240} \text{ U/g} \cdot \text{min}$  FW. The superoxide dismutase (SOD) activity was as-

sayed according to ZHU et al. (2010), one unit of the SOD activity was defined as the amount of enzyme that inhibited 50% of the photochemical reduction of NBT. The eperoxidase (POD) activity was spectrophotometrically assayed according to VANDER et al. (1998) and expressed as  $\Delta OD_{470}$  U/g·min FW. The polyphenol oxidase (PPO) activity was determined by measuring the conversion of catechol to quinine mediated by PPO (LIU et al. 2007), and was expressed as U/g·min FW.

**Scanning electron microscopy of the kiwifruit pericarp.** The pericarp tissues of the harvested kiwifruit were analysed by scanning electron microscopy (SEM) using the following method (MOHAMMAD et al. 2016): the kiwifruit samples were fixed in a 4% glutaraldehyde solution for 2–3 h at 4°C and then washed three times for 15 min each in a 0.1 mol cacodylate buffer (pH 7.4). The washed samples were fixed in 1% osmic acid solution for 2–3 h at 4°C and then washed three times for 15 min each in a 0.1 mol cacodylate buffer (pH 7.4). The samples were then dipped in 50% ethanol → 70% ethanol → 90% ethanol → 90% ethanol and 90% acetone: 1 : 1 → 90% acetone → 100% acetone twice every 20 minutes. Finally, the samples were dipped in the following chemicals: 100% ethanol and 100% isoamyl acetate: 2 : 1; 100% ethanol and 100% isoamyl acetate: 1 : 1; 100% ethanol and 100% isoamyl acetate: 1 : 2; and 100% isoamyl acetate every 20 minutes. After the chemical dipping, the samples were dried in a freeze drier (LGJ-10D, Beijing Fourth Ring Scientific Instrument Co., Ltd., Beijing, China), an ion sputter coated with gold, and examined by SEM (JEM1230, JEOL, Japan) at an accelerating voltage of 40 kV.

**The statistical analyses.** The data were presented as  $\bar{x} \pm s.d.$  (standard deviation) of three replications. A one-way analysis of variance (ANOVA) followed by Duncan's test was performed. All analyses were performed using the SPSS statistical software package release 18.0 (SPSS Inc., Chicago, USA).

## RESULTS AND DISCUSSION

### The effects of preharvest 28.6% CTS-Fh sprays on the yield and quality of kiwifruit

The longitudinal diameter, transverse diameter, lateral diameter and fruit shape index of the *Actinidia deliciosa*. cv. Guichang were not significantly influenced by the preharvest 28.6% CTS-Fh, Ca-N

and 80% Thiopsin-M WP sprays ( $P < 0.05$ ). Furthermore, we calculated the single fruit volume, the single fruit weight and the equivalent yield of kiwifruit, they were significantly increased by the preharvest 28.6% CTS-Fh 100-800 dilut-t and Ca-N 0.5 g/l, compared with the chemical fungicide and control ( $P < 0.05$ ); And the effective effects of the 28.6% CTS-Fh 100-400 dilut-t were more than the Ca-N 0.5 g/l (Table 1). More related studies indicate that chitosan could effectively stimulate plant growth and enhance the germination index, increase the yield of plants, such as maize and rice (BOONLERTNIRUN et al. 2008; MONDAL et al. 2013). Additionally, calcium nutrition played an active role in promoting plant growth and improving the quality (NESLIHAN et al. 2016; MOHAMMAD et al. 2016). Based on the present results, the preharvest chitosan and the calcium composite film application could contribute to the growth and yield of the kiwifruit.

The nutritional parameters in the fruits of the kiwifruit are shown in Table. 2, with the increasing levels of 28.6% CTS-Fh, all the quality parameters in the fruits gradually increased. After the preharvest 28.6% CTS-Fh (from 400 to 100 dilut-t) the spray, Vitamin C, soluble solid, total soluble sugar, dry matter, titratable acidity, soluble protein and chlorophyll content of the kiwifruit were significantly increased as compared to the control ( $P < 0.05$ ). Similarly, with promoting growth, the effective effects of the 28.6% CTS-Fh 100–400 dilut-t were more than the Ca-N 0.5 g/l. BOONLERTNIRUN et al. (2008) reported that chitosan could increase the dry matter content in several ornamental plants, and another study showed that the application of chitosan in agriculture as a fertilizer supplement improved the plant products' quality (HENGAMEH, MEHDI 2016). These findings imply that chitosan and calcium should have a favourable synergistic effect on improving the plant products' quality in the preharvest application.

### The effects of the preharvest 28.6% CTS-Fh sprays on the fresh-keeping of the kiwifruit

As shown in Fig. 2a, the preharvest application of 28.6% CTS-Fh was significantly effective for increasing the Ca content in the kiwifruit ( $P < 0.01$ ), which increased gradually with the increasing input of 28.6% CTS-Fh and the CTS-Fh treatments were more effective than the Ca-N treatment ( $P < 0.01$ ). Firmness is one of the most important parameters

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Table 1. The effects of the preharvest 28.6% CTS-Fh sprays on the development and yield of the kiwifruit

