Effect of ferulic acid and natamycin treatments on quality and reactive oxygen species metabolism of postharvest blackberry

HONGXIA LIU – ZHENGJIN HUANG – LIANFEI LYU – SUFAN FAN – WENLONG WU – WEILIN LI

Summary
A series of physiological changes takes place in the postharvest blackberry (*Rubus* sp.) fruit. We investigated the effects of ferulic acid and natamycin on quality and antioxidant capacity in the fruit. The results showed that the nutrients consumption was delayed by their action. The skin and flesh of fruit had changed after treatments as seen by microstructural observation. The combined treatment group had smooth skins and clear flesh folds, while the other groups showed varying levels of disruption. The single and combined treatments increased the relative electrolyte leakage and the malondialdehyde content of the fruit. The content of reactive oxygen species increased with time, including the superoxide radical and hydrogen peroxide, this increase being the lowest in the combined treatment group. The combined treatment significantly increased the superoxide dismutase, catalase and peroxidase activities, and also reduced cell membrane damage, which causes the postharvest caducity of the blackberry fruit, thereby increasing its shelf life and maintaining its quality. This study provides a basis for improvement in postharvest storage of blackberry fruit.

Keywords
ferulic acid; natamycin; blackberry; quality; reactive oxygen species

The production and consumption of blackberries are increasing substantially, not only because of their unique flavour and high nutrient content, but also for their medicinal efficacy [1]. However, the shelf life of the mature blackberry is less than a week at 0 °C, because of both rapid changes in its physicochemical properties and pathogen growth after harvest [2]. In recent years, postharvest handling and packaging methods have been adapted to preserve fresh blackberries, including the use of low temperatures [3], modified atmosphere packaging [4] and various packaging materials [5]. In our previous study, we found that the combination of ferulic acid and natamycin showed synergistic antifungal activity against two pathogenic fungal isolates, and improved the fresh blackberry fruit quality together with extension of the shelf life for up to 12–15 days [6].

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2) and the hydroxyl radical($^{\bullet}OH$), have been implicated in the postharvest senescence of fresh products [6]. Various postharvest treatments have been tested to effectively improve the antioxidant defense systems of fruit by inhibiting their ROS production, including UV-C irradiation [7], hypobaric treatment in peach fruit [8] and allyl isothiocyanate treatment of blueberries [9]. Ferulic acid mainly occurs in the seeds and leaves of fruits, vegetables [10]. It exerts a wide range of biomedical effects, including antioxidant, antiallergic, hepatoprotective, anticarcinogenic, anti-inflammatory, and antimicrobial effects [11]. It is also used as a cross-linking agent in food preservation [12]. Natamycin has been used as a natural food additive to control the growth of many microorganisms. When a chitosan-water solution containing natamycin was added to ground cherry, it reduced the levels of ROS and increased the activities of antioxidant enzymes, thus improving the quality during storage [13].

The aim of this study was to evaluate

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the effects of a combined treatment by ferulic acid and natamycin on the postharvest free radical metabolism and the antioxidation system of the blackberry fruit.

MATERIALS AND METHODS

Sample preparation, treatment and storage

Variety 'Hull' blackberry fruits were grown in Lishui, Nanjing, China, and brought to the laboratory within 2 h after harvest. The selected fruits were of uniform size and colour, and were also free from damage and disease. The blackberry fruits were treated with ferulic acid at 0.512 g·l^{-1}, by natamycin at 0.001 g·l^{-1} and by the combination of them at 0.128 g·l^{-1} and 3.125 × 10^{-5} g·l^{-1}, respectively [5]. Eighty fruits were randomly separated into four groups and dipped for 5 min in solutions. After treatment, the fruits were stored at 4 °C and 90 % ± 5 % relative humidity (*RH*) for 12 days. Fruits treated by sterile water were used as the control group.

Analysis of nutrient contents

The blackberries for each treatment group were ground into a paste in a mortar. The mass fraction of soluble solids (*SSC*) in the juice were determined with a PAL-1 hand-held refractometer (Atago, Tokyo, Japan), which was calibrated before each measurement and the results were expressed as percentage.

