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Dryopteris crassirhizoma Dryocrassin ABBA for Postharvest Control of the Potato Dry Rot Pathogen *Fusarium solani* var. *coeruleum*

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Abstract

Dryopteris crassirhizoma dryocrassin ABBA treatment was tested for its effectiveness in controlling *Fusarium solani* var. *coeruleum* growth *in vitro* and for prevention of postharvest dry rot of potato tubers and slices. Dryocrassin ABBA strongly inhibited mycelial growth, resulting in reductions in both mycelium dry weight and per cent spore germination of *F. solani* var. *coeruleum* at concentrations of 2.0, 0.5 and 0.1 mg/ml. Scanning electron microscopy (SEM) observations showed that treatment induced abnormal, tightly twisted morphological changes in hyphae. Moreover, *in vivo* experiments demonstrated that dryocrassin ABBA treatment at 2 mg/ml effectively controlled dry rot of potato tubers inoculated with mycelial discs of *F. solani* var. *coeruleum*. After dryocrassin ABBA treatment, the content of soluble proteins, peroxidase (POD) and superoxide dismutase (SOD) activity increased, and malondialdehyde (MDA) content remained the stable situation. In addition, the expression level of plant lipid-transfer proteins (LTPs) genes – *StLTPa1*, *StLTPa7*, *StLTPb1* and *StLTPb3* – was upregulated. These results collectively demonstrate that dryocrassin ABBA can inhibit growth of *Fusarium* pathogens to induce disease resistance. On the other side, dryocrassin ABBA maybe induce potato defence responses.

Introduction

Potato dry rot, which is caused by *Fusarium* spp., is one of the most important economic postharvest and storage diseases affecting potato tuber production worldwide. Huge crop losses caused by *Fusarium* pathogens range from 6 to 25% during storage periods, with up to 60% of tubers affected in some cases (Stevenson et al. 2001). *Fusarium solani* var. *coeruleum* has been demonstrated to be one of the main causative pathogens of dry rot in Heilongjiang Province (Min et al. 2010), the largest seed potato and ware potato production area in China (Xie 2012). *Fusarium* pathogens enter tubers through wounds incurred during harvest. Subsequently, the fungus causes potato dry rot damage that first appears on tubers as shallow, small brown lesions at wound sites beginning 3–4 weeks after harvest. Once formed, lesions continue to grow in all directions on tubers throughout the storage period (Dean 1994).

More than ten species of *Fusarium* pathogens exist, including *F. solani*, *Fusarium sambucinum*, *Fusarium avenaceum*, *Fusarium culmorum* and *Fusarium oxysporum* (Stevenson et al. 2001; Mecteau et al. 2002; Cullen et al. 2005; Gachango et al. 2012). The majority of dry rot is caused by *F. sambucinum* and *F. solani* var. *coeruleum*. Of additional concern, these *Fusarium* species produce trichothecene toxins that may contaminate tubers and pose a serious health hazard to humans and animals (Delgado et al. 2010).

The most effective control of potato dry rot has been achieved using fungicides such as thiabendazole

(TBZ). However, strains resistant to TBZ have become widespread (Desjardins et al. 1993; Gachango et al. 2012). Fortunately, recently heightened food and environmental safety considerations have spurred discovery of new fungicides that are less detrimental to human health and to the environment. As a result, several natural fungicides for biocontrol of plant pathogens have been discovered and should serve as safer alternatives for control of many infectious diseases (Tripathi and Dubey 2004).

Dryopteris crassirhizoma is a species of fern belonging to the *Dryopteridaceae* family and has been used as a Chinese medicinal crop for centuries. *Dryopteris crassirhizoma* contains large amounts of phloroglucinol compounds (Gao et al. 2008) such as dryocrassin ABBA, filixic acid ABA, albaspidin AA, albaspidin AP, albaspidin PP, flavaspidic acid AB and flavaspidic acid PB (Lee et al. 2003). These compounds have been reported to exhibit antibacterial activities (Lee et al. 2009), anthelmintic activities (Lu et al. 2012) and antivirus activities (Ou et al. 2015). However, to our knowledge, no research is currently underway to elucidate the antifungal activity of *D. crassirhizoma* extract dryocrassin ABBA extract against *F. solani* var. *coeruleum*.

The goals of this work include (1) to research the antifungal properties of dryocrassin ABBA against *F. solani* var. *coeruleum,* (2) to study dryocrassin ABBA control of potato dry rot mechanism by physiology and molecular method, (3) to evaluate the efficacy of dryocrassin ABBA as a newly discovered natural fungicide to control dry rot of potato tubers.

Materials and Methods

Pathogen

Fusarium solani var. *coeruleum* was obtained from the Virus-free Seedling Research Institute of Heilongjiang Academy of Agriculture Sciences (VSRI-HAAS), Harbin, China. *Fusarium solani* var. *coeruleum* was maintained on potato dextrose agar (PDA) at 25°C. Fungal spores were isolated from a 7-day-old PDA culture and suspended in 5 ml of sterile distilled water containing 0.05% (v/v) Tween-80. The suspension was adjusted after counting of spores using a haemocytometer to 1×10^5 spores/ml.

