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Physiological response of chestnuts (*Castanea mollissima Blume*) infected by pathogenic fungi and their correlation with fruit decay

Anyan wen [a], Yiyi Zhu [b], Yangyang Geng [c], Likang Qin [a,d,e,*]

[a] School of Liquor and Food Engineering, Guizhou University, Guiyang, 550025, China
[b] Zhijin County Market and Quality Supervision Administration, Zhijin, 552100, China
[c] Guizhou Institute of Walnut, Guiyang 550005, China
[d] Key Laboratory of Agricultural and Animal Products Storage and Processing of Guizhou Province, Guiyang 550025, China
[e] National & Local Joint Engineering Research Center for the Exploitation of Homology Resources of Medicine and Food, Guiyang 550025, China

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ABSTRACT

The postharvest decay of chestnuts (*Castanea mollissima Blume*) limits their industrial utilization, and pathogenic fungi are the main cause for chestnut decay. In this study, the physiological changes and their correlation with the rotting degree of chestnuts were investigated during single or mixed infection by *Fusarium proliferatum*, *Penicillium crustosum*, and *Alternaria alternata*. During the infection period, the activities of cell wall degrading enzymes (CWDEs) and antioxidant enzymes firstly increased and then decreased, the contents of nutrients decreased, but the levels of physiological indexes increased. The mycelium and spores of pathogenic fungi colonized the intercellular space, and then the mycelium covered the entire tissue surface of chestnuts, leading to the disappearance of cell structure. Notably, the most prominent changes in physiological indexes and ultrastructure were observed in chestnuts infected with three pathogenic fungi. Furthermore, the rotting degree of chestnuts was positively correlated with the levels of CWDEs, antioxidant enzymes and malondialdehyde.

1. Introduction

Food security is related to national security, human security, and sustainable development (Fei, Shuang, & Xiaolin, 2023). Given the growing variety of food preferences, it is necessary to actively investigate the development potential for forest foods and ensure the security of national grain and oil supplies. Chestnuts (*Castanea mollissima Blume*) are a popular forest food crop in Asia, Europe, and America due to their nutritional value, unique taste, and pleasant aroma (Massantini, Moscetti, & Frangipane, 2021). Chestnut fruit is rich in starch, protein, functional polysaccharides, essential fatty acids, vitamins, and minerals (Santos et al., 2023). Additionally, chestnuts have various health benefits such as lowering cholesterol, preventing obesity and diabetes, fighting tumours, and improving the immune system (Li et al., 2022a). However, chestnut fruit is prone to rot and deteriorate during the harvest and storage periods because of high moisture content and rich nutrients, thus seriously affecting their quality and production value (Rodrigues, Driss, Gomes-Laranjo, & Sampaio, 2022). At present, approximately >50% of chestnut production loss is caused by rot and deterioration during storage and transportation (Vettraino et al., 2019). Therefore, it is crucial to investigate the underlying causes for chestnut rot and deterioration to reduce decay and economic losses in chestnut industry.

One of the main causes for chestnut rot during storage is the infection of pathogenic fungi in chestnut fruit. Various fungal pathogens have been identified in chestnuts, including *Penicillium*, *Alternaria*, *Mucor*, *Rhizopus*, *Botryosphaeria*, *Sclerotinia*, *Fusarium*, *Parvum*, and *Trichothecium* (Li et al., 2022b). These fungi can be divided into two main categories. The first category of fungi infects chestnut fruit during their growth period in the planting environment. These fungi firstly remain dormant and then induce diseases after fruit harvest or during storage (Galsurker, Diskin, Maurer, Feygenberg, & Alkan, 2018). The second category of fungi infects fruit after harvest, and they usually enter the fruit through wounds on the surface of the kernel or are introduced in the water spraying process during storage (Zakaria, 2022). However, the types of pathogenic fungi causing rot in chestnuts vary across different production areas due to the differences in geographical environment, climate conditions, soil types, and cultivation levels (Al-Tayyar, Youssef, & Al-Hindi, 2020). *Penicillium* sp. is the most commonly isolated genus in European sweet chestnuts (*Castanea sativa*) and American chestnuts

* Corresponding author at: College of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China.
E-mail address: lkqin@gzu.edu.cn (L. Qin).

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Table 1
Results of the activities of cell wall degrading enzymes produced by pathogenic fungi in different induction culture medium(U/mL).

Samples	Exogenous inducers	Carboxymethyl cellulase	β-Glucosidase	β-Galactosidase	Xylanase	Pectate lyase	Polygalacturonase
Fusarium proliferatum	fresh chestnut	21.58 ± 0.17de	135.16 ± 0.24d	111.77 ± 0.92e	13.62 ± 0.45f	26.19 ± 0.74e	58.99 ± 0.54e
	CCS	24.54 ± 0.33b	145.64 ± 0.47b	108.76 ± 0.60f	15.47 ± 0.43c	28.08 ± 0.32 cd	52.03 ± 0.43i
	xylan	24.00 ± 0.27b	114.60 ± 1.82j	127.48 ± 0.60b	18.32 ± 0.47a	24.81 ± 0.46f	53.81 ± 0.19 h
	pectin	25.55 ± 0.40a	127.66 ± 1.18f	68.76 ± 0.61j	16.71 ± 0.60b	31.90 ± 0.72b	63.82 ± 0.39b
Penicillium crustosum	fresh chestnut	21.23 ± 0.46de	131.65 ± 0.51e	108.02 ± 0.64f	14.04 ± 0.34def	27.77 ± 0.57d	57.16 ± 0.42f
	CCS	25.17 ± 0.38a	141.27 ± 0.49c	84.51 ± 0.42 h	14.62 ± 0.27de	28.65 ± 0.29c	55.64 ± 0.43 g
	xylan	23.40 ± 0.15c	122.17 ± 0.29 h	129.10 ± 0.38a	15.88 ± 0.44c	27.54 ± 0.42d	53.80 ± 0.69 h
	pectin	24.45 ± 0.31b	124.78 ± 0.24 g	98.56 ± 0.24 g	14.64 ± 0.37de	31.06 ± 0.76b	64.85 ± 0.38a
Alternaria alternata	fresh chestnut	21.78 ± 0.15d	135.14 ± 0.30d	118.88 ± 0.42c	13.83 ± 0.60ef	28.81 ± 0.44c	56.15 ± 0.78 fg
	CCS	24.56 ± 0.33b	150.10 ± 0.59a	116.37 ± 0.55d	16.11 ± 0.86b	31.22 ± 0.66b	59.97 ± 0.61d
	xylan	21.81 ± 0.13d	126.39 ± 0.44f	126.37 ± 0.51b	18.74 ± 0.41a	23.36 ± 0.40 g	56.57 ± 0.39f
	pectin	21.16 ± 0.30e	120.87 ± 0.50i	72.83 ± 0.39i	14.94 ± 0.69 cd	33.71 ± 0.35a	61.84 ± 0.59c

CCS: carboxymethyl cellulose sodium. All of the data were expressed as means ± standard deviations. Different letters (a - m) in the same composition indicate significant difference ($P < 0.05$).

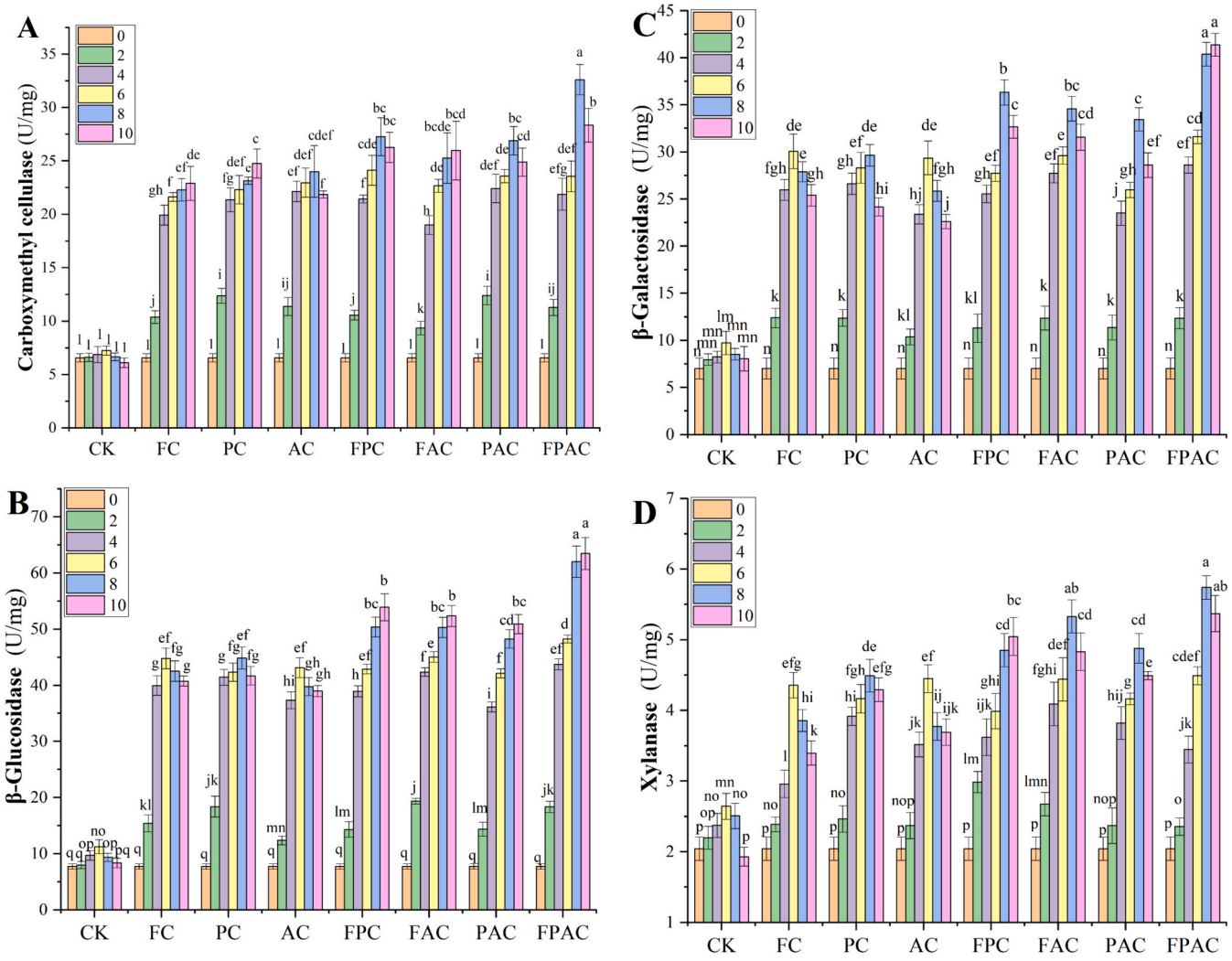


Fig. 1. Changes of the activities of carboxymethyl cellulase (A), β-glucosidase (B), β-Galactosidase (C), xylanase (D), Pectate lyase (E) and polygalacturonase (F) in chestnuts during storage. Different letters in the same composition indicate significant difference ($P < 0.05$). CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