Titratable acidity (*TA*) of the juice was measured by titration with 0.1 mol·l^{-1} NaOH to an end-point of pH 8.2 with a ZD-2 automatic potentiometric titrator (Shanghai Jinmai Instrument, Shanghai, China) [14]. The pH meter was calibrated before each measurement. *TA* was calculated with the Eq. 1 and expressed as the percentage of malic acid.

$$TA = \frac{C_1(V_1 - V_2)KF}{m} \times 100 \quad (1)$$

where C_1 is concentration of NaOH standard titration solution (in moles per litre), V_1 is volume of NaOH standard titrated solution consumed during titration (in millilitres), V_2 is volume of standard titration solution of NaOH consumed in blank test (in millilitres), K is conversion factor for acid, F is dilution ratio of test solution, m is sample mass (in grams or millilitres).

The protein content was determined with a quantitative assay kit for soluble protein (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) and expressed as gram of fresh fruit weight per kilogram.

3 ml coomash bright blue solution (Nanjing Jiancheng Bioengineering Institute) was added to 0.05 ml double steamed water, protein standard and sample, mixed well, and standed for 10 min, and then measured absorbance.

$$C = \frac{(A_2 - A_0)}{(A_1 - A_0)} \times C_1 \times d \quad (2)$$

where C is protein content in sample, C_1 is the protein content in standard, d is dilution times, A_0 is absorbance of double steamed water, A_1 is is absorbance of protein standard pipe, A_2 is is absorbance of the test sample.

The *SSC*/*TA* ratio was calculated for each type of treatment and sampling day from the results for *SSC* and *TA*.

The total anthocyanin content (*TAC*) was determined by the pH-differential method [15]. The blackberries were ground and blended to a homogeneous mixture in a mortar. Anthocyanin was extracted by mixing 1 ml of the mixture with 9 ml of acidified (0.01% HCl) ethanol in a ratio 80 : 20 at 45 °C for 2.5 h, and then centrifuged for 15 min at 5 000 ×g. The samples were diluted 1 : 10 with HCl-acidified 8.2% sodium acetate of pH 1.0 or HCl-acidified 1.5% KCl of pH 4.5 before the absorbance was read at wavelengths of 510 nm and 700 nm with a 759 UV spectrophotometer (Shanghai Jinghua Technology Instrument, Shanghai, China). *TAC* was calculated using Eq. 3 and expressed as milligrams of fresh fruit weight per kilogram of fresh weight.

$$TAC = \frac{A \cdot M_w \cdot DF}{\varepsilon \cdot m \cdot L} \times V \quad (3)$$

where M_w is the molecular weight of cyanidin 3-glucoside (449.38 g·mol^{-1}); DF is the dilution factor; ε is the extinction coefficient (26 900 l·cm^{-1}mol^{-1}); m is the weight of fresh fruit; L is the path length of the spectrophotometer in centimetres; and V is the total volume of the solvent required to extract the blackberry fruits.

$$A = (A_{510} - A_{700})_{\text{pH1.0}} - (A_{510} - A_{700})_{\text{pH4.5}} \quad (4)$$

where A_{510} is the absorbance value of the test sample at 510 nm, A_{700} is the absorbance value of the test sample at 700 nm,

Five to ten blackberries per treatment were measured, and the values were averaged.

Microstructural observation

The four groups of blackberries were cut into pieces of 3 × 3 × 1 mm, and fixed overnight with 2.5% glutaraldehyde. They were then dried with a K850 critical point dryer (Quorum Technologies, Laughton, United Kingdom). The morphol-

ogy of the sections was analysed with a Quanta 200 scanning electron microscopy (FEI, Hillsboro, Oregon, USA). Samples K1 and K2 were the skin and flesh of control group, respectively. Samples L1 and L2 were the skin and flesh of of ferulic acid-treated group, respectively. Samples M1 and M2 were the skin and flesh of atamycin-treated group, respectively. Samples N1 and N2 were the skin and flesh of the group treated with a combination of ferulic acid and natamycin, respectively.