Plant material

Dryopteris crassirhizoma was collected in Shangzhi, China (127°17′N, 44°28′E). The plant material was verified taxonomically using morphological characteristics.

The virus-free tubers of a major potato variety grown in Heilongjiang Province, Kexin No. 13 were used as the source of treated potatoes. Potato tubers were harvested from a farm within the VSRI. Tubers were packed in string bags and transported to the laboratory within 24 h of harvest. Tubers without physical injuries or visible infections were stored at 5–8°C.

Dryopteris crassirhizoma dryocrassin ABBA extraction

Total crude phloroglucinol extraction from D. crassirhizoma

A crude phloroglucinol extraction method was performed as described previously (Jin et al. (2015). Briefly, 0.5 kg of *D. crassirhizoma* whole plants was air-dried, powdered and passed through a 40-mesh sieve. The resulting powder was next soaked in 5 l 80% ethanol for 24 h, avoiding high temperature and light. Ultrasonic extraction of the powder suspension was performed using 800 W of power applied at 30°C for 30 min for two extraction cycles. The ultrasonicated suspension was then centrifuged at 3000 ***g*** for 10 min at 4°C by Thermo Sorvall LYNX 4000 (Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant decanted. The supernatant was extracted using an equal volume of hexane then concentrated using a rotary evaporator at 40°C and finally dried into an extractum. The extractum was dissolved in a 10-fold volume of 15% ethanol and the pH adjusted with 5% NaOH and 5% H_2SO_4 to 7.

Purification and enrichment of total phloroglucinol

Total crude phloroglucinol was purified using DM-130 macroporous adsorption resin to achieve a final sample concentration and final volume of 1.5 mg/ ml and 7 bed volumes (7 BV, 210 ml), respectively. The target compound was eluted with 80% ethanol at 1.0 ml/min (Wang et al. 2008; Jin et al. 2015).

Total phloroglucinol measurements

The total phloroglucinol content in the ethanol extract of *D. crassirhizoma* was determined using a colorimetric method (Lv et al. 1988), and total phloroglucinol level was determined using a calibration curve. The final results are expressed as milligram (mg) per millilitre (ml) of ethanol extract.

High-performance liquid chromatography (HPLC)

Purification of phloroglucinol from the ethanol extract of *D. crassirhizoma* was performed using a Waters 600 HPLC system equipped with a C18 column (250 mm × 4.6 mm, 10 μm), a quaternary pump and a UV detector set to 295 nm. Gradient elution was performed using a flow speed of 1.0 ml/min

and a mobile phase consisting of acetonitrile-0.5% phosphoric acid. Collection of the phloroglucinol was observed within the 80~100% gradient elution range. The column was maintained at room temperature, and the injection volume was 20 μl. Identification of dryocrassin ABBA was based on retention time and UV spectra comparisons to available standards. Quantification of dryocrassin ABBA was performed using the volume within the peak areas recorded at 295 nm. Yields are expressed as mg per ml of *D. crassirhizoma* culture volume (Xiao et al. 2005).

Measurements of effects of dryocrassin ABBA on spore germination and mycelial growth

Spore germination rate assay

The effects of dryocrassin ABBA on spore germination of *F. solani* var. *coeruleum* were assayed using method described previously by Mu (1994); 20 μl of aliquots of a conidial suspension of 1×10^5 spores/ml was plated onto the surface of PDA plates containing 0.1, 0.5 or 2.0 mg/ml of dryocrassin ABBA dissolved in distilled water (DW). PDA plates containing distilled water alone were used as a control. After incubation for 24 h at 25°C, germination rates were recorded after counting germination events under a light microscope. The germination rate was measured for a sample of approximately 100 spores per treatment. Each treatment was replicated three times, and the experiment was repeated twice. The spore germination rate was determined and expressed as a percentage using the following formula:

$$\begin{aligned}&\text{Percentage of inhibition of spore germination}\\&= \big[(\text{mean number of germinated control conidia}\\&\quad - \text{mean number of germinated treated conidia})/\\&\quad \text{mean number of germinated control conidia}\big]\\&\quad \times 100\%\end{aligned} \tag{1}$$

Mycelial growth assay and viability tests

The effect of dryocrassin ABBA on mycelial growth of *F. solani* var. *coeruleum* was measured as previously described as Yao and Tian (2005). Mycelial discs (6 mm in diameter) from 1-week-old fungal cultures were placed in the centre of the Petri dishes (90 mm in diameter) with 10 ml of PDA containing various concentrations of dryocrassin ABBA (0.1, 0.5 or 2.0 mg/ml) and then incubated at 25°C. PDA containing only DW was used as the control. Mycelial growth was determined by measuring colony diameter 7 days after inoculation. Each treatment was replicated three times, and the experiment was repeated twice. Mycelial growth inhibition of treatment relative to control was calculated using the following equation:

$$\begin{aligned}&\text{Mycelial growth inhibition}\\&= \big[(\text{mycelial growth of control} -\\&\quad \text{mean mycelial growth of treatment})/\\&\quad \text{mycelial growth of control}\big] \times 100\%\end{aligned} \tag{2}$$