(*Castanea americana*). *Alternaria* sp., *Mucor* sp., *Rhizopus* sp., and other pathogenic fungi are also prevalent (Rodrigues et al., 2022). In Asia, common pathogenic fungi include *Alternaria* sp., *Fusarium* sp., *Parvum* sp., *Trichothecium* sp., *Penicillium* sp., *Ozonium* sp., *Aspergillus* sp., *Colletotrichum* sp., and *Nigrospora sp* (Jiang & Tian, 2019; Zhang et al., 2022). The community structure and isolation of pathogenic fungi have been

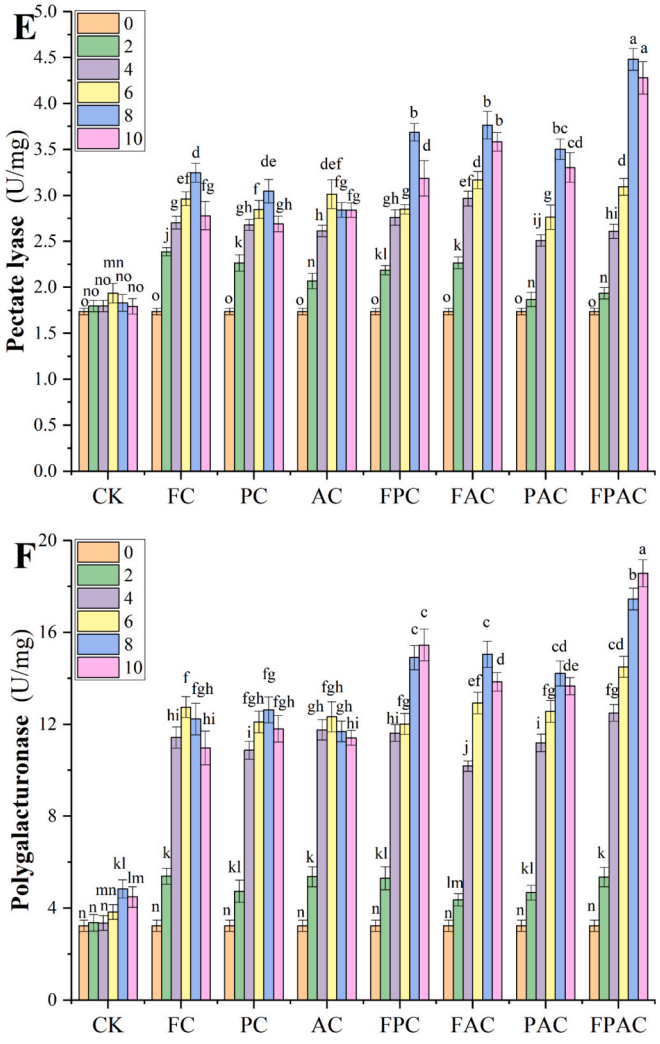


Fig. 1. (*continued*).

extensively explored, but the mechanisms of pathogenic fungal invasion in chestnuts were seldom reported.

Chestnut fruit provides favourable conditions for the invasion and reproduction of pathogens due to their high nutrient contents and strong respiratory intensity (Possamai, Dallemole-Giaretta, Gomes-Laranjo, Sampaio, & Rodrigues, 2023). After being harvested, chestnuts remain viable seeds with a well-functioning defence enzyme and active oxygen-free radical clearance system. However, if the regulation of defence enzymes is insufficient to eliminate harmful substances produced in the body, chestnuts undergo a series of physiological and biochemical changes, thus resulting in metabolic disorders and weakened disease resistance (Xiao et al., 2021). These changes increase the likelihood of pathogen invasion, worsen fungal diseases, and finally lead to decay. For instance, the contents of total soluble solid, soluble protein, starch, moisture, and malondialdehyde (MDA) gradually decreased in the decay process of chestnut fruit, whereas the content of soluble sugar increased. The contents of MAD, starch, yeast and mold count were associated with chestnut fruit decay (Li et al., 2022b). Therefore, it is necessary to explore the physiological and biochemical changes of chestnuts to prevent fungal diseases.

The planting area and production of chestnuts in China ranked first in the world (Qin et al., 2023). Wangmo chestnuts from China have been certified as a National Geographical Indication product, due to their large fruit size, high water content, and rich nutrients such as high-quality carbohydrates, proteins, vitamins, and minerals (Ban et al., 2020). In our previous study, we isolated the major pathogenic fungi from Wangmo chestnuts, including *Fusarium proliferatum*, *Penicillium crustosum*, and *Alternaria alternata* (Zhu, Wen, Wang, Hu, & Qin, 2023). Nevertheless, the physiological mechanisms underlying the invasion of the three pathogenic fungi in chestnuts remain unknown. Hence, we analyzed the variations in the activities of CWDEs and antioxidant enzymes during the invasion process of three pathogenic fungi in chestnuts at 25 °C and examined the changes in physiological and quality indicators as well as ultrastructure. The study aims to establish the correlation between pathogenic fungal invasion and fruit quality and provide valuable references for the development of post-harvest disease prevention and control technology for chestnuts.

2. Materials and methods

2.1. Samples and treatment

Chestnuts (*Castanea mollissima Blume*, Guizhou wild chestnuts) came from Guizhou Guangxiu Ecological Food Co., Ltd., and were produced in Wangmo County, Guizhou Province. *Fusarium proliferatum*, *Penicillium crustosum*, and *Alternaria alternata* were isolated from Wangmo rotten chestnuts and stored in the laboratory (Zhu et al., 2023). β-Galactosidase, superoxide dismutase, and α-amylase activity detection reagent kits were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All fungi in this experiment were cultured in potato

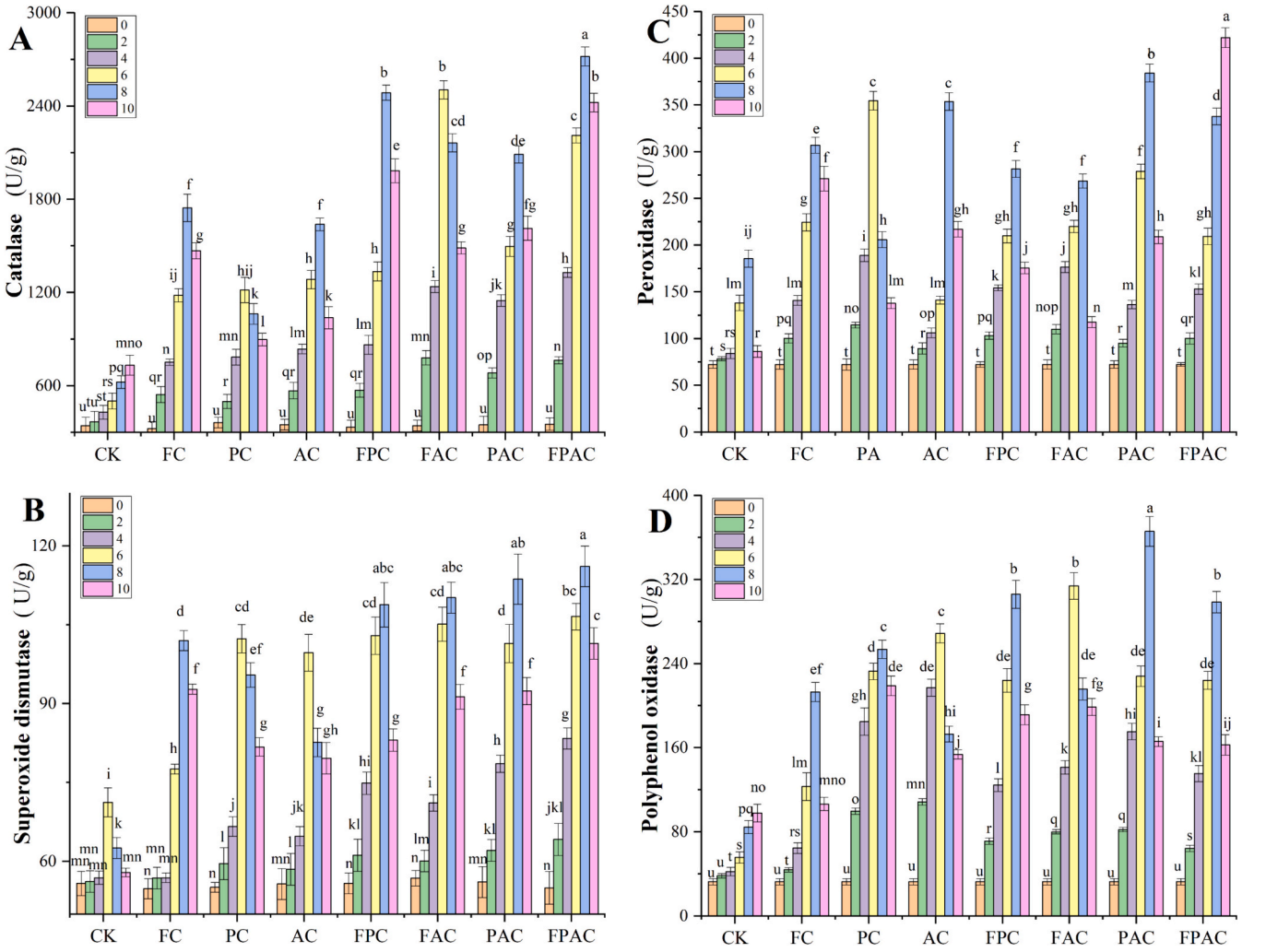


Fig. 2. Changes of the activities of Catalase (A), superoxide dismutase (B), peroxidase (C), and Polyphenol oxidase (D) in chestnuts during storage. Different letters in the same composition indicate significant difference ($P < 0.05$). CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