Membrane permeability and lipid peroxidation

Membrane permeability was determined as the relative electrolyte leakage, as measured by a modification of the previously published method [15]. The level of lipid peroxidation was expressed as content of malondialdehyde (MDA), which was determined with a malondialdehyde assay kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Content of MDA was expressed as micromoles per kilogram of blackberry tissue.

Superoxide anion and H_2O_2 levels

Superoxide anion content was determined by the modified method of Joo et al. [2]. Blackberry fruit (5.0 g) were sliced and homogenized in 45 ml of 50 mmol·l^{-1} K-phosphate buffer (pH 7.8) for 30 s at 4 °C. After centrifugation at 8 000 ×*g* for 20 min at 4 °C, the free radical levels were determined in the supernatant. The H_2O_2 content was determined with a hydrogen peroxide assay kit (Nanjing Jiancheng Bioengineering Institute) by a colorimetric method, according to the manufacturer's instructions. Superoxide anion and H_2O_2 contents were both expressed as micromoles per kilogram of blackberry tissue.

Antioxidant enzyme activities

To assay the antioxidant enzyme activities, 5 g of blackberry fruits was homogenized with 45 ml of 8.5 g·l^{-1} NaCl solution containing 20 g·l^{-1} insoluble polyvinylpyrrolidone on ice. The homogenate was centrifuged at 6 000 ×g for 10 min at 4 °C, and the supernatant was used as the crude enzyme extract. The superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities were determined with the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. The SOD, CAT and POD activities were all expressed as enzyme activity units per gram of blackberry tissue. In the reaction system, the amount of enzyme corresponding to the inhibition rate of 50% was a SOD activity unit. Decomposition of 1 μmol of H_2O_2 per second per milligram of tissue protein was a CAT enzyme activity unit. A POD enzyme activity unit was defined as the amount of enzyme catalysing 1 μg of the substrate per milligram of tissue protein per minute at 37 °C.

Statistical analysis

All statistical analyses were performed with SPSS version 19.0 (IBM, Armonk, New York, USA). Values within a column followed by the same lower-case letter were not statistically significantly different according to Duncan's multiple range test at $P < 0.05$.

RESULTS AND DISCUSSION

Nutrient contents

The initial values and evolution of *SSC*, *TA*, the soluble protein and *TAC* during storage at 4 °C and 90% relative humidity with the different treatments are shown in Tab. 1. *SSC* and soluble protein increased but *TA* and *TAC* declined during storage. The mass fraction of *SSC* after the combined treatment was lower than after the other treatments at the end of the storage period. However, it did not differ significantly from the results for the control and natamycin treatment groups. Application of combined treatment significantly affected *TA*, which changed from 1.8 % to 1.4 % at the end of the storage period. This reduction was associated with the ripening process [16]. The *SSC*/*TA* ratios increased in all the groups during storage, primarily due to the large reduction in *TA* during this period. *TA* (1.8 %), the content of soluble protein (3.1 g·kg^{-1}) and *TAC* (1.12 g·kg^{-1}) were higher in the combined treatment group than in the other groups, and the differences were significant.

A series of physiological and biochemical changes occur in postharvest fruit, and postharvest decay causes considerable economic losses [17]. Postharvest treatment is necessary to maintain fruit quality. The combined application of ferulic acid and natamycin better improved the fruit quality than all the other treatments [6]. Its effects on firmness, weight loss and the rot ratio were discussed in our previous study. It was reported that little or no change in *SSC* occurs after packaging and storage at low temperatures [3]. However, *SSC* in three berries (blackberry, raspberry and strawberry) declined after treatment with calcium chloride [18]. The change in *SSC* was also dependent on the containers used [2]. The increase in *SSC* in apples was attributed to the dehydration process during storage and to the enzymatic conversion of larger polysaccharides, such as starches and

Tab. 1. Content of nutritional components in blackberry fruits.