The fungi viability was used the method of Coley-Smith and Javed (1970) and Yingjun et al. (2006). After mycelial growth determination spore germination rate were tested, dryocrassin ABBA and DW on mycelial growth of *F. solani* var. *coeruleum* were used as treated and control, respectively. The viability was measured for a sample of approximately 100 spores per treatment, after incubation for 24 h at 25°C. Each treatment was replicated three times, and the experiment was repeated.

$$\begin{aligned}&\text{Percentage of fungi viability}\\&= \big[(\text{mean number of germinated control conidia} -\\&\quad \text{mean number of germinated treated conidia})/\\&\quad \text{mean number of germinated control conidia}\big] \times 100\%\end{aligned} \tag{3}$$

Mycelial dry weight assay

The effect of dryocrassin ABBA on mycelial dry weight of *F. solani* var. *coeruleum* was tested using the method of Yu and Chen (2002). Five mycelial discs (6 mm in diameter) from 1-week-old fungal cultures were placed in flasks with 50 ml of PD containing various concentrations of dryocrassin ABBA (0.1, 0.5 or 2.0 mg/ml) and then incubated at 25°C and shaken at 1.4534 ***g*** by CRYSTAL IS-RDS3C INCUBATOR SHAKER (Crystal Technology & Industries, Inc., Addison Texas, USA). Flasks containing PD with only added sterile distilled water were used as controls; 15 days later, the mycelial cultures were filtered through two layers of sterile cheesecloth to remove the culture supernatants and the solids were dried and their weights were determined. Each treatment was replicated three times, and the experiment was repeated twice.

$$\begin{aligned}&\text{Mycelial dry weight inhibition}\\&= \big[(\text{mycelial dryweight of control} - \text{mean dry weight}\\&\quad \text{of treated cultures})/(\text{mycelial dry weight of control})\big]\\&\quad \times 100\%\end{aligned} \tag{4}$$

Scanning electron microscopy (SEM)

After 7-day culture, the effect of dryocrassin ABBA on hyphal development of *F. solani* var. *coeruleum* was

observed using SEM according to the method of Benhamou et al. (1999). The fungal cultures were treated with dryocrassin ABBA at concentrations of 0.1 or 0.5 mg/ml in PDA and then incubated at 25°C for 7 days. A culture treated with sterile distilled water was used as a control. The samples were vapour-fixed with 2% (w/v) aqueous osmium tetroxide for 2 h at 4°C, air-dried and sputter coated with gold palladium using a Polaron E 500 sputter coater (Polaron, Cambridge, UK). Samples were then kept in a desiccator until examination using a Cambridge Stereoscan 5–150 SEM (LEO Electron Microscopy Ltd., Cambridge, UK) operating at 20 kV. Micrographs were taken by CCD-Camera (Gatan, Inc., Pleasanton, CA, USA). The experiment was repeated three times using two replicate plates for each treatment. For each replicate, 10 agar blocks were examined using SEM.

Effect of dryocrassin ABBA on potato tubers and slices

Before treatments, potato tubers were surface-sterilized by soaking them in a 5% sodium hypochlorite solution for 5 min then rinsed with distilled water and air-dried. To assess the antifungal effects of dryocrassin ABBA, the method described by Mvuemba et al. (2009) was used with minor modifications. The tubers were first wounded by creating circular wounds (5 mm in diameter and 3 mm deep) using a sterile hole punch. Mycelial discs (5 mm in diameter) of *F. solani* var. *coeruleum* from 1-week-old cultures were placed hyphal side down in each wound and labelled T1. To the wound holes introduced into other tubers, 200 μl of 2 mg/ml dryocrassin ABBA was added per wound and incubated for 2 h in darkness, and these samples were labelled T2. To the other wound holes introduced into other tubers, 200 μl of 2 mg/ml dryocrassin ABBA was added per wound introduced into other tubers, 200 μl of 2 mg/ml dryocrassin ABBA was added per wound and incubated at 25°C for 2 h in darkness, and then mycelial discs (5 mm in diameter) of *F. solani* var. *coeruleum* from 1-week-old cultures were placed hyphal side down in each wound, and these wounds were labelled T3. Sterile distilled water control was added to other wounds and labelled CK. All treated potato tubers were individually transferred to plastic containers lined with sterile moistened paper towels to maintain high relative humidity. Each lesion size was expressed as the mean average of two diameters of lesions after 16-day incubation at 25°C. Each treatment included three replicates with 10 tubers per replicate, and the entire experiment was repeated twice.