dextrose agar (PDA) medium (Haibo Biotechnology Co., Ltd., Qingdao, Shandong, China). All other chemicals and reagents were of analytical grade and purchased from Sinopharm Chemical Reageant Co. Ltd. (Suzhou, Jiangsu, China) and were of analytical grade.

2.2. Infection of chestnuts by pathogenic Fungi

The previous infection method of chestnuts with some modifications was adopted in the study (Zhang et al., 2022). The pathogenic fungi were inoculated on PDA medium at 26 °C for 4 d. The fungus blocks (5 mm in diameter) were taken from the edge of the colony for use. Healthy chestnuts were soaked in 75% ethanol for 300 s, then rinsed with sterile water three times, and air-dried on the stay-clean workbench (Jiangsu Jinghua Purification Equipment Co. Ltd., Shaoxing, Zhejiang, China). The shell and testa at the terminal bud of chestnuts were stripped off and a wound was made with a sterile corkborer on the terminal bud of each chestnut. The total inoculation of chestnuts was six mycelium blocks, and the spore concentrations of each mycelium block (5 mm in diameter) of *Fusarium proliferatum*, *Penicillium crustosum*, and *Alternaria alternata* were 4.90×10^6, 1.31×10^7, 4.75×10^5 CFU/mm^2, respectively. Chestnuts were inoculated with 6 mycelium blocks of a single fungal species to prepare three samples (Table S1): *Fusarium proliferatum* (FC), *Penicillium crustosum* (PC) and *Alternaria alternata* infected chestnut samples (AC). Chestnuts were inoculated with 6 mycelium blocks of every two fungal species (at a ratio of 1:1) to prepare three samples: *Fusarium proliferatum* and *Penicillium crustosum* (FPC), *Fusarium proliferatum* and *Alternaria alternata* (FAC), as well as *Penicillium crustosum* and *Alternaria alternata* infected chestnut samples (PAC). Chestnuts were inoculated with 6 mycelium blocks of three fungal species at a ratio of 1:1:1 to prepare FPAC samples: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnut samples. Chestnuts were treated with sterile PDA medium to prepare control check samples (CK). All treated samples were incubated at 25 °C for 10 d and were collected at an interval of 2 d. The tissue blocks of 5 mm × 5 mm size were obtained from the disease-health junction of all collected chestnut samples in an ultra-clean bench, respectively. Then, tissue blocks were sterilised with 75% ethanol for 30 s, rinsed three times with sterile water and dried with sterilised filter paper. Finally, the tissue blocks were rapidly ground and crushed with liquid nitrogen, and stored in an ultra-low temperature refrigerator at −80 °C for further analysis.

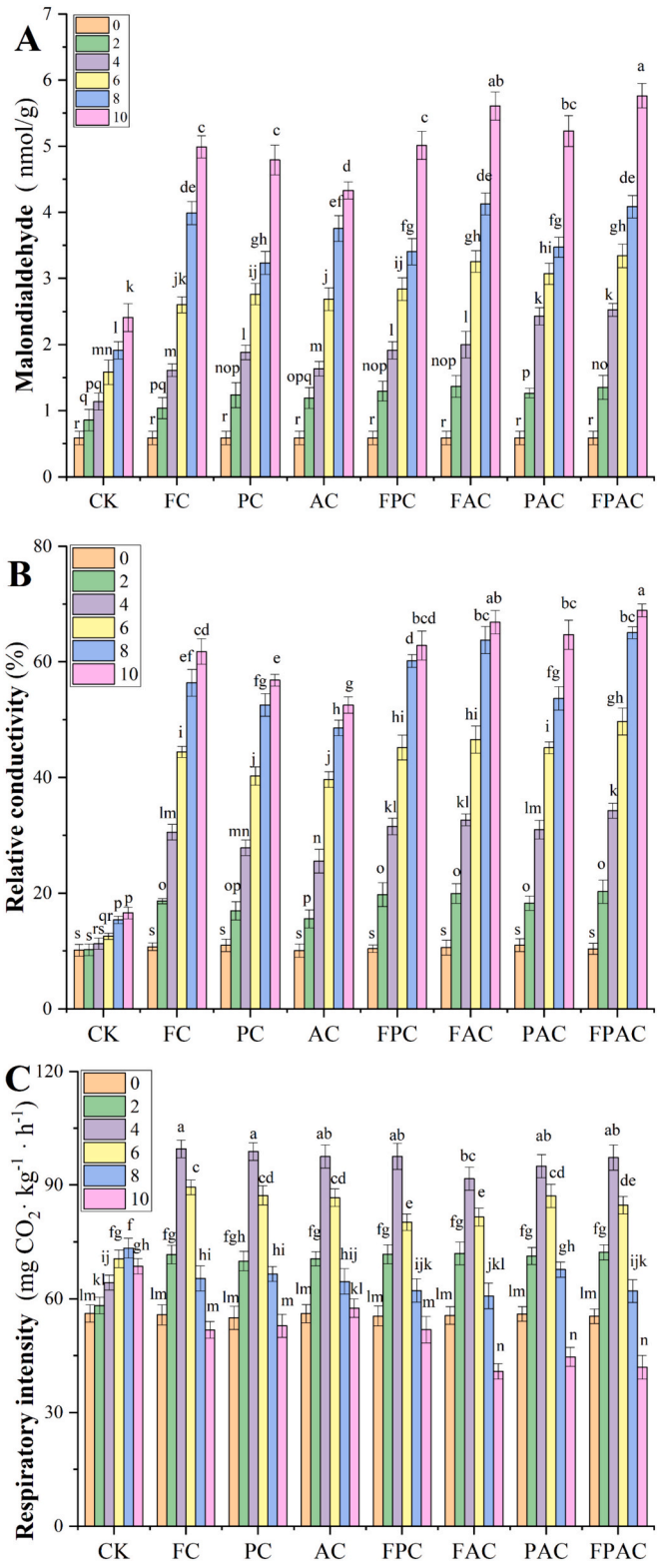


Fig. 3. Changes of malondialdehyde content (A), relative conductivity (B), and respiratory intensity (C) in chestnuts during storage. Different letters in the same composition indicate significant difference ($P < 0.05$). CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

2.3. Determination of cell wall degrading enzymes (CWDEs)

2.3.1. Induction of CWDEs in vitro

The induction of cell wall degrading enzymes in vitro was performed with the method described by Wang, Hao, Wang, and Ling (2019). Czapek liquid medium was prepared with 3 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, 0.01 g/L $FeSO_4$ 1 g/L K_2HPO_4, and 10 g/L inducer. The used inducers were chestnut fruit, sodium carboxymethyl cellulose, pectin and xylan, respectively. Subsequently, 5 mycelial blocks of pathogenic fungi with 5 mm diameter were obtained from PDA medium and included in different induction media, and cultured at 25 °C under shaking conditions for 7 d. The excess mycelium and spores were removed by vacuum filtration. Then, the filtrate was centrifuged at 7000*g* for 30 min at 4 °C, and the supernatant was obtained as the crude enzyme solution for further analysis.

2.3.2. Extraction of CWDEs in chestnuts infected by pathogenic fungi

Approximately 10.0 g of the chestnut sample was placed in a mortar, and then 40 mL of 1 mol/L NaCl was added. The mixed sample was ground at 4 °C, collected in a centrifuge tube and centrifuged at 8000*g* for 600 s at 4 °C. The supernatant was collected for enzyme activity determination.

2.3.3. Determination of CWDEs

The activities of polygalacturonase (PG), carboxymethyl cellulase (Cx), β-glucosidase (β-Glu) and xylanase were determined by the method of Li, Li, and Wang (2021). The enzyme activities were calculated based on reducing sugar released in enzyme reactions, the absorbance values of the reaction mixtures were measured at 540 nm with a UV–visible spectrophotometer (Shanghai Yidian Analytical Instrument Co., Ltd., Shanghai, China), and the enzyme activities were calculated based on reducing sugar released by the enzyme reaction. Pectate lyase (PL) activity was determined according to the method of Ge et al. (2017). β-Galactosidase (β-Gal) activity was assayed according to the instructions of the β-Gal Activity Assay Kit. PG, Cx, β-Glu and xylanase activities were expressed as U/g or U/mL, where one enzyme activity unit was defined as 1 μmol of reducing sugar per minute per millilitre of enzyme solution (per gram of tissue fresh weight) catalyzing the release of the substrate.