Treatment	Storage time [d]				
	0	3	6	9	12
Mass fraction of soluble solid [%]					
K	9.7 ± 0.2^{a}	9.6 ± 0.6^{a}	9.7 ± 0.2^{a}	9.9 ± 0.1^{b}	10.6 ± 0.7^{b}
L	9.7 ± 0.2^{a}	9.7 ± 0.2^{a}	9.6 ± 0.3^{a}	9.5 ± 0.1^{a}	9.5 ± 0.2^{a}
M	9.7 ± 0.2^{a}	9.9 ± 0.1^{a}	10.1 ± 0.3^{a}	9.9 ± 0.1^{b}	10.3 ± 0.7^{b}
N	9.7 ± 0.2^{a}	9.6 ± 0.3^{a}	9.9 ± 0.1^{a}	9.8 ± 0.1^{b}	10.0 ± 0.3^{b}
Titratable acidity [%]					
K	2.0 ± 0.3^{a}	1.8 ± 0.0^{a}	1.6 ± 0.1^{a}	1.5 ± 0.1^{a}	1.4 ± 0.1^{a}
L	2.0 ± 0.3^{a}	1.8 ± 0.1^{a}	1.8 ± 0.1^{b}	1.7 ± 0.4^{b}	1.6 ± 0.1^{b}
M	2.0 ± 0.3^{a}	1.9 ± 0.1^{a}	1.6 ± 0.1^{a}	1.5 ± 0.1^{a}	1.4 ± 0.0^{a}
N	2.0 ± 0.3^{a}	1.9 ± 0.1^{b}	1.8 ± 0.0^{b}	1.8 ± 0.1^{c}	1.8 ± 0.2^{c}
***SSC*/*TA* ratio**					
K	5.0 ± 0.8^{a}	5.4 ± 0.1^{b}	6.1 ± 0.3^{b}	6.6 ± 0.1^{c}	7.6 ± 0.0^{d}
L	5.0 ± 0.8^{a}	5.3 ± 0.0^{b}	5.4 ± 0.2^{a}	5.6 ± 0.1^{b}	6.0 ± 0.1^{b}
M	5.0 ± 0.8^{a}	5.3 ± 0.0^{b}	6.2 ± 0.2	6.5 ± 0.2^{c}	7.1 ± 0.1^{c}
N	5.0 ± 0.8^{a}	5.1 ± 0.1^{a}	5.5 ± 0.1^{a}	5.4 ± 0.2^{a}	5.5 ± 0.3^{a}
Soluble proteins [g·kg^{-1}]					
K	2.5 ± 0.2^{a}	2.6 ± 0.3^{a}	2.8 ± 0.1^{a}	3.2 ± 0.3^{a}	2.8 ± 0.2^{a}
L	2.5 ± 0.2^{a}	3.2 ± 0.1^{a}	3.2 ± 0.3^{ab}	3.6 ± 0.1^{a}	3.0 ± 0.2^{b}
M	2.5 ± 0.2^{a}	2.8 ± 0.5^{a}	3.6 ± 0.1^{b}	3.4 ± 0.7^{a}	3.1 ± 0.1^{b}
N	2.5 ± 0.2^{a}	3.1 ± 0.5^{a}	3.3 ± 0.2^{ab}	3.6 ± 0.3^{a}	3.3 ± 0.1^{c}
Anthocyanin contents [g·kg^{-1}]					
K	1.18 ± 0.02^{a}	1.04 ± 0.01^{a}	0.86 ± 0.02^{a}	0.82 ± 0.8^{a}	0.77 ± 0.02^{a}
L	1.18 ± 0.02^{a}	1.08 ± 0.04^{b}	0.93 ± 0.02^{b}	0.86 ± 11.0^{b}	0.81 ± 0.02^{b}
M	1.18 ± 0.02^{a}	1.01 ± 0.02^{a}	0.85 ± 0.01^{a}	0.80 ± 6.7^{a}	0.74 ± 0.02^{a}
N	1.18 ± 0.02^{a}	1.12 ± 0.01^{c}	0.98 ± 0.02^{c}	0.90 ± 10.9^{b}	0.86 ± 0.02^{c}

Values represent mean ± standard deviation.
SSC – mass fraction of soluble solid, *TA* – titratable acidity. K – control group, L – ferulic acid treatment group, M – natamycin treatment group, N – combined treatment group utilizing ferulic acid and natamycin.