Treatments	Longitudinal diameter (mm)		Transverse diameter (mm)	Lateral diameter (mm)	Fruit shape index	Single fruit volume (cm <sup>3</sup> )	Single fruit weight (g)	Equivalent yield (kg/667 m <sup>2</sup> )
Pharmacy	dilution times							
28.6% CTS-Fh	100	80.06 ± 3.02 <sup>a</sup>	53.89 ± 5.79 <sup>a</sup>	41.37 ± 2.07 <sup>a</sup>	1.68 ± 0.05 <sup>a</sup>	74.36 ± 1.05 <sup>a</sup>	104.80 ± 2.34 <sup>a</sup>	1966.05 ± 13.83 <sup>a</sup>
	200	77.55 ± 0.67 <sup>ab</sup>	53.71 ± 4.36 <sup>a</sup>	40.76 ± 2.17 <sup>a</sup>	1.64 ± 0.05 <sup>a</sup>	70.88 ± 1.52 <sup>b</sup>	103.16 ± 0.47 <sup>ab</sup>	1935.20 ± 8.78 <sup>ab</sup>
	400	78.26 ± 2.10 <sup>ab</sup>	49.60 ± 2.90 <sup>a</sup>	41.84 ± 1.01 <sup>a</sup>	1.71 ± 0.04 <sup>a</sup>	68.74 ± 3.69 <sup>b</sup>	102.36 ± 0.15 <sup>bc</sup>	1920.19 ± 2.89 <sup>bc</sup>
	800	75.20 ± 1.70 <sup>b</sup>	50.82 ± 3.47 <sup>a</sup>	39.92 ± 1.89 <sup>a</sup>	1.66 ± 0.06 <sup>a</sup>	68.47 ± 0.27 <sup>b</sup>	100.84 ± 0.08 <sup>c</sup>	1891.84 ± 1.44 <sup>c</sup>
Calcium Nitrate	0.5 g/l	77.33 ± 2.40 <sup>ab</sup>	52.00 ± 2.33 <sup>a</sup>	41.28 ± 1.21 <sup>a</sup>	1.66 ± 0.04 <sup>a</sup>	69.52 ± 1.11 <sup>b</sup>	101.96 ± 0.66 <sup>bc</sup>	1912.68 ± 1.23 <sup>4bc</sup>
80% Thiopsin-M WP	500	78.03 ± 2.10 <sup>ab</sup>	50.05 ± 2.13 <sup>a</sup>	41.85 ± 1.01 <sup>a</sup>	1.70 ± 0.04 <sup>a</sup>	67.89 ± 1.75 <sup>b</sup>	96.62 ± 0.28 <sup>d</sup>	1812.63 ± 5.21 <sup>d</sup>
Control		77.33 ± 2.40 <sup>ab</sup>	51.66 ± 2.55 <sup>a</sup>	41.06 ± 1.56 <sup>a</sup>	1.67 ± 0.03 <sup>a</sup>	64.08 ± 0.23 <sup>c</sup>	96.53 ± 0.27 <sup>d</sup>	1810.97 ± 5.01 <sup>d</sup>

values represent the mean ± s.d., *n* = 3; different lowercases mean significant differences between different treatments at 5% level (*P* < 0.05)

Table 2. The effects of the preharvest 28.6% CTS-Fh sprays on the nutritional quality of the kiwifruit

Treatments	Vitamin C (mg/100 g)		Soluble solids (%)	Dry matter (%)	Total soluble sugar (%)	Titrate acidity (%)	Soluble protein (mg/g)	Chlorophyll (μg/g)
Pharmacy	dilution times							
28.6% CTS-Fh	100	200.30 ± 6.29 <sup>a</sup>	15.60 ± 0.00 <sup>a</sup>	19.46 ± 0.02 <sup>a</sup>	12.85 ± 0.03 <sup>a</sup>	1.02 ± 0.09 <sup>a</sup>	0.69 ± 0.01 <sup>a</sup>	11.75 ± 0.03 <sup>a</sup>
	200	190.50 ± 0.47 <sup>b</sup>	14.70 ± 0.10 <sup>c</sup>	19.19 ± 0.03 <sup>b</sup>	12.66 ± 0.03 <sup>b</sup>	1.10 ± 0.11 <sup>a</sup>	0.63 ± 0.09 <sup>b</sup>	11.02 ± 0.00 <sup>b</sup>
	400	186.20 ± 5.20 <sup>b</sup>	14.53 ± 0.06 <sup>d</sup>	18.06 ± 0.05 <sup>b</sup>	12.47 ± 0.02 <sup>c</sup>	1.01 ± 0.09 <sup>a</sup>	0.32 ± 0.04 <sup>c</sup>	10.18 ± 0.09 <sup>c</sup>
	800	185.72 ± 1.35 <sup>b</sup>	14.43 ± 0.06 <sup>de</sup>	17.98 ± 0.01 <sup>b</sup>	11.91 ± 0.04 <sup>d</sup>	1.06 ± 0.14 <sup>a</sup>	0.30 ± 0.07 <sup>d</sup>	8.88 ± 0.02 <sup>d</sup>
Calcium Nitrate	0.5 g/l	185.38 ± 1.26 <sup>b</sup>	14.90 ± 0.10 <sup>b</sup>	17.49 ± 0.10 <sup>c</sup>	12.67 ± 0.00 <sup>b</sup>	1.11 ± 0.05 <sup>a</sup>	0.30 ± 0.02 <sup>d</sup>	10.14 ± 0.04 <sup>c</sup>
80% Thiopsin-M WP	500	188.11 ± 0.66 <sup>b</sup>	14.30 ± 0.10 <sup>e</sup>	15.91 ± 0.29 <sup>d</sup>	11.82 ± 0.01 <sup>e</sup>	0.71 ± 0.08 <sup>b</sup>	0.31 ± 0.02 <sup>d</sup>	7.47 ± 0.08 <sup>e</sup>
Control		184.37 ± 3.35 <sup>b</sup>	14.10 ± 0.00 <sup>f</sup>	15.67 ± 0.37 <sup>d</sup>	11.83 ± 0.01 <sup>e</sup>	0.72 ± 0.07 <sup>b</sup>	0.31 ± 0.01 <sup>d</sup>	7.39 ± 0.08 <sup>f</sup>

values represent the mean ± s.d., *n* = 3; different lowercases mean significant differences between different treatments at 5% level (*P* < 0.05)