2.4. Determination of antioxidant enzyme activity

Catalase (CAT) activity was determined according to the method by Yu et al. (2008). The peroxidase (POD) activity was determined according to the method described by Wang (2007). Polyphenol oxidase (PPO) activity was determined according to the method of Xia et al. (2022). Superoxide dismutase (SOD) activity was determined according to the instructions of the SOD activity assay kit.

2.5. Determination of physiological indexes

Malondialdehyde (MAD) content was determined according to the method of Liu (2016). The determination method of the relative conductivity referred to Jin et al. (2016). The respiration intensity of chestnuts was determined with an FT-GX20 Fruit and Vegetable Respirometer (Shandong Anpal Environmental Technology Co., Ltd., Shandong, China). The experimental and control groups were analyzed after 0, 2, 4, 6, 8, and 10 d of storage, respectively. The dosage of chestnuts in each group was 300 g. The testing time was 300 s, and the tests were repeated three times. The unit of respiration intensity was expressed in mg $CO_2/(kg \cdot h)^{-1}$.

2.6. Determination of quality indexes

The moisture content of chestnuts was determined with an MT-C fully automatic moisture tester (Brabender GmbH & Co KG, Duisburg,

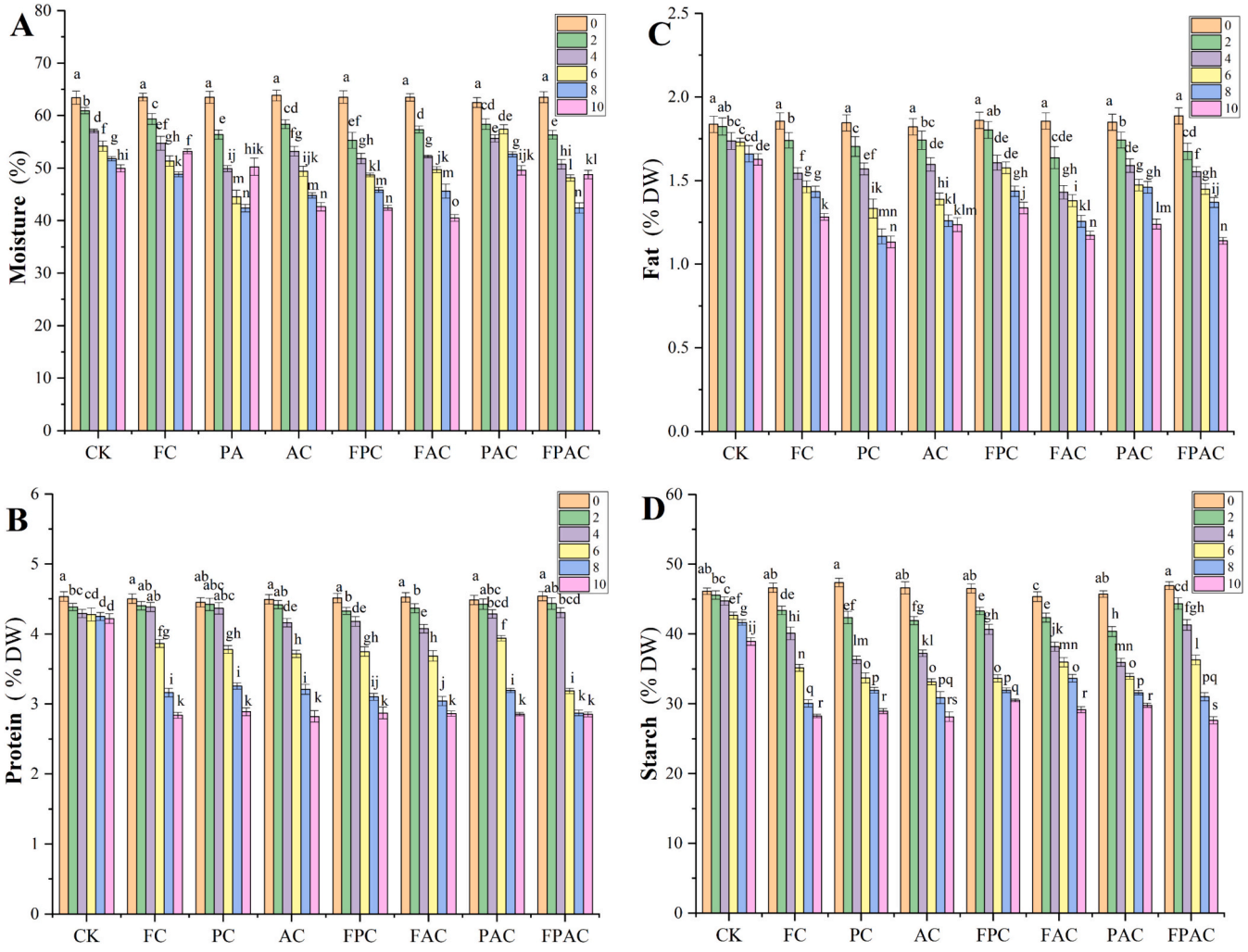


Fig. 4. Changes of the contents of moisture (A), protein (B), fat (C), starch (D), reducing sugar (F), and α-amylase activity (E) in chestnuts during storage. Different letters in the same composition indicate significant difference ($P < 0.05$). CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

Germany). Fat content was determined regarding the Soxhlet extraction method in the Chinese national standard GB5009.6-2016 "Determination of fat in foods" (2016). Protein content was detected concerning the Kjeldahl method in Chinese National Standard GB5009.5-2016 "Determination of Protein in Foods" (2016). Starch content was determined with the acid hydrolysis method and expressed as glucose equivalents (Li et al., 2022b). α-Amylase activity was measured according to the instructions of the Solebo α-Amylase Activity Assay Kit. Reducing sugar content was determined with the DNS method (Deshavath, Mukherjee, Goud, Veeranki, & Sastri, 2020).

2.7. Observation on the ultrastructure of chestnut cells

Based on the method described by Han et al. (2021), chestnut samples were placed in 2.5% glutaraldehyde solution and fixed at 4 °C for 12 h. The samples were washed three times with 0.1 mol/L PBS buffer, subjected to dehydration of gradient ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 min at each stage, and then substituted with isoamyl acetate at 4 °C for 15 min. The replaced samples were placed in a sample cage and dried in a CO_2 critical point dryer. Finally, the dried samples were pasted onto the sample stage with conductive adhesive, and the samples were sprayed with gold particles by using an ion sputtering spraying instrument for 30 s and then observed under a scanning electron microscope.

2.8. Statistical analysis

All the samples were analyzed with three replicates at each time point and the results were expressed as means ± standard deviation. The data were analyzed with the one-way ANOVA method in SPSS 20.0 software (SPSS Inc., Chicago, United States of America). The correlation was evaluated with the Pearson correlation coefficient (r).

3. Results and discussion

3.1. Effect of fungal infection on CWDEs of chestnuts

CWDEs are key pathogenic factors for the successful infection of host plants by pathogenic fungi (Kim, Lee, Lee, Nam, & Seo, 2018). Most of CWDEs are adaptively secreted by pathogenic fungi in the presence of appropriate substrates. The addition of exogenous inducers could assess the type and activity of CWDEs and analyze the correlation between the

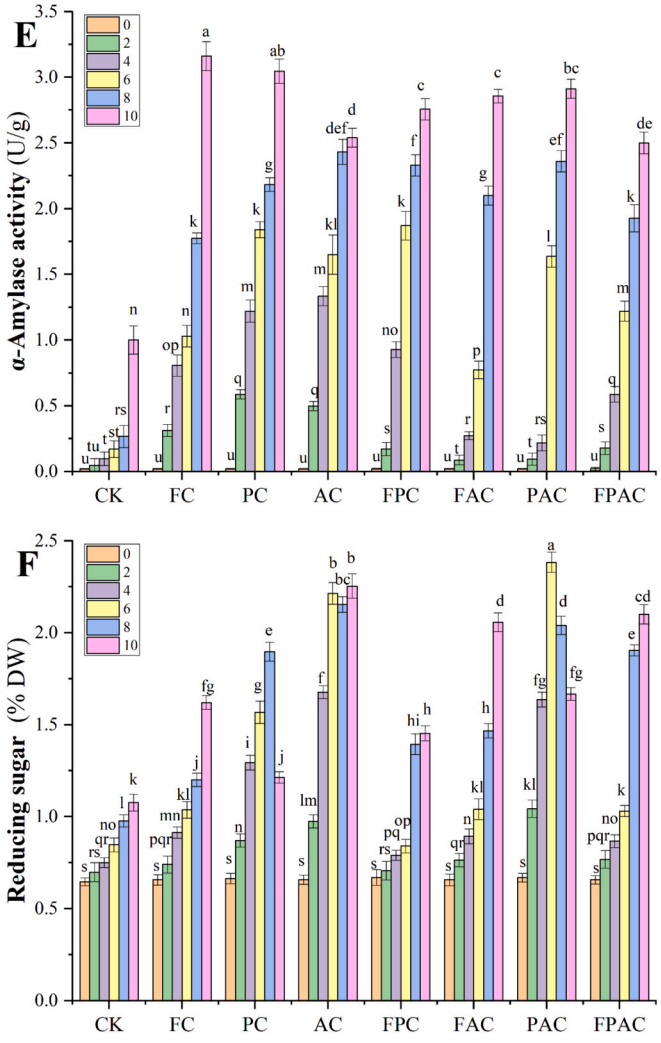


Fig. 4. (*continued*).

enzyme-producing and colonising abilities of pathogenic fungi (Sharafaddin et al., 2019). Plant cell walls are mainly composed of lignin, cellulose, pectin, and hemicellulose (Dabravolski & Isayenkov, 2023). Thus, to determine the types and activies of CWDEs secreted by three pathogenic fungi, the enzyme activities were detected in four induction culture media (fresh chestnut, carboxymethyl cellulose sodium, pectin, and xylan). Cellulases (Cx, β-Glu), hemicellulases (β-Gal, xylanase), and pectinases (PL, PG) were detected in all four induction culture media (Table 1). The activities of CWDEs produced by the same pathogenic fungi in different induction culture media showed significant differences ($P < 0.05$), and the activities of CWDEs ranked in the following order: β-Glu > β-Gal > PG > PL > Cx > xylanase. The genes encoding CWDEs in pathogenic fungi are divided into major genes and redundant genes. When the major genes are suppressed, the related redundant genes are rapidly expressed to produce the same enzymes or enzymes with the same functions. Therefore, three pathogenic fungi could secrete six CWDEs in media with different inducers (Li, Yuan, Jiang, & Li, 2019). To sum up, three pathogenic fungi produced six CWDEs in four induction culture media, β-Glu and β-Gal were the main causes of plant cell wall degradation by pathogenic fungi.