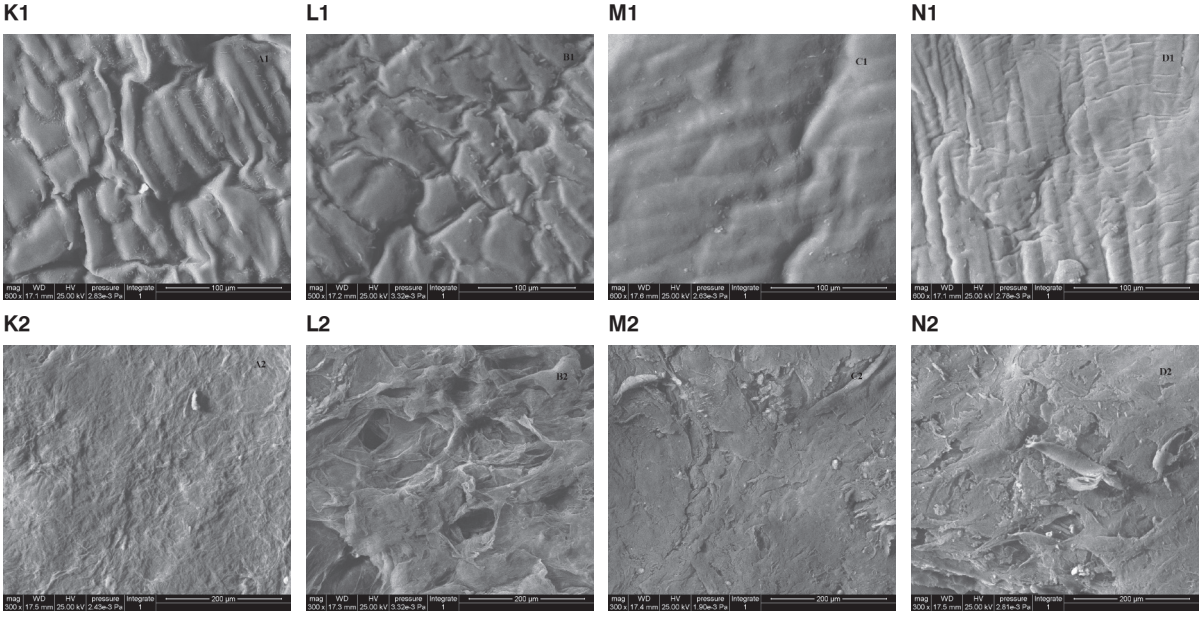

Fig. 1. Cell microstructure of blackberry fruits visualized by scanning electron microscopy.

K1 – skin of control group, K2 – flesh of control group, L1 – skin of ferulic acid treatment group, L2 – flesh of ferulic acid treatment group, M1 – skin of natamycin treatment group, M2 – flesh of natamycin treatment group, N1 – skin of combined treatment group, N2 – flesh of combined treatment group.

pectins, to simple sugars [19]. It was reported that *TA* and the *SSC/TA* ratio are better indicators of blackberry maturity than *SSC* alone [20]. A higher *SSC/TA* ratio was used to indicate good taste [21]. However, a higher *SSC/TA* ratio was associated with faster decay in blueberries [16]. A reduction in *TAC* in 'Chester' blackberries was also observed during storage at 3 °C [22]. The main anthocyanin of blackberries, cyanidin 3-*O*-monoglucoside, showed a smaller reduction in calcium-treated samples [18]. Anthocyanins are relatively unstable and tend to break down over time [23]. The growing conditions of the fruit and various laboratory procedures means differing laboratory methods of extraction and analysis contribute to these changes [24].