Before treatments, potato tubers were surface-sterilized by soaking them in a 5% sodium hypochlorite solution for 5 min then rinsed with distilled water and air-dried. To assess the antifungal effects of dryocrassin ABBA, a method described previously was used (Ray and Hammerschmidt 1998). After disinfection, tubers were cut into slices (5 mm in thickness) with a sterile knife and each slice was placed onto a wet sterile filter paper. The mycelial discs (5 mm in diameter) of *F. solani* var. *coeruleum* from 1-week-old cultures were obtained and placed hyphal side down in the centre of each potato piece in sterile dishes, and these samples were labelled T1. 200 μl of 2 mg/ml dryocrassin ABBA was added to other potato slices, which were inoculated at 25°C for 2 h in the dark and labelled T2. Other slices were treated with 200 μl of 2 mg/ml dryocrassin ABBA at 25°C for 2 h in the dark, before placement of mycelial discs of *F. solani* var. *coeruleum* from 1-week-old cultures onto each slice and labelled T3. Sterile distilled water control was added to other potato slices and labelled CK. All treated potato slices were individually covered and inoculated at 25°C. The lesion size was expressed as the mean of average of two diameters of the lesions after 3- and 6-day incubations. Each treatment contained three replicates, and the experiment was repeated twice.

Measurement of defence-related enzymes and antifungal compounds

For enzyme assays, potato tubers were surface-sterilized as mentioned before. The tubers were first made into slices (5 mm in thickness); 200 μl DW and 2 mg/ml *D. crassirhizoma* dryocrassin ABBA were added to the centre potato slices, respectively. After treatment, the potato slices were inoculated at 25°C. The crude enzyme extraction as Yin et al. (2010) described and made minor modifications; the samples were ground with 1% polyvinyl polyvinylpyrrolidone (PVPP), 2 mM pH 8.0 EDTA, 0.04% β-mercaptoethanol and then centrifuged at 4000 ***g*** at 4°C for 20 min. The supernatants were used to assay enzymatic activities and antifungal compounds by enzyme-protein combined assay (Zhang and Zhai 2003). Each treatment contained three replicates. All the collected samples were determined for superoxide dismutase (SOD), peroxidase (POD), malondialdehyde (MDA) and soluble protein (Wang and Win 2002) from 0 to 6 days. SOD activity was determined using nitroblue tetrazolium (NBT). The reaction mixture consisted of 0.1 ml crude extract, 3.1 ml phosphate buffer (0.05 M, pH 7.8), 0.2 ml EDTA-Na (1 mg/ml), 0.2 ml L-methionine (20 mg/ml), 0.2 ml riboflavin (0.1 mg/ml) and 0.2 ml NBT (1 mg/ml).

POD activity was determined using guaiacol as substrate. The reaction mixture consisted of 50 μl crude extract, 4 ml of 0.3% guaiacol (0.02 M in phosphate buffer, pH 6.0), 50 μl of 0.3% H_2O_2. Mixing well, the increase in absorbance at 470 nm was measured after 1 min and continuous 5 min.

The total MDA content in extracts was measured by a colorimetric assay. 0.6 g thiobarbituric acid was added to 10 ml NaOH (1 M); 11 ml HCL (1 M) was added and then transfer to 100 ml volumetric flask and mixed well. After mixed well, took 2 ml solution in a new flask, 1 ml crude extract and 1 ml phosphate buffer (0.05 M) were added, then put the flask in boiling water for 15 min. At last rapid cooling, all the mixture were centrifuged at 12 000 ***g*** by Thermo Sorvall LYNX 4000 (Thermo Fisher Scientific) for 10 min. The supernatants were used to assay for absorbance at 532, 600 and 450 nm.

Soluble protein content was determined by Coomassie Brilliant Blue G-250 and measured absorption at 595 nm, and the content was estimated using calibration curves (Bradford 1976).

RNA extraction and real-time PCR

Total RNA (200 mg) was isolated and purified using OminiPlant RNA Kit (DNase I) (CW2598M; CWBIO, Beijing, China) according to the manufacturer manual. From DW and dryocrassin ABBA treatment samples, mRNA was reverse-transcribed into single-strand cDNAs using Super Quick RT cDNA Kit and reverse transcriptase (CW2381, CWBIO) according to the manufacturer instruction. The gene expression levels of *StLTPa1* (GenBank: EU057716), *StLTPa7* (GenBank: EU057712), *StLTPb1* (GenBank: EU057715), *StLTPb3* (GenBank: EU057717) and *StLTPf10* (GenBank: EU057714) (Gang 2008) were designed by Primer Premier 5.0, and *EF1-α* was used internal gene. These genes were analysed using a real-time PCR (RT-PCR) detection system. Gene-specific primers were designed using Primer Premier 5.0 based on the gene sequences. Then, primers and internal reference were synthesized commercially by BSGENE (Harbin, China). The gene-specific primers (Table 1) were diluted to a final concentration of 25 M each and used for RT-PCR. For RT-PCR, amplification was performed in a 20-μl reaction system containing 10 μl SYBR Green Mixture (CW0957; CWBIO), 0.5 μl of each forward and reverse primers, and 0.5 μl cDNA templates. The reaction was performed in a Roche LightCycler 480 Real-Time PCR cycler using the following program: 95°C for 10 min, 60°C for 1 min, 95°C 15 s and 72°C 15 s. The experiments were each repeated three times. Calculation of the relative amounts of amplification products was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analyses

Statistical analyses were all performed using PASW Statistics 18 (http://www.spss.com.hk/statistics/). One-way analysis of variance was used to evaluate the effect of each treatment, and the means of multiple comparisons were determined using the Least Significant Difference (LSD) method. The data are expressed as mean ± standard error (SE).