The cell wall of chestnuts is a complex polysaccharide network structure, which is the first line of defence against pathogen invasion and expansion. To disrupt the cell wall barrier, pathogenic fungi utilize the mechanical force of appressorium or secrete CWDEs, so as to colonize plant tissues, take over nutrients from host tissues, and destroy host cells (Foster, Ryder, Kershaw, & Talbot, 2017). The activities of six CWDEs in all samples after 0 to 4 d of storage exhibited a linear increase trend and then experienced a slow rising trend from 6 to 8 d (Fig. 1). However, after 8 d, the activities of CWDEs tended to slightly decrease or be stabilized. Chestnuts secrete CWDEs, which can hydrolyse glycosidic bonds to produce reducing sugar and maintain the normal operation of vital activity. Hence, the activities of CWDEs in CK samples gradually increased slowly. Similarly, the activities of CWDEs were higher in chestnuts infected with fungi than those in CK samples, as reported by Qian et al. (2019). The difference might be interpreted as follows. Most pathogenic fungi secrete numerous CWDEs to penetrate the cell wall and thus infect fruit and the fruit infected with phytopathogens are stimulated to release more CWDEs (Lagaert, Beliën, & Volckaert, 2009).

Notably, the enzyme activities of chestnuts with mixed inoculation were higher than those of samples with single inoculation, and the enzyme activities of β-Glu, β-Gal, and Cx were higher. The activities of CWDEs were positively correlated with the strain virulence and infectivity. The higher the enzyme activity was, the stronger the invasion ability of the CWDEs, indicating that β-Glu, β-Gal and Cx had the stronger invasion ability to chestnuts. Among them, the activity of β-Glu was higher than that of the other five CWDEs, as reported by Farian, Cholewa, Cholewa, Matczuk, and Angelina (2020), indicating that β-Glu played a crucial role in the fungal infection process of chestnuts. Additionally, the activities of CWDEs of FPAC samples were higher than those of other samples after 8 d, inferring that three spoilage fungi could synergistically induce the secretion of CWDEs. However, the exact

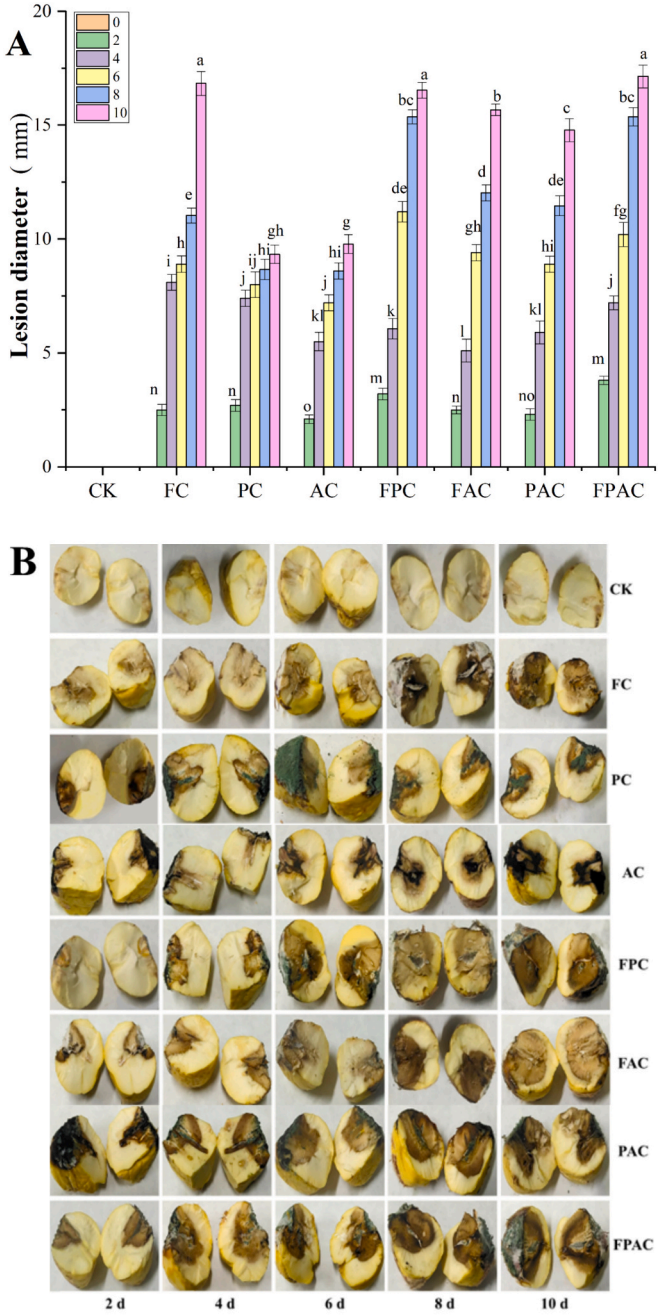


Fig. 5. Changes of lesion diameter (A) and surface Appearance (B) in chestnuts during storage. Different letters in the same composition indicate significant difference ($P < 0.05$). CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

synergistic effect of three spoilage fungi is uncertain and needs to be further explored.

3.2. Effect of fungal infection on antioxidant enzymes of chestnuts

One of the initial responses of fruit to the invasion of pathogens is the increase of reactive oxygen species (ROS), but excessive ROS may lead to peroxidation damage of membrane lipids, and affect cell integrity and physiological functions. To minimize ROS damage, chestnuts have evolved a complex antioxidant defence system in which antioxidant enzymes can scavenge ROS within chestnut fruit, thereby reducing the damage caused by ROS to chestnut tissues (Xiao et al., 2021). The antioxidant enzyme activities increased first and then decreased during the invasion process of pathogenic fungi (Fig. 2). The enzyme activities were ranked as CAT > POD > PPO > SOD. SOD catalyzes superoxides to O_2·- and H_2O_2, and then POD and CAT convert H_2O_2 into H_2O, thereby alleviating the toxic effects of H_2O_2. Except that the highest CAT activities of PC and FAC samples were observed at 6 d, the highest CAT activity of the remaining samples was at 8 d (Fig. 2A), and the CAT activity of FPAC samples was the highest (2718.78 U/g). When fruit is infected by pathogens, the increased activities of antioxidant enzymes indicate the more serious damage to the fruit. FPAC samples had the highest activities of CAT, POD, and SOD, implying that they had higher ROS contents and were most severely attacked by pathogenic fungi.

3.3. Effect of fungal infection on physiological indexes of chestnuts

MDA content and relative conductivity are physiological indicators of the degree of membrane lipid peroxidation and plasma membrane disruption, as well as the degree of damage to the cell membrane system (Xie et al., 2021). As shown in Fig. 3A, the MDA content of chestnuts continuously increased from 0 to 10 d, and the MDA content of inoculated chestnuts was higher than that in the control group after 4 d. In 10 d, the MDA contents in FC, PC, and AC (inoculated with single fungus) were respectively increased by 4.4, 4.2, and 3.74 nmol/g, and in FPC, FAC, PAC, and FPAC (inoculated with mixed fungi) were increased by 4.42, 5.02, 4.64, and 5.17 nmol/g, respectively. MDA, a highly toxic molecule, can bind to proteins causing polymerisation and cross-linking of proteins and enzymes, thereby disrupting the structure and catalytic function of biological membranes (Elsherbiny, Dawood, & Safwat, 2021). Meanwhilw, MDA also inhibits the activities of cellular resistance enzymes and reduces the oxide content, thereby intensifying membrane lipid peroxidation. Due to the higher MDA content, the relative conductivity increases rapidly (Yu et al., 2022). Therefore, the relative conductivity of chestnuts in pathogenic fungal invasion gradually increased and reached 52.3% to 68.91% in 10 d (Fig. 3B). Notably, the relative conductivity of chestnuts inoculated with mixed fungi, especially in FPAC samples, was always higher than that of chestnuts inoculated with single fungus.

The change in respiratory intensity is also an important feature of fruit in the interaction between pathogenic fungi and fruit, and the enhancement of respiratory intensity is a typical early response of fruit to the infection of pathogenic fungi (Gong, Bi, Li, Li, & Wang, 2019). The respiratory intensity in the control samples remained high in the prestorage period and then decreased after 8 d. However, the respiratory intensity of chestnuts inoculated with fungi increased rapidly from 0 to 4 d. The respiratory intensity of chestnuts inoculated with single fungus was higher than that of chestnuts inoculated with mixed fungi in 4 d. Furthermore, the respiratory intensity of chestnuts inoculated with fungi increased slowly or became stable from 6 to 10 d. Generally, the respiratory intensity of chestnuts exhibited a consistent upward trend. After the interaction of fruit with the pathogenic fungi, the increase in respiration rate was ascribed to the host in the early stage and pathogenic fungi in the late stage. When fruit is attacked by pathogenic fungi, the wounded respiration of the host is caused by the energy and intermediate metabolites in establishing the defences of cells (Gong et al., 2019). Moreover, the pathogenic fungi induce the release of ethylene from the host, thus stimulating respiration and increasing the respiratory intensity of pathogenic fungi.