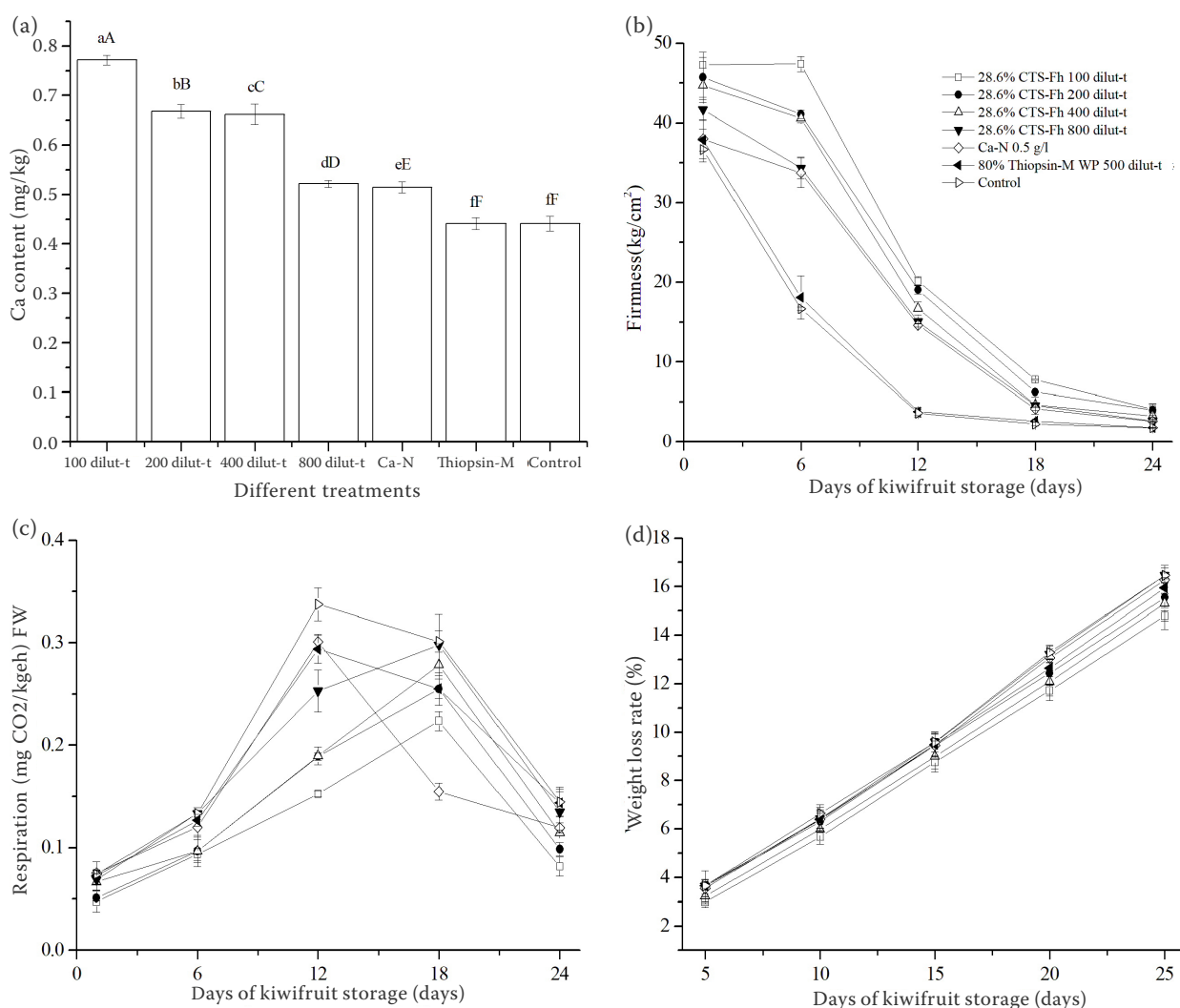


Fig. 2. The effects of the preharvest 28.6% CTS-Fh sprays on the Ca content (a), flesh firmness (2b), respiration (c), and weight loss rate (d) of the kiwifruit during storage

values represent the means of the replicates; the error bars represent the standard deviations of the mean ( $n = 3$ ); columns followed by the different lower cases and capital letters between the different treatments were the significant differences at a 5% level ( $P < 0.05$ ) and a 1% level ( $P < 0.01$ ), respectively

used to indicate the degree of a fruit's ripening and softening, we compared these to the samples of the chemical fungicide treated and control. The samples treated with the preharvest application of 28.6% CTS-Fh and Ca-N showed a significant increase and maintained flesh firmness (Fig. 2b) in the kiwifruit and a reduction in the weight loss during storage (Fig. 2d). The preharvest 28.6% CTS-Fh spray also effectively delayed the time at which the peak respiration rate occurs and lowered the magnitude of this peak respiration rate (Fig. 2c). The above results show that the CTS-Fh was better than the Ca-N treatment in the aspect of increasing the calcium content, firmness and decreasing the weight

loss rate in the kiwifruit. The possible reasons are that the chitosan and calcium composite film both have high antimicrobial activities and barrier properties in the fruit growth period to prevent the softening and decay. These results were in agreement with previous reports on postharvest chitosan and calcium treatments (YANG et al. 2010; YANG et al. 2012; NESLIHAN et al. 2016). Therefore, the preharvest chitosan and calcium composite film spray could be practical for maintaining the fruit quality and reducing the post-harvest losses of the kiwifruit during storage.