Result

Dryocrassin ABBA content

The extraction process yielded an ethanol extract with a concentration of 8.10 mg/g. The retention time was determined to be 18 min, and total dryocrassin ABBA content was determined from UV spectra to be 15.30 mg/ml. These values were consistent with the results reported by Jin et al. (2015), which demonstrated the feasibility of this extraction method for achieving reasonable yields.

Effect of dryocrassin ABBA on spore germination, mycelial growth and viability

Spore germination and mycelial growth of *F. solani* var. *coeruleum* were significantly inhibited ($P < 0.05$) by dryocrassin ABBA after 12 h of treatment. The

Table 1 The primers used in this research

Gene name	Forward primers (5′–3′)	Reverse primers (5′–3′)
StLTPa1	TGGCTCCCTGCCTCCCTTATC	CCATCTTATTCTTCATCTCCG
StLTPa7	TGGCTCCCTGCCTCCCTTATC	CCATCTTATTCTTCATCTCCG
StLTPb1	GGCGAG GTTACATCTG GCTTG	GGTCTTCTGGGGTTTTGGCTG
StLTPb3	GGCGA GGTTACATCT GGCTT	GTCTTCTGGGGTCTTGGCTG
StLTPf10	GCTCCTTGCC TCCCTTATCTT	GGCTTTTCCCGTATCAATTCC
Internal reference	TGCCCAGAAGAAGGGAAAGTGA	AATCATGCTCGCCACCGCCTAT

mycelium growth per cent inhibition was elevated from 45.3 to 98.0%, the percentage of fungi viability were from 4.5 to 99.8%, the spore germination inhibition per cent ranged from 4.07 to 68.13% (Table 2), and the mycelium growth per cent inhibition was elevated from 45.3 to 98.0%. Inhibitory effects varied in direct correlation with increase of dryocrassin ABBA concentration from 0.1 to 2.0 mg/ml (Table 2).

*D*ryocrassin ABBA at various concentrations also significantly inhibited ($P < 0.05$) the mycelial dry weight results for *F. solani* var. *coeruleum* after 15-day treatment, with greater inhibitory effects at higher concentrations (Table 2). For concentrations of dryocrassin ABBA of 0.5 and 2.0 mg/ml, mycelium dry weight inhibition values were 69.95 and 79.31%, respectively. Likewise, dryocrassin ABBA exhibited strong inhibitory effects on *F. solani* var. *coeruleum* mycelial growth. The greatest effect was observed using 2.0 mg/ml, which completely suppressed mycelium growth, as exhibited by reduction in pathogen mycelial dry weight.

SEM observations

SEM observations of *F. solani* var. *coeruleum* treated with dryocrassin ABBA revealed effects on hyphal morphology (Fig. 1). Hyphae of the control samples were normal, evenly distributed and were regular with a smooth surface (Fig. 1a,b). Hyphae treated with dryocrassin ABBA at 0.1 and 0.5 mg/ml showed an obvious effect on hyphal morphology (Fig. 1c-f). Hyphae of samples treated with 0.1 mg/ml dryocrassin ABBA were abnormal in morphology and were disordered, sparse, blasted and wizened (Fig. 1c, d). Hyphae of the sample treated with 0.5 mg/ml were tightly twisted and were much more blasted and wizened than observed for the lower concentration treatment (Fig. 1e,f).

Table 2 The mycelial growth inhibition of *Fusarium solani* var. *coeruleum* by *Dryopteris crassirhizoma* dryocrassin ABBA

Concentration (mg/ ml)	Mycelial growth inhibition (%)	Spore germination inhibition (%)	Mycelial dry weight inhibition (%)	Percentage of fungi viability (%)
0	0a	0a	0a	99.8a
0.1	45.3b	4.07b	31.36b	44.7b
0.5	68.3c	20.14c	66.95c	20.3c
2	98.0d	68.13d	79.31d	4.5d

Significant differences are shown by dissimilar letters ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

Effects of dryocrassin ABBA on dry rot of potato tubers and slices

In contrast to untreated potato tubers inoculated with *F. solani* var. *coeruleum,* after a 16-day incubation at 25°C (T1), tubers treated with dryocrassin ABBA at 2 mg/ml showed an extremely significant ($P \leq 0.01$) inhibition of lesion development by *F. solani* var. *coeruleum* (T3), with 53.76% inhibition (Fig. 2). In contrast to the control treatment (CK), potato tubers treated with dryocrassin ABBA (T2) showed no significant increase in lesion development, thus demonstrating that dryocrassin ABBA alone did not damage potato tubers.