3.4. Effect of fungal infection on quality indexes of chestnuts

Moisture, protein, and fat play vital roles in maintaining normal physiological activities and ensuring excellent quality of chestnuts. The

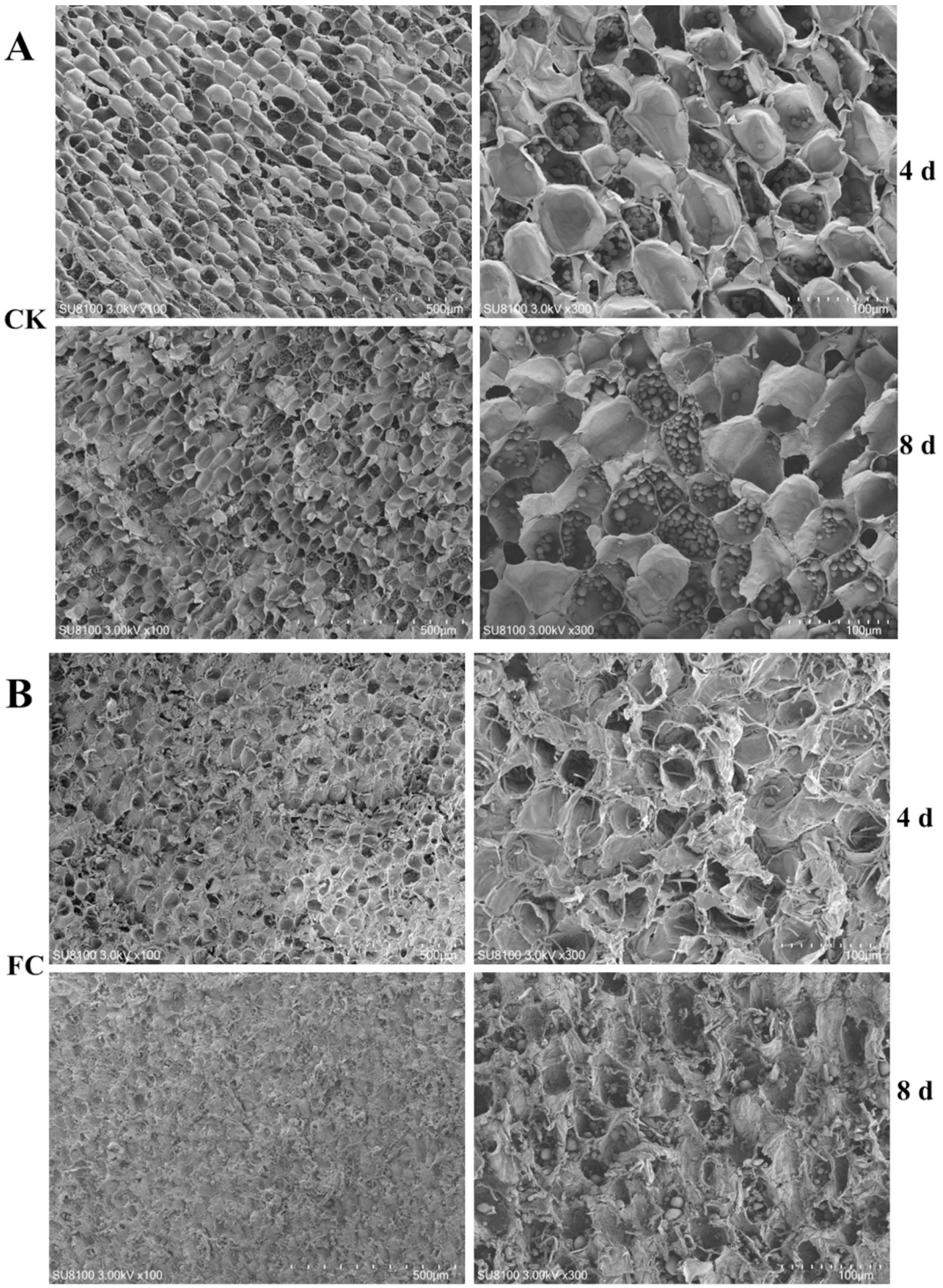
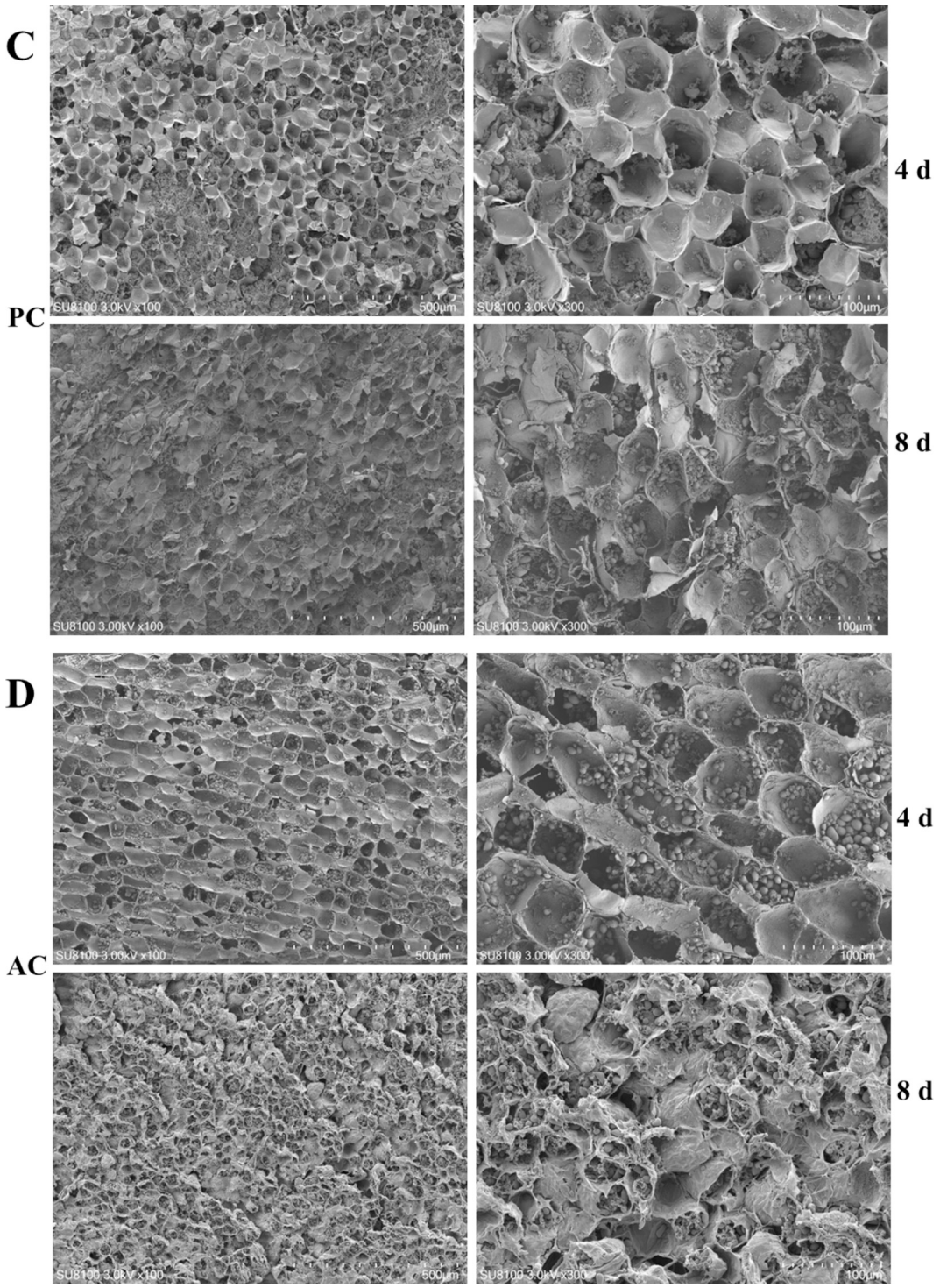


Fig. 6. Ultrastructural changes of chestnuts in 4 and 8 d. CK: control check samples; FC: *Fusarium proliferatum* infected chestnuts; PC: *Penicillium crustosum* infected chestnuts; AC: *Alternaria alternata* infected chestnuts; FPC: *Fusarium proliferatum* and *Penicillium crustosum* infected chestnuts; FAC: *Fusarium proliferatum* and *Alternaria alternata* infected chestnuts; PAC: *Penicillium crustosum* and *Alternaria alternata* infected chestnuts; FPAC: *Fusarium proliferatum*, *Penicillium crustosum* and *Alternaria alternata* infected chestnuts.

moisture content in raw chestnuts ranged from 62.47% to 63.84% in 0 d (Fig. 4A). The moisture content in chestnut samples gradually decreased during storage, ranging from 42.42% to 53.19% in 10 d. Water loss damages the organelle membrane structure within the chestnut tissue and disperses protoplasm into clumps, thus leading to an irreversible reaction (Liu, 2020). In addition, due to water loss, chestnuts can not maintain water required for life activities, thus resulting in reduced vitality, weakened disease resistance, and accelerated chestnut rot (Gu, Li, & Yin, 2013). Similarly, the contents of protein and fat in chestnut samples declined steadily (Fig. 4B and C). The protein content in raw chestnuts was 4.45% DW to 4.54% DW (dry weight) at 0 d. The protein content in chestnut samples inoculated with fungi decreased after 4 d, and reached 2.82% DW ~ 2.89% DW in 10 d (Fig. 4B). Compared with CK samples, the fat contents in chestnuts with single