The vitamin C and chlorophyll content in all the treatments increased continuously during the storage (Fig. 3a, f), and both the content and the

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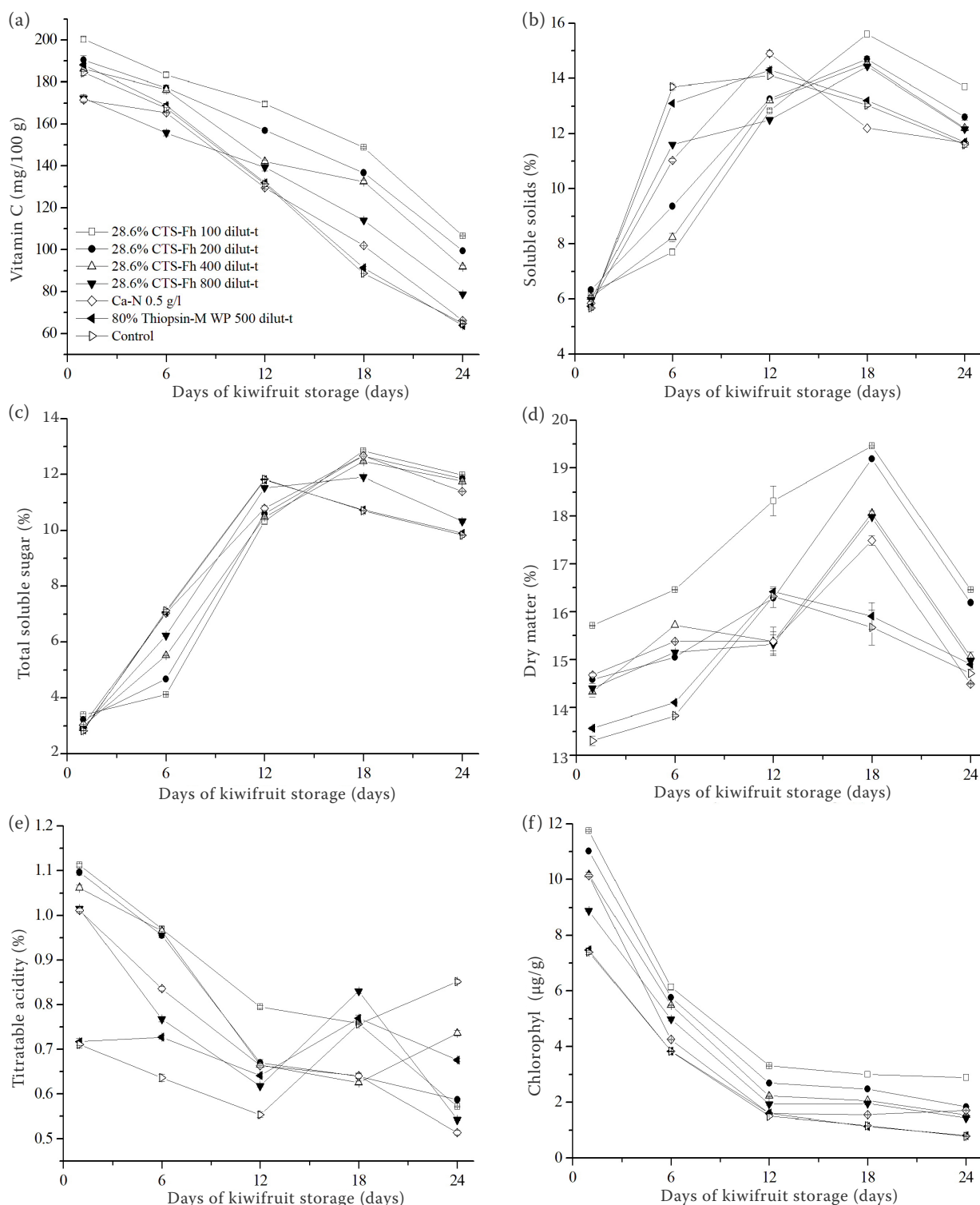


Fig. 3. The effects of the preharvest 28.6% CTS-Fh sprays on the changes in the vitamin C (a), soluble solid (b), total soluble sugar (c), dry matter (d), titratable acidity (e), and chlorophyll (f) content of the kiwifruit during storage the values represent the means of the replicates; the error bars represent the standard deviations of the mean ( $n = 3$ )

decreasing trend of the 28.6% CTS-Fh and Ca-N treatments were significantly higher and lower than the chemical fungicide treatment and the control

( $P < 0.05$ ), respectively. The preharvest 28.6% CTS-Fh sprays effectively delayed the time at which the peak soluble solid, the total soluble sugar and the

Table 3. The inhibition of the different CTS-Fhs to mycelial growth of *Botryosphaeria dothidea* and *Phomopsis sp.*