Potato slices inoculated with *F. solani* var. *coeruleum* for 3 and 6 days at 25°C (T1). Slices treated with dryocrassin ABBA at 2 mg/ml showed an extremely significant ($P \leq 0.01$) inhibition of lesion development by *F. solani* var. *coeruleum* (T3). The lesion diameters of T3 relative to T1 samples were 66.28 and 47.56% at 3 and 6 days, respectively (Fig. 3). These results therefore show that resistance to the pathogen not only continued to 6 days, but also increased.

Collectively, the results described in this work conclusively demonstrate that potato tubers and slices treated with dryocrassin ABBA exhibited inhibition of *F. solani* var. *coeruleum* growth to achieve control of dry rot in inoculated tubers or slices. Moreover, by decreasing the incidence of potato dry rot, treatment ultimately maintained improved potato quality. Because few reports have yet focused on the efficacy of plant extracts for control of dry rot, much more work is needed in this research area. Furthermore, the results of this study, the first report describing the use of dryocrassin ABBA to control potato dry rot, are especially noteworthy, as few reports exist to date that demonstrate efficacy of plant extracts for control of dry rot on tubers or slices. Of the few studies in the literature, one study demonstrated that treatment of potato slices with the oil and α-pinene of *Zanthoxylum bungeanum* significantly reduced lesion diameter after inoculation with *Fusarium sulphureum* (Li and Xue 2014). Moreover, another study showed that treatment of potato tubers with a methanol extract solution of *Punica granatum* significantly reduced lesion diameter in potato tubers after inoculation with *F. sulphureum* (Elsherbiny et al. 2016). However, much more work is needed to control potato dry rot.

Enzyme activity after *D. crassirhizoma* dryocrassin ABBA treatment

The SOD activity, POD activity, MDA content and soluble protein content of Kexin No. 13 potato slices

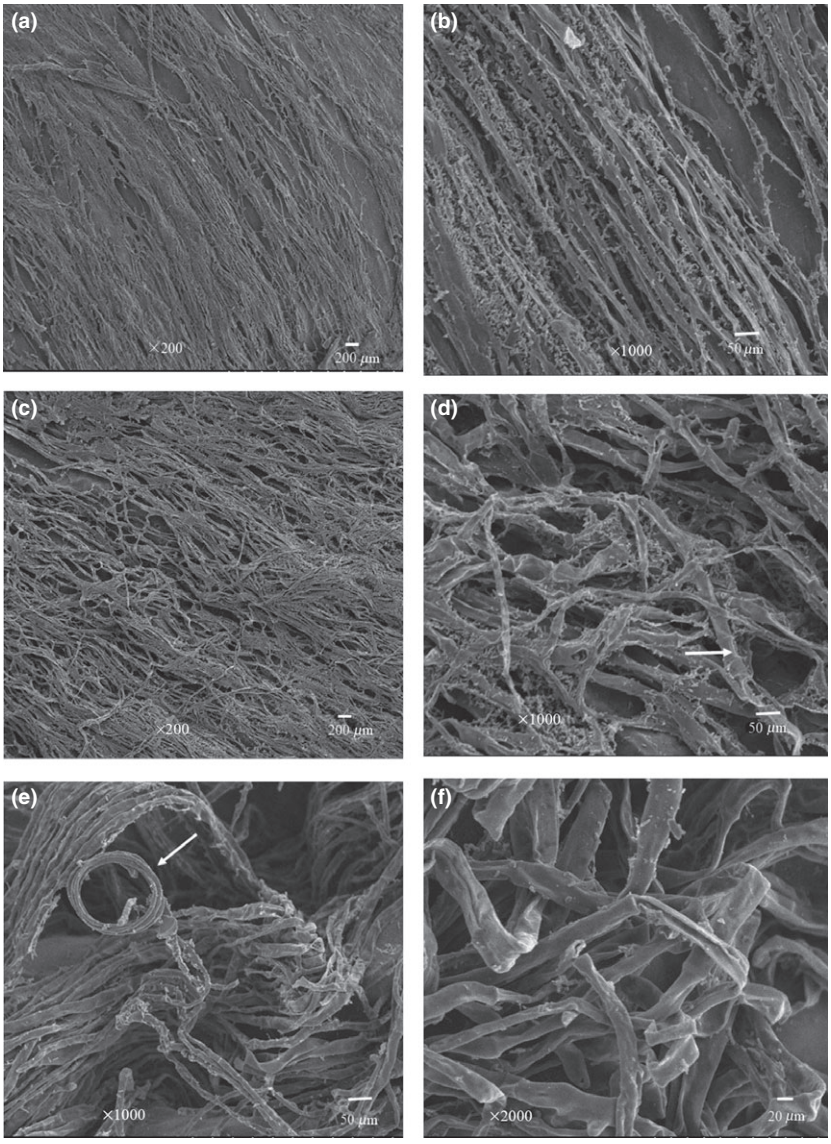

Fig. 1 Morphological change in hyphae of *Fusarium solani* var. *coeruleum* treated with *Dryopteris crassirhizoma* dryocrassin ABBA of 0.1 mg/ml (c,d), 0.5 mg/ml (e,f) and untreated control (a,b), removed from 7-day PDA cultures as revealed by SEM. Scale bars represent 200 μm in (a) and (c); 50 μm in (b), (d) and (e); 20 μm in (f). Hyphae were normal, evenly distributed and were regular with a smooth surface (a and b) in the untreated control. Hyphae of samples treated with 0.1 mg/ml *D. crassirhizoma* dryocrassin ABBA were abnormal in morphology (c) and were disordered, sparse, blasted and wizened (arrowhead) (d). Hyphae of the sample treated with 0.5 mg/ml *D. crassirhizoma* dryocrassin ABBA were tightly twisted (arrowhead) (e), blasted and wizened (arrowhead) (f).