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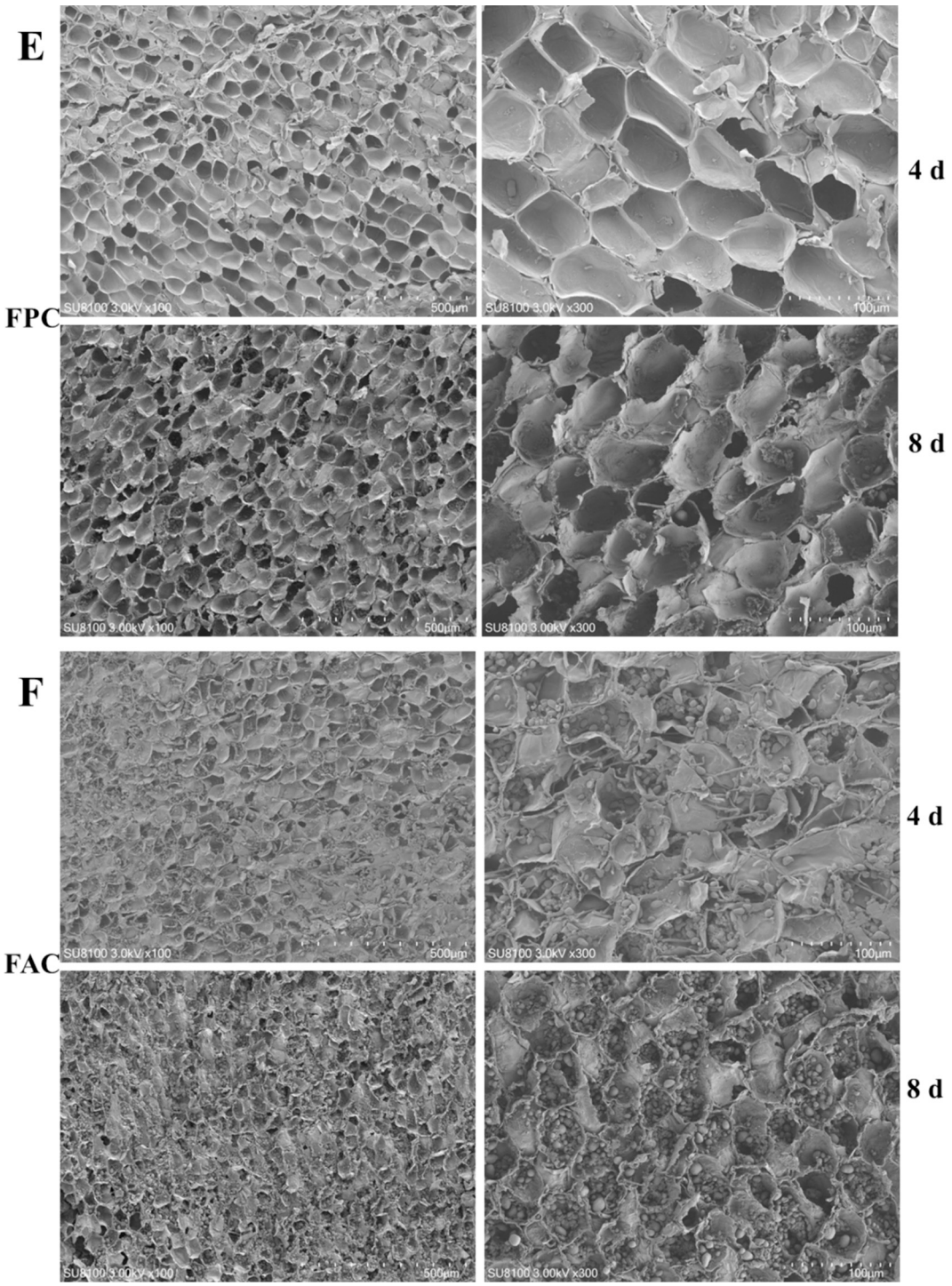
inoculation and mixed inoculation were significantly decreased, especially PC and FPAC ($P < 0.05$, Fig. 4C). Notably, the fat contents in PC and FPAC samples were reduced by 39.24% DW and 38.71% DW in 10 d, respectively.

Starch and reducing sugar contents are related to chestnut decay. The starch content of chestnuts exhibited a decreasing trend during storage (Fig. 4D). Initially, the starch content of chestnuts in 0 d was 45.36% DW to 47.37% DW. In 10 d, FPAC samples had the lowest starch content of 27.62% DW. The 1,4-D-glucoside bond of starch could be catalyzed by α-amylase, and α-amylase activity had a direct impact on the starch content of chestnuts. During the storage process of chestnuts, the α-amylase activity generally increased (Fig. 4E). The increase in α-amylase activity of chestnut samples inoculated with single fungus was higher than that of chestnuts inoculated with mixed fungi from 0 to 4 d, and FC samples had the highest activity (3.16 U/g) in 10 d. The growth of rotting-related fungi required nutrients and starch was the main nutrient in chestnuts. The water loss of chestnuts induced an increase in α-amylase activity, thus accelerating starch hydrolysis. The decomposition of starch made the reducing sugar content increase, which was conducive to the growth and reproduction of pathogens.



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Therefore, the reducing sugar contents in chestnut samples increased. Among the chestnut samples inoculated with single fungus, AC had the highest reducing sugar content (2.25% DW). Among the chestnut samples inoculated with mixed fungi, FPAC had the highest content in 10 d (2.10% DW). The nutrients from chestnuts were gradually consumed by pathogens, and then the energy was not enough to supply their life activities, thus leading to cell death and intensified decay of chestnuts.

3.5. Effect of fungal infection on surface appearance of chestnuts

3.5.1. Surface appearance changes

The lesion diameter of chestnuts gradually increased in the invasion period of pathogenic fungi, and the lesion diameter was smaller in PC and AC and the largest lesion diameter in FPAC in 10 d (Fig. 5A). The rotting appearance caused by the pathogenic fungal invasion in chestnuts is shown in Fig. 5B. The control groups showed no rotting sign during storage. FC samples showed obvious rotting in 2 d, and the rotting spots expanded gradually during storage. The rotting spots were gradually darkened from light brown to dark brown from 8 to 10 d,

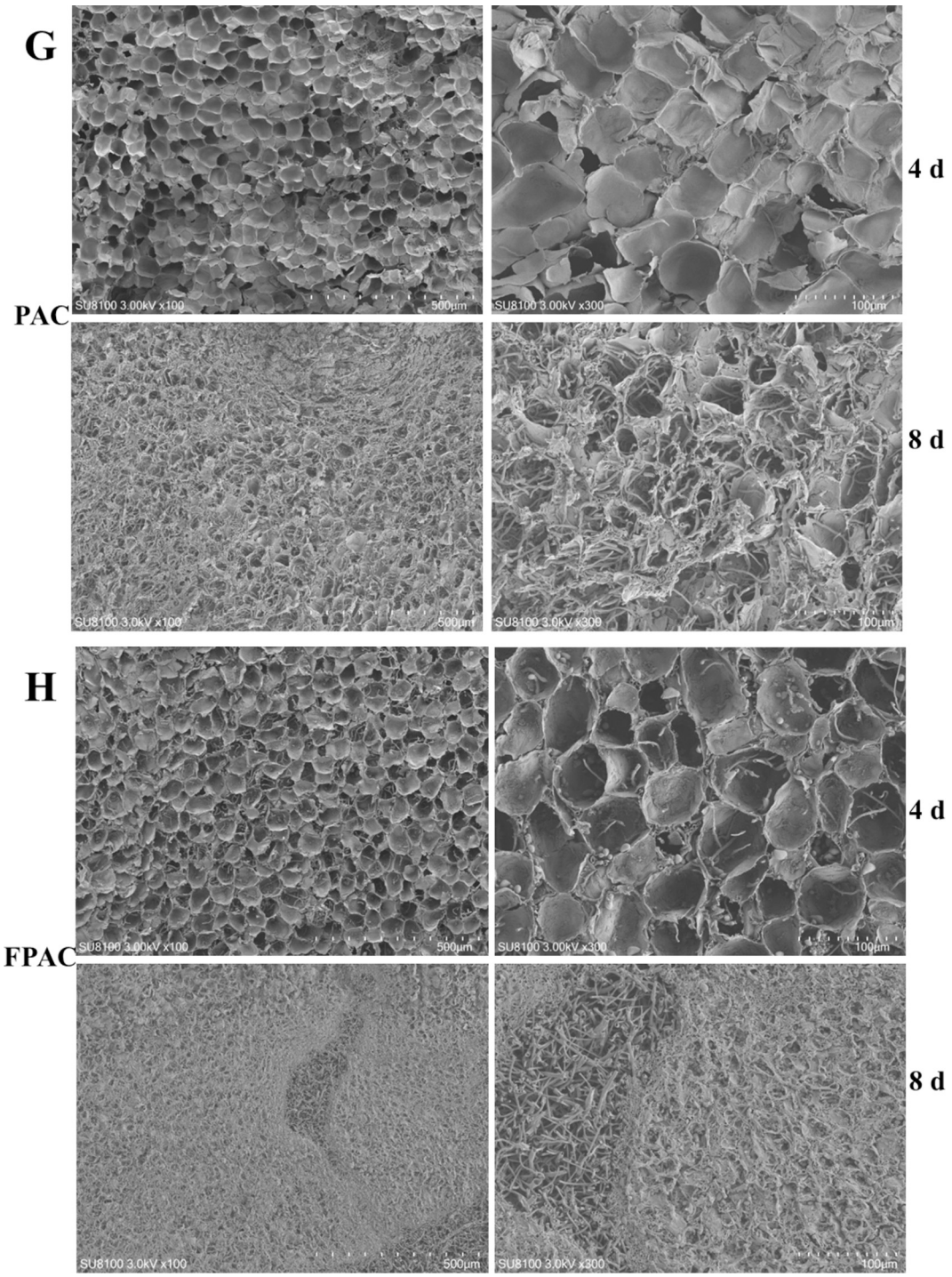

Fig. 6. (*continued*).