Pathogenic fungi	Tested pharmacy	Toxic regression equation	Correlatin coefficient (r)	EC <sub>50</sub> (mg/l)
<i>B. dothidea</i>	28.6% CTS-Fh	$y = 6.0206 + 0.8746x$	0.9990	68.11
	24% CTS-Fh	$y = 6.0213 + 0.8795x$	0.9993	69.00
	19% CTS-Fh	$y = 6.3511 + 1.2645x$	0.9901	85.40
	18% CTS-Fh	$y = 5.1942 + 0.8207x$	0.9960	579.89
<i>Phomopsis sp.</i>	28.6% CTS-Fh	$y = 5.8783 + 0.6764x$	0.9878	50.34
	24% CTS-Fh	$y = 5.7794 + 0.6363x$	0.9913	59.52
	19% CTS-Fh	$y = 5.9323 + 1.1281x$	0.9949	149.11
	18% CTS-Fh	$y = 5.4267 + 0.9425x$	0.9882	352.45

results are the average of three replicates

dry matter occurs and significantly increased the peak ( $P < 0.05$ , Fig. 3b–d). The titratable acidity of the chemical fungicide treatment and the control decreased substantially in the first 12 days of storage and increased afterwards (Fig. 3e). These results indicated that the preharvest 28.6% CTS-Fh sprays distinctly delayed the softening process and indirectly prolonged the storage period of the kiwifruit. It was reported that chitosan could delay the ripening of horticultural products by slowing down the production of anthocyanin and ethylene, it could retain the vitamin C, soluble carbohydrates, titratable acidity and fresh weight of the fruits (YANG et al. 2012; NESLIHAN et al. 2016). Our experiments demonstrated that the preharvest 28.6% CTS-Fh sprays had a similar effect on the postharvest kiwifruit.

The effects of the preharvest 28.6% CTS-Fh sprays on the soft rot development in the kiwifruit

The effect of the CTS-Fhs on the mycelial growth of the pathogenic fungi *in vitro* is shown in Table 3. As shown, the different CTS-Fhs were able to inhibit

the growth of two pathogenic fungi. Furthermore, this effect exhibited a dose-dependent effect. Among all the tested phytopathogenic fungi, 28.6% CTS-Fh caused the greatest inhibition of mycelium growth in both *B. dothidea* and *Phomopsis sp.* with the mycelial EC<sub>50</sub> values of 68.11 mg/l and 50.34 mg/l, respectively. Chitosan exhibited strong antifungal activity against several plant fungal pathogens (MENG et al. 2010; LIU et al. 2012), the remarkable antibacterial activity of 28.6% CTS-Fh should originate from the synergistic action of chitosan, ferulic acid and other components of composite films (GARCIA et al. 2005; SHUKLA et al. 2012; YANG et al. 2012; WILLIAM et al. 2016).

The kiwifruit soft rot in the harvested fruit caused by *B. dothidea* and *Phomopsis sp.* was effectively inhibited by the preharvest sprayed with the different concentrations of 28.6% CTS-Fh (Table 4). For instance, the cumulative incidence of soft rot was significantly reduced from 64.70 to 79.42% ( $P < 0.05$ ) after 25 days of storage by the preharvest sprayed with 28.6% CTS-Fh 100–400 dilut-t, compared with

Table 4. The effects of the preharvest 28.6% CTS-Fh sprays on the control of the kiwifruit soft rot

Treatments		Cumulative incidence (%)	Disease index	Control effect(%)
Pharmacy	Dilution times			
28.6% CTS-Fh	100	15.55 ± 2.22 <sup>e</sup>	4.44 ± 0.94 <sup>e</sup>	88.79 ± 2.29 <sup>a</sup>
	200	22.22 ± 2.22 <sup>de</sup>	7.04 ± 3.90 <sup>d</sup>	82.24 ± 2.68 <sup>b</sup>
	400	26.67 ± 3.85 <sup>cd</sup>	11.48 ± 1.71 <sup>c</sup>	71.03 ± 2.81 <sup>c</sup>
	800	35.55 ± 2.22 <sup>c</sup>	15.19 ± 4.21 <sup>bc</sup>	61.68 ± 2.13 <sup>d</sup>
Calcium nitrate	0.5 g/l	48.89 ± 4.44 <sup>b</sup>	22.96 ± 7.40 <sup>b</sup>	42.06 ± 2.78 <sup>e</sup>
80% Thiopsin-M WP	500	28.89 ± 2.22 <sup>cd</sup>	11.11 ± 1.92 <sup>c</sup>	71.96 ± 2.13 <sup>c</sup>
Control		75.55 ± 2.22 <sup>a</sup>	39.63 ± 1.98 <sup>a</sup>	—

values represent the mean ± s.d.;  $n = 3$ ; different upppercases mean significant differences between different treatments at 5% level ( $P < 0.05$ )

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the control fruit. Moreover, the samples treated with the preharvest application of 28.6% CTS-Fh 100–400 dilut-t showed higher control effects on the soft rot than the chemical fungicide and the Ca-N treatment. Studies have shown that calcium and dextrin had good film-forming and antibacterial properties (LANDMAN and FOCK 2006; SHUKLA et al. 2012; WILLIAM et al. 2016), and ferulic acid had a good antibacterial property (GARCIA et al. 2005). Our experiments demonstrated that 28.6% CTS-Fh would have better film-forming and antibacterial properties. It is possible that a natural barrier was formed on the fruits and leaves' surface by spraying 28.6% CTS-Fh 100–800 dilut-t on the surface of the kiwifruit plants, and it resulted in reducing the infection by pathogenic fungi. Our results are consistent with the findings in the preharvest oligochitosan sprays on jujube (MENG et al. 2010; YAN et al. 2012; LIU et al. 2012).