were measured in both treated and untreated potatoes at different times (Fig. 4). The results of SOD activity were showed in Fig. 4a, increased gradually and peaked at 3 days in *D. crassirhizoma* dryocrassin ABBA-treated potato. The activity level was approximately 2.92-fold compare treated with control at 3 days. The SOD activity in control maintained a relatively low level. POD activity was showed in Fig. 4b; dryocrassin ABBA treatment caused a progressive and significantly increase and peaked at 4 days. The level was approximately 4.19-fold compare treated with control at 4 days. The POD activity in control increased slowly. After dryocrassin ABBA treatment, MDA content increased slightly from the early stage, then decreased and remained the stable situation as control (Fig. 4c). The soluble protein content increased slightly and the maximum content appeared at 6 days (Fig. 4d).

Specific expression of resistance-related genes

Lipid-transfer proteins (LTPs) are secreted proteins and reported involved in different aspects of plant physiology and cell biology, including formation of cutin, flowering, defence reactions and the adaptation of plants to various environmental conditions. It was reported, after *Ralstonia solanacearum* treated, the expression of *StLTPa1*, *StLTPa7*, *StLTPb1*, *StLTPb3* and *StLTPf10* (Gang 2008; Gang et al. 2008, 2009) was upregulated, which may be pathogen-responsive gene in plant defences and involve in a complex gene regulation network in potato.

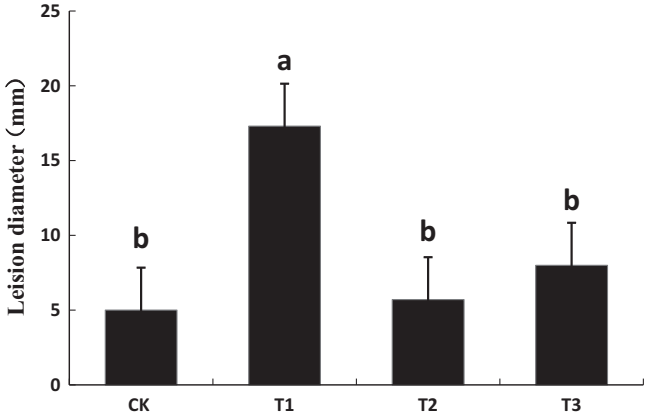

Fig. 2 Effects of *Dryopteris crassirhizoma* dryocrassin ABBA on dry rot of different varieties potato tubers inoculated with *Fusarium solani* var. *coeruleum* after 16-day incubation at 25°C. The mean and standard error are expressed by the values and error bars as indicated, respectively, from thrice independent measurements. Significant differences are shown by dissimilar letters ($P < 0.05$, one-way ANOVA, followed by Duncan's test). CK, potato tubers treated by sterile distilled water; T1, potato tubers inoculated by mycelial discs of *F. solani* var. *coeruleum* from 7-day-old cultures; T2, potato tubers treated by 200 μl of 2 mg/ml *D. crassirhizoma* dryocrassin ABBA; T3, potato tubers treated by 200 μl of 2 mg/ml *D. crassirhizoma* dryocrassin ABBA then added the mycelial discs of *F. solani* var. *coeruleum* from 7-day-old cultures.