Microstructural evaluation

The changes in the skin and flesh of the fruit are shown in Fig.1. The control group (K1) had severe skin shrinkage and the skin was not smooth. The epidermis of the fruit in the ferulic acid-treated group (L1) was slightly shrunken and was relatively smoother than that in the control group. The epidermal shrinkage was more severe in the natamycin-treated group (M1) than in the ferulic acid-treated group, but milder than that in the control group. The fruits in the combined treatment group (N1) had smooth skin, which was consistent with the original characteristics of the variety. In the view of the flesh, the skin and flesh were severely separated in the control group, resulting in a relatively smooth cross-section (K2). The ferulic acid-treated group had clear flesh folds (L2) and the combined treatment group had similar characteristics (N2). However, in the natamycin-treated group, the skin was markedly separated from the pulp (M2), but less so than in the control group. The findings of our SEM analysis of differently treated blackberries were consistent with the results for firmness [6].

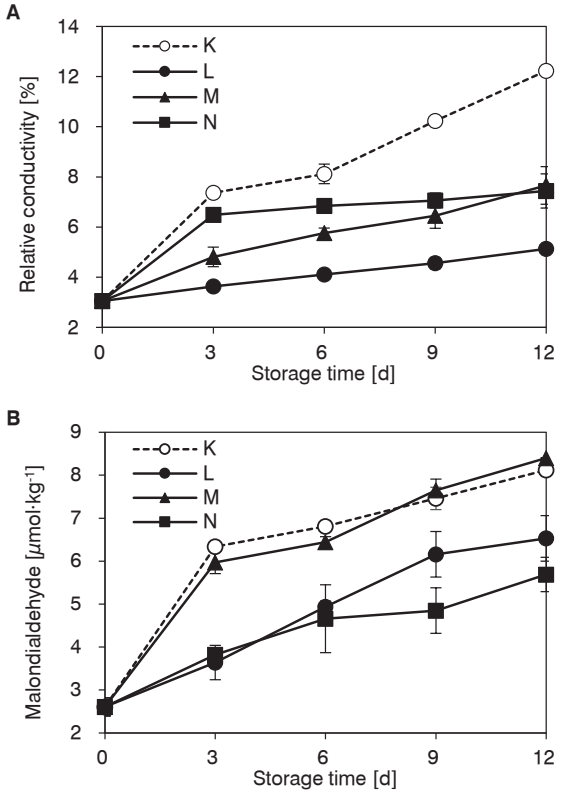

Fig. 2. Relative electrolyte leakage and malondialdehyde content of blackberry fruits.

A – relative conductivity, B – malondialdehyde content.
K – control group, L – ferulic acid treatment group, M – natamycin treatment group, N – combined treatment group (ferulic acid and natamycin).

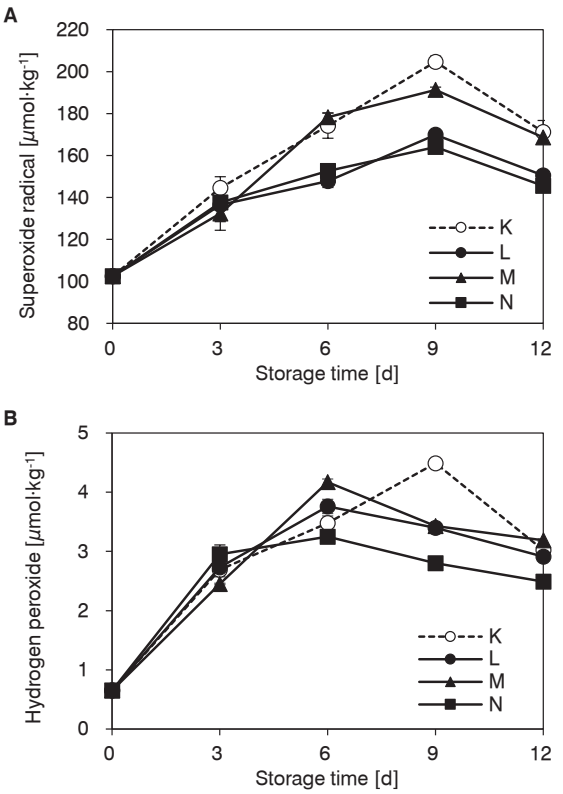

Fig. 3. The superoxide anion and H_2O_2 contents of blackberry fruits.