#### **The effects of the preharvest 28.6% CTS-Fh sprays on the defence responses in the kiwifruit**

The total phenolics and flavonoids are important secondary metabolites in plants, which themselves have resistance and play an extremely important role in plant defence reactions. The total phenolics and flavonoids showed a decreasing trend in the fruits during storage (Fig. 4a, b). The preharvest 28.6% CTS-Fh spray could help to increase and maintain the content of the total phenolics and flavonoids in the kiwifruit during storage ( $P < 0.05$ ). MDA was an important index to reflect the degree of the membrane lipid peroxidation, as shown in Fig. 4c, the preharvest 28.6% CTS-Fh treatment obviously decreased the accumulation of MDA ( $P < 0.05$ ). The soluble protein, the basis of the material and energy metabolism in the plant, firstly increased then decreased in all the samples, while the preharvest application of 28.6% CTS-Fh significantly increased its content ( $P < 0.05$ , Fig. 4d). SOD, POD and CAT were the key enzymes to remove the free radicals and the protective enzyme system in the plants, and PPO contributed to the reinforcement of the cell wall structure preventing the penetration of the pathogens. As shown in Fig. 4e, f, g, an increase in the SOD, POD and CAT activity in all the preharvest 28.6% CTS-Fh treatments' samples were observed during the first 18 days of stor-

age, afterwards it gradually decreased, and the peak values were later than the chemical fungicide treatment and the control. In addition, the preharvest 28.6% CTS-Fh treatment could effectively increase the SOD, POD, CAT and PPO activity ( $P < 0.05$ ).

Numerous studies have shown that chitosan and its derivatives can induce a resistance protein to resist pathogens in plants (YU et al. 2007; MENG et al. 2008; YAN et al. 2012). KIM et al. (2005) reported that the total amount of phenolic compounds significantly increased after the chitosan treatment, and the corresponding antioxidant activity increased at least 3.5-fold. Besides, the chitosan treatment significantly increased POD activity in the flesh around the wound of the pear fruit (MENG et al. 2010), the POD gene expression in the chitosan-treated fruit maintained relatively higher than that in the control fruit (MA et al. 2013). A similar increase of the chitosan inducing CAT activity in peaches suggested that chitosan exhibited an antioxidant capability (MA et al. 2013), due to the enhancement of CAT, which is helpful to eliminate the free radicals (CHEN 2008). Chitosan significantly increased the PPO activity in rice seedlings following the inoculation of the pathogens (LI et al. 2013). As an essential nutrient element for plants, calcium has the function of stabilising the membrane and wall of the cell and inducing plant disease resistance (NESLIHAN et al. 2016). Similarly, to the above results, the effective effects for eliciting plant defence responses of 28.6% CTS-Fh 100–400 dilut-t treatments were more than the Ca-N 0.5 g/l. In fact, the applied amount of calcium was equivalent for the 28.6% CTS-Fh 100 dilut-t with the Ca-N 0.5 g/l. The results presented here also show that the mixed application of chitosan and calcium should have obvious synergistic effects to induce the enhancement of defence responses in the kiwifruit.

#### **The effects of the preharvest 28.6% CTS-Fh sprays on the ultra-structural of the kiwifruit pericarp cell wall"**

The preharvest 28.6% CTS-Fh treatment (Fig. 5a–h): the cell wall structures were compact, tidy, large and had uniform thickness, a thickening phenomenon. The middle lamellae were obvious and had a bright-dark-bright district, with no intercellular space. The micro fibre filaments were arranged neatly and highly dense, and the kiwifruits