displaying white or light purple mycelium, unpleasant odour and sticky substances. PC samples began to decay in 2 d, and a large number of grey-green spores were observed on the chestnut surface. The degree of decay increased with the invasion of spores from 6 to 10 d, while rotting spots did not expand obviously. In AC samples, the rotting spots were dark grey and expanded slowly, and the tissues were dry and firm, accompanied by black mycelium. The degree of decay was increased. FPC samples exhibited an obvious rotting phenomenon with dark brown spots in 2 d. The spots were gradually enlarged, along with moist tissue, white mycelium, and grey-green spores. In FAC samples, the spots were light brown in 2 d and gradually expanded with time. In 10 d, the chestnuts were completely rotted, accompanied by a large number of white mycelium and a small amount of black mycelium. PAC samples severely decayed with black spots in 4 d, and the spots turned brown in 6 d, accompanied by severe browning of tissues. The spots gradually expanded, displaying black mycelium and grey-green spores. The spotted area of FPAC samples in 4 d was larger than that of other samples. In the later stage, the spots of FPAC samples further expanded. The whole chestnut was completely rotten in 10 d. In summary, lesion diameter and spot area of chestnuts gradually increased in pathogenic fungal invasion and were the most notable in FPAC samples.

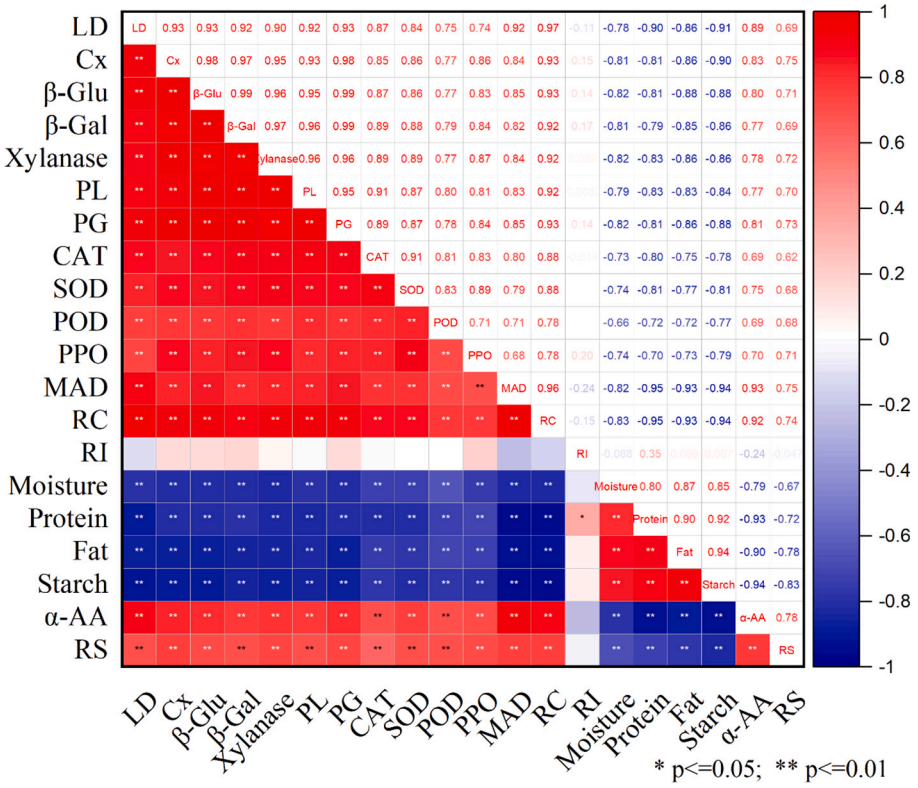

Fig. 7. The correlation between the lesion diameter and relevant indexes in chestnut samples inoculated with fungi. LD: lesion diameter; Cx: carboxymethyl cellulase; β-Glu: β-glucosidase; β-Gal: β-galactosidase; PL: pectate lyase; PG: polygalacturonase; CAT: Catalase; SOD: superoxide dismutase; POD: peroxidase; PPO: Polyphenol oxidase; MAD: malondialdehyde content; RC: relative conductivity; RI: respiratory intensity; α-AA: α-amylase activity; RS: reducing sugar.

3.5.2. *Ultrastructural changes of chestnuts*

The structural changes on the surface of chestnut tissues in 4 d and 8 d were observed under the electron microscope at 100× and 300×, respectively (Fig. 6). Shrinkage or plasmolysis was not observed in the raw tissue, and the cellular structure was intact with a smooth cell surface and homogeneous cytoplasm in CK samples (Fig. 6A). CK samples had no change in cellular structure in 4 d. Then the cellular structure of marginal tissue began to crumple in 8 d, while the cellular structure of internal tissues remained mostly unaltered. In 4 d, the tissue cells of FC samples were ruptured with a small number of mycelium on the surface, and in 8 d the tissue cells were completely disintegrated with a substantial quantity of mycelium on the surface (Fig. 6B). In 4 d, the tissue cells of PC and AC samples collapsed with spores on some tissue surfaces (Fig. 6C and D). In 8 d, the tissue was heavily covered with spores of saprophytic fungi. Cell walls were severely disintegrated, and the cellular structure completely disappeared. In 4 d, FPC and FAC samples had a minority of spores and mycelium expanding into the internal pulp tissues (Fig. 6E and F). The majority of mycelium was distributed in the inter-cellular space and a small quantity of mycelium was in the cell wall. In 8 d, a large quantity of mycelium existed in the inter cellular and intracellular spaces of the outer and inner pulp tissues. Cell wall was disintegrated and cells were fragmented. In 4 d, a large number of mycelium and spores were observed on the tissues of PAC and FPAC samples (Fig. 6G and H). Cells displayed obvious plasmolysis and the cytoplasm was severely wrinkled. In 8 d, mycelium expanded to form a dense web in PAC and FPAC, and the spores covered the entire surface of chestnut tissues. The cytoplasmic wall of chestnut tissues was completely separated, and cells were fully degraded. In summary, a large quantity of mycelium was mainly distributed in the intercellular space of chestnuts in 4 d, and a small quantity of mycelium was located in the cell wall, indicating that pathogenic fungi colonized and survived in the intercellular space of the outer tissue after invading the wound tissue in the early stage. In 8d, the chestnut tissue was covered with a large number of spores, and the entire surface was covered with mycelium, thus further verifying that the pathogenic fungi could infect the chestnut pulp tissue.

3.6. *Correlation analysis*

The correlations between the lesion diameter of chestnuts infected with fungi and CWDEs, antioxidant enzymes, quality indexes, and physicochemical indexes were analyzed. As shown in Fig. 7, the lesion diameter of chestnuts showed significant positive correlations with Cx, β-Glu, β-Gal, xylanase, PL, PG, CAT, SOD, α-amylase, MAD, and relative conductivity ($P < 0.01$ and $r > 0.80$). However, it had negative correlations ($P > 0.05$) with moisture, protein, fat, and starch, and showed no significant correlation with respiratory intensity.

4. Conclusion

In summary, during the infection process of chestnuts, pathogenic fungi firstly survived in the intercellular spaces of the outer tissue, secreted various CWDEs to degrade the chestnut cell wall and destroy the cell wall structure of the internal tissue cells of the chestnut. The invasion of pathogenic fungi induced an increase in reactive oxygen species, thus leading to a continuous enhancement in antioxidant enzyme activity. Subsequently, the content of MAD further rose, thus exacerbating membrane lipid peroxidation, and increasing the permeability of the biological membrane and the conductivity. Chestnuts lose a large quantity of water, inducing an increase in the activity of α-amylase and the content of reducing sugar. Pathogenic fungi utilized the nutritional components of chestnuts to continuously grow and reproduce, thus decreasing the nutrient content (starch, protein, and fat). The tissue was filled with a large number of pathogenic fungal spores and hyphae and the tissue cell wall was severely dissolved as the infection time increased. Finally, the cells ruptured, and the cell

structure disappeared, thus ultimately leading to the decay of chestnuts.

At present, how to effectively control postharvest decay has always been an urgent problem for the chestnut industry.Chestnut fruit is prone to infection by pathogenic microorganisms during postharvest storage, transport and marketing periods because of high moisture content, rich nutrients, and vigorous respiration. Therefore, the mechanical damage in picking and post-harvest circulation should first be reduced to decrease the chance of pathogen infection. Furthermore, the physical methods such as low temperature, modified atmosphere, and edible film (Fernandes, Pereira, Fidalgo, Gomes, & Ramalhosa, 2020; Huang et al., 2020), or the chemical methods such as ClO2 and O3 (Vettraino et al., 2019), or the biological methods such as natural extracts, antifungal bacteria, and microbial metabolites (Silva-Campos, Callahan, & Cahill, 2022) should be properly used during storage and transportation. These techniques mainly played a role in the following two aspects: on the one hand, the use of substances with antifungal effect or the creation of an environment unfavourable to the growth of pathogenic fungi to reduce the infection; on the other hand, the increase of resistance of chestnut fruits to resist the infection of pathogenic fungi. In future studies, according to the growth, reproduction and toxin production characteristics of pathogenic fungi in chestnut, we will develop a method of polysaccharide film with a natural antibacterial substance combined with low temperature, so as to meet the comprehensive requirements of chestnut preservation and anticorrosion.

CRediT authorship contribution statement

Anyan wen: Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Yiyi Zhu:** Visualization, Validation, Methodology, Investigation, Conceptualization. **Yangyang Geng:** Writing – review & editing, Funding acquisition. **Likang Qin:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101450.

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