A – superoxide anion content, B – H_2O_2 content.
K – control group, L – ferulic acid treatment group, M – natamycin treatment group, N – combined treatment group (ferulic acid and natamycin).

Relative electrolyte leakage and malondialdehyde content

As shown in Fig. 2, the relative electrolyte leakage of the blackberry fruit gradually increased in all treatment groups during the whole storage period, but it increased rapidly in the control group. The combined treatment group had the lowest value of relative conductivity (7.4 %) of all the groups. The MDA content increased rapidly during the storage period in all groups. The combined treatment caused the fruit to maintain the significantly lowest MDA content (5.69 μmol·kg^{-1}) compared with the other groups. The level of MDA indirectly reflects the severity of free radical attack on cells [25]. These results indicated that the combined treatment protected the cells of blackberries from membrane damage during storage. A study of 'Daw' longan fruit treated with chlorine dioxide demonstrated that ROS levels correlated positively with the MDA content [25]. Therefore, the combined treatment might have improved the antioxidant activity that protects membrane integrity.

Superoxide anion and H_2O_2 contents

The superoxide anion content increased significantly in the first 6 days of storage in all the groups, and then began to fall in the treatment groups, whereas in the control group, it continued to rise until day 9 (Fig. 3). In the combined treatment group, the H_2O_2 content showed the same trend as superoxide anion. The superoxide anion and H_2O_2 contents reached 145 × 10^{-3} mmol·kg^{-1} and 2.49 mmol·kg^{-1}, respectively, on day 12, which were lower than those in the control and other treatment groups. These results indicated that the postharvest treatments, especially the combined treatment, inhibited the accumulation of superoxide anion and H_2O_2 in the fruit during storage and protected the cells from ROS-induced injury by scavenging ROS. The processes of fruit ripening and senescence are accompanied by a continuous increase in oxidation, which may lead to the accumulation of ROS, producing oxidative damage. This results from the imbalance between the production and scavenging of ROS, and reduces the shelf life and marketability of fruits and vegetables [26]. H_2O_2 is a key regulator of the physiological processes of senescence, excessive H_2O_2 triggering oxidative stress in plant cells [27]. Generation of superoxide anion may cause peroxidation of membrane lipids, and the product of superoxide anion combined with hydroxyl caused damage to DNA of cell. [28], and the superoxide anion and H_2O_2 levels were consistent with the MDA contents.

Antioxidant enzyme activities

The changes in SOD, CAT and POD activities are shown in Fig. 4. The SOD activity decreased during storage but remained higher in the combined treatment group (46.54 U·g^{-1}) than in the other groups. The POD activity increased stably in the first 6 days and continued to grow rapidly over