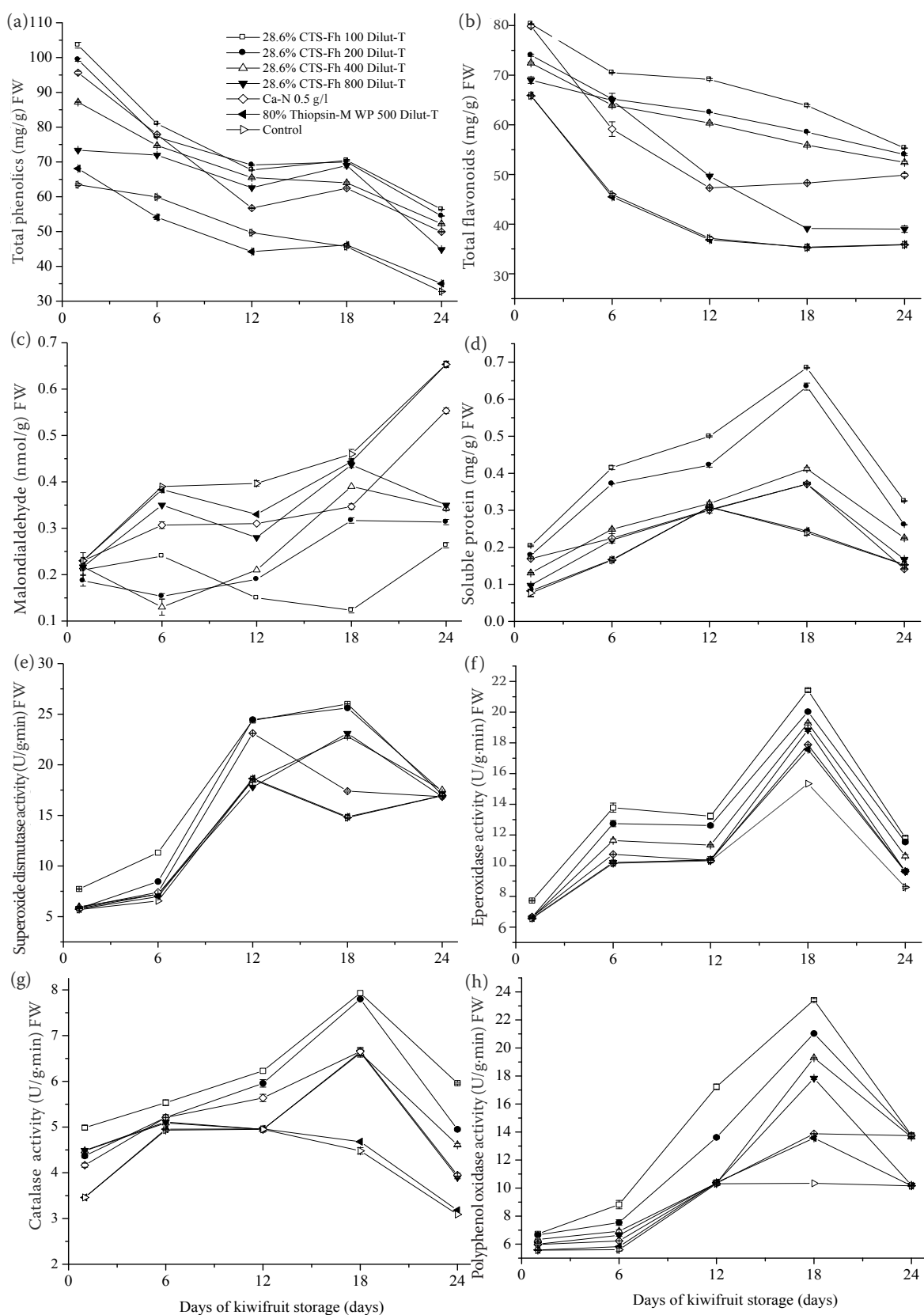


Fig. 4. The effects of the preharvest 28.6% CTS-Fh sprays on the changes in the total phenolic (a), total flavonoid (b), MDA (c) and soluble protein (d) content, the superoxide dismutase (e), eperoxidase (f), catalase (g), and polyphenol oxidase (h) activity of the kiwifruit during storage. Values are means of the replicates; the error bars - standard deviations of the mean ( $n = 3$ )

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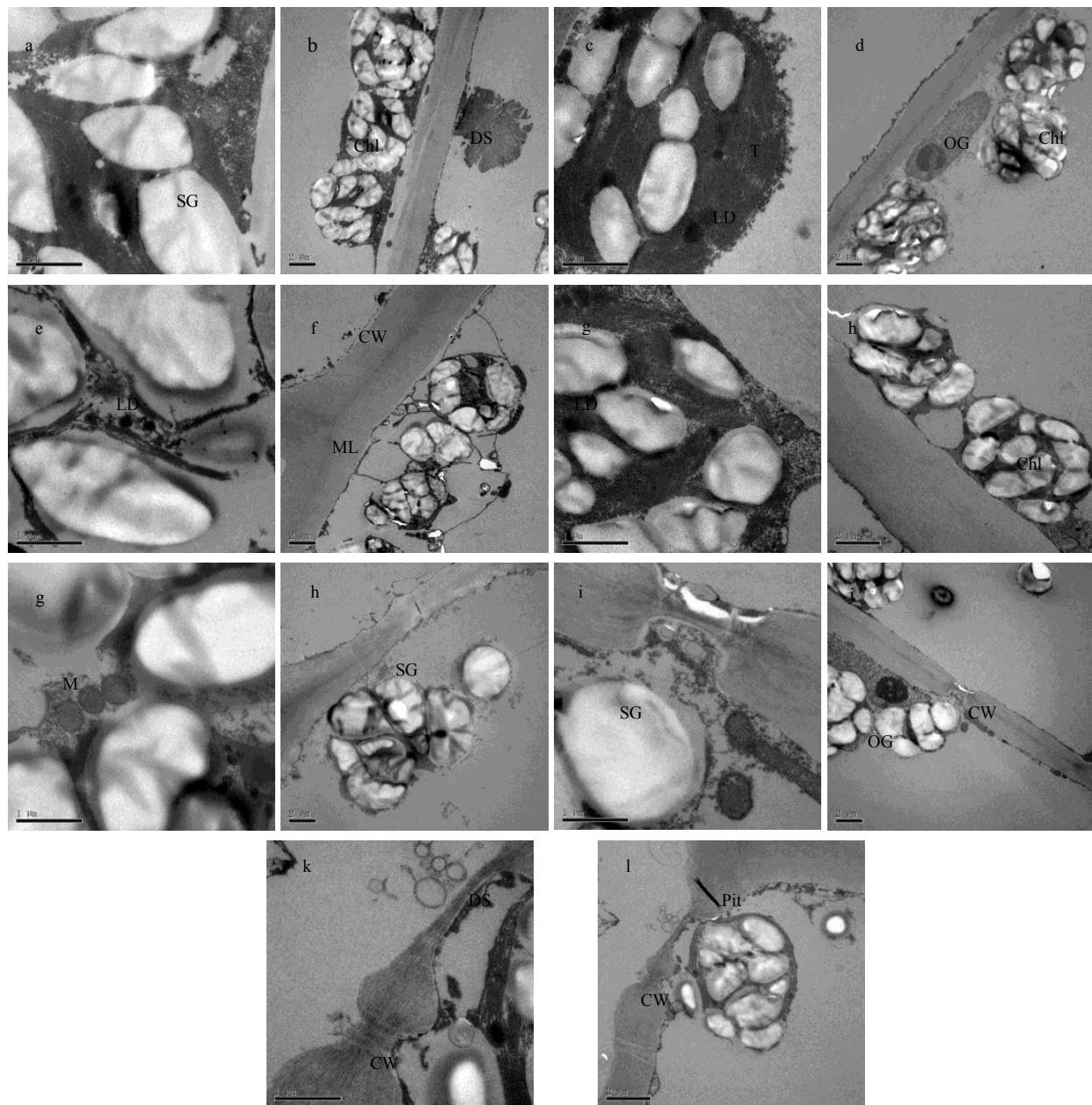