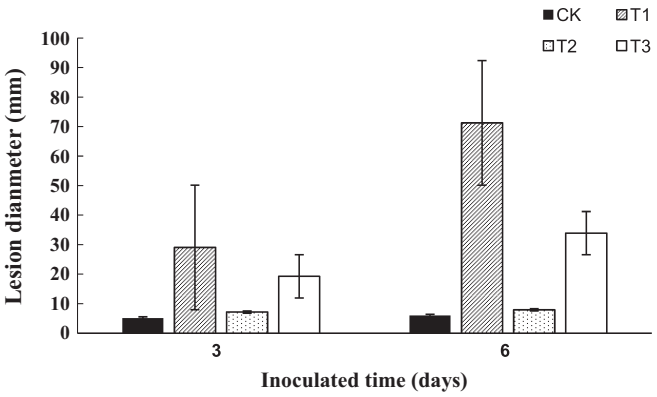

Fig. 3 Effects of *Dryopteris crassirhizoma* dryocrassin ABBA on dry rot of potato slices inoculated with *Fusarium solani* var. *coeruleum* after 3- and 6-day incubation at 25°C. The mean and standard error are expressed by the values and error bars as indicated, respectively, from thrice independent measurements. Significant differences are shown by dissimilar letters ($P < 0.05$, one-way ANOVA, followed by Duncan's test). (a) Superoxide dismutase (SOD) activity of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (b) Peroxidase (POD) activity of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (c) Malondialdehyde (MDA) content of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (d) Soluble protein content in Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. CK, Kexin No. 13 potato slices treated by distilled water (DW); Treatment, Kexin No. 13 potato slices treated by 200 μl of 2 mg/ml *D. crassirhizoma* dryocrassin ABBA; CK, potato slices treated by sterile distilled water; T1, potato slices inoculated by mycelial discs of *F. solani* var. *coeruleum* from 7-day-old cultures; T2, potato slices treated by 200 μl of 2 mg/ml *D. crassirhizoma* dryocrassin ABBA; T3, potato slices treated by 200 μl of 2 mg/ml *D. crassirhizoma* dryocrassin ABBA then added the mycelial discs of *F. solani* var. *coeruleum* from 7-day-old cultures.

The expression levels of *StLTPa1*, *StLTPa7*, *StLTPb1*, *StLTPb3* and *StLTPf10* genes on dry rot of potato slices Kexin No. 13 treated with dryocrassin ABBA were analysed using RT-PCR after 1, 3 and 6 days at 25°C. The transcripts of these five genes could be detected, but showed different expression levels (Fig. 5). After the treatment, the transcript accumulation of *StLTPa1*, *StLTPa7*, *StLTPb1* and *StLTPb3* increased and peaked at 3 days, *StLTPf10* increased along with the treatment time and peaked at the end of treatment (6 days). Among them, *StLTPa1* and *StLTPa7* were induced to approximately 4.4-fold and showed the quickest response, *StLTPb1* and *StLTPb3* were 3.5- and 2.7-fold after 3-day treatment. However, *StLTPf10* was only 0.9-fold after 6-day treatment. These results suggest that *StLTPa1*, *StLTPa7*, *StLTPb1* and *StLTPb3* were induced by dryocrassin ABBA. Considering

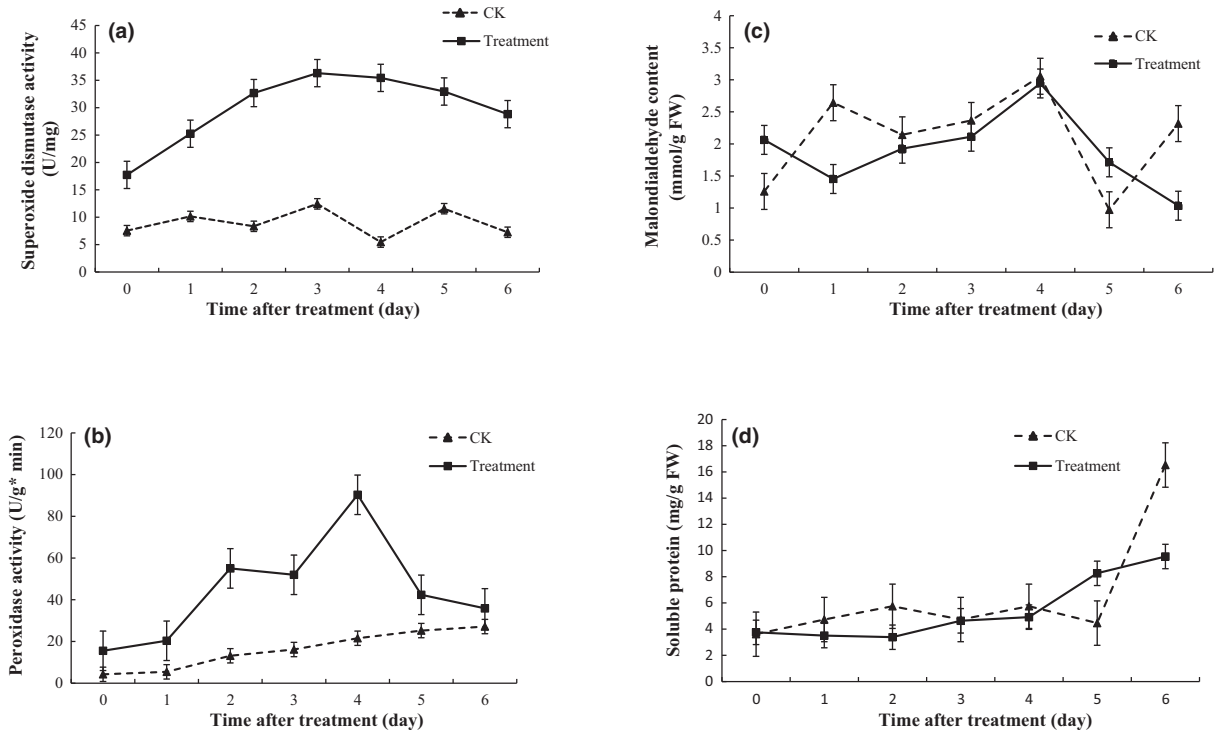

Fig. 4 Results of defence-related enzymes and antifungal compounds content in Kexin No. 13 potato slices after *Dryopteris crassirhizoma