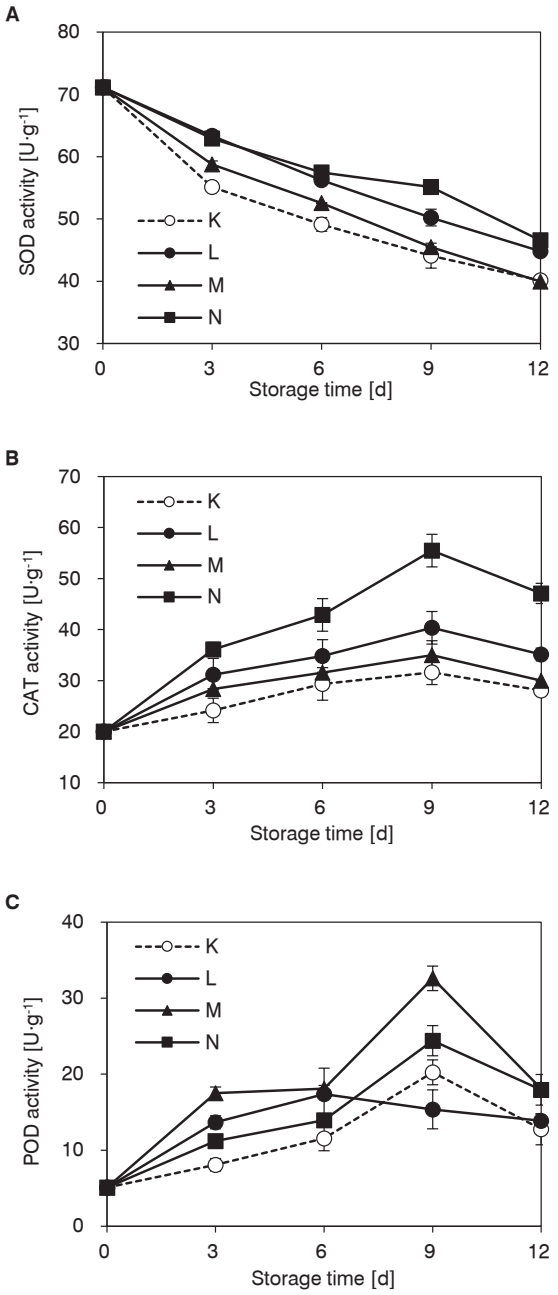

Fig. 4. Antioxidant enzyme activities of blackberry fruits.
A – superoxide dismutase activity (SOD), B – catalase activity (CAT), C – peroxidase activity (POD).
K – control group, L – ferulic acid treatment group, M – natamycin treatment group, N – combined treatment group (ferulic acid and natamycin).

the next 3 days, except for the ferulic acid treatment group, in which it decreased in last 6 days. The activities of POD were higher in the natamycin treatment (18.11 $U \cdot g^{-1}$) and combined treatment (17.94 $U \cdot g^{-1}$) groups than in the other two groups. The CAT activity increased steadily in first 9 days, after which it decreased. The highest CAT activity was detected in the combined treatment group (47.10 $U \cdot g^{-1}$). The enzymatic antioxidant system plays a key role in controlling the production of ROS [29], thus regulating, in part, the degree of lipid peroxidation, which reduces membrane fluidity and increases the leakiness of the membrane to various substances [30]. SOD converts superoxide anion to H_2O_2 to protect cells from oxidant stress, whereas CAT and POD decompose H_2O_2 [6]. The actions of antioxidant enzymes have been regarded as a part of mechanism in lipid peroxidation and senescence in many horticultural crops. The induction of SOD, ascorbate peroxidase (APX), and CAT to scavenge superoxide anion and H_2O_2 caused a delay in UV-C-induced senescence in peach fruit [7]. In the present study, the activities of SOD, POD and CAT increased simultaneously in the combined treatment group, corresponding to the lower superoxide anion, H_2O_2 and MDA levels detected. These results suggest that the combined treatment induces ROS scavenging by enhancing the activities of antioxidant enzymes in the blackberry fruit, thus reducing lipid peroxidation and delaying senescence.

CONCLUSION

The quality of postharvest blackberry fruit treated with natural preservative agents and their ROS metabolism were investigated. In general, all the treatments had a good effect on the fruit quality, in particular the combined treatment. The combined treatment protected the quality of the blackberry fruit, which delayed the nutrients consumption and reduced the degradation rate of anthocyanin. The ROS (superoxide anion and H_2O_2) levels decreased and the activities of antioxidant enzymes (SOD, CAT and POD) increased after the combined treatment. Therefore, the combined treatment induced the scavenging of ROS to some extent, thus alleviating lipid peroxidation and delaying the senescence of blackberry fruit.

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