Fig. 5. The effects of the preharvest 28.6% CTS-Fh sprays on the ultra-structural changes of the kiwifruit pericarp cell wall. Abbreviations: CW – cell wall; ML – middle lamella; V – vacuole; SG – starch granule; Chl – chloroplast; M – mitochondrion; OG – osmiophilic granules; LD – lipid droplets; T – thylakoids layer. (a, b) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the 28.6% CTS-Fh 100 dilut-t( $\times 10\,000$ ;  $\times 30\,000$ ); (c, d) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the 28.6% CTS-Fh 200 dilut-t( $\times 10\,000$ ;  $\times 30\,000$ ); (e, f) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the 28.6% CTS-Fh 400 dilut-t( $\times 10\,000$ ;  $\times 30\,000$ ); (g, h) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the 28.6% CTS-Fh 800 dilut-t( $\times 10\,000$ ;  $\times 30\,000$ ); (i, j) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the Ca-N 0.5 g/l ( $\times 10\,000$ ;  $\times 30\,000$ ); (k, l) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the 80% Thiopsin-M WP 500 dilut-t( $\times 1\,000$ ;  $\times 30\,000$ ); (m, n) the ultra-structural of the cell of the kiwifruit preharvest sprayed with the control ( $\times 10\,000$ ;  $\times 30\,000$ )

were harder. The inclusions were very abundant, the structure of the starch granules was complete and

the quantity was more and larger. The structure and membrane of the chloroplast were complete and

the grana lamellae could be clearly seen. These effects exhibited a dose-dependent effect, among all the treatments, the 28.6% CTS-Fh 100–400 dilution was obvious. The Ca-N treatment (Fig. 5.i, j): the effects were roughly similar with the 28.6% CTS-Fh treatment, but the inclusions and starch grains were less. The chemical fungicide treatment and control (Fig. 5k–n): the cell walls had malformations, relaxation and were bent, gradually melted and fractured. The inclusions were very exiguous, and the starch granule quantity was few.

In the plant-pathogen interaction, the lignification of the cell wall was induced around the infection point of the pathogen by the chitosan treatment, a mechanism for the disease resistance and it provides plants with effective protection against pathogens (RAJAN et al. 2005; KIM et al. 2005). Moreover, cucumber fruits with calcium chloride treatment maintain the cell wall structure and membrane integrity in the fruit during storage (KIM et al. 2005). Our findings were in agreement with the previous reports, the preharvest chitosan and calcium composite film spray increased the cell wall strength by forming a network that controls the formation of the intercellular spaces, a process that contributes to the disease resistance and storage of the kiwifruit.

## CONCLUSION

This study shows that the preharvest plant 28.6% CTS-Fh sprays in the fruit growing season could reliably increase the yield, significantly improve the quality, notably delay the fruit ripening and softening, and consequently extend the storage life and reduce the postharvest losses of the kiwifruit during storage. The preharvest 28.6% CTS-Fh spray effectively inhibited the soft rot caused by the infection of *B. dothidea* and *Phomopsis* sp., noticeably induced the kiwifruit to produce a strong disease resistance system before or at harvest by increasing the defence-related secondary metabolites and enhancing the activity of some defence enzymes which are involved in the antioxidant metabolism such as SOD, POD, CAT and PPO. In addition, the preharvest 28.6% CTS-Fh sprays also increased the cell wall strength that contributes to the disease resistance and storage of the kiwifruit. Moreover, the main components of 28.6% CTS-Fh, chitosan and calcium should have favourable synergistic effects on the storage quality, control of the soft rot, and defence responses in the kiwifruit.

With the emergence of more and more food safety problems, alternative technologies for the wide use of chemical pesticides has been of great concern. The results from this study suggest that a composite application of chitosan and calcium could become a promising alternative to control the postharvest diseases in horticultural products. Though some work in the mechanisms of the chitosan composite film that inhibited the growth of pathogens and induced the plant immunity has been undertaken, implementation of the technology still faces some challenges, e.g., a combined transcriptome and proteome analysis of the key defence genes and the proteins that will enhance our understanding of the complicated chitosan-mediated signal pathway. An appropriate chemical modification and formula adjustment could significantly enhance its antimicrobial activities, and make it more suitable for field applications. Moreover, one can explore its application effect on more crop diseases and obtain a registered production license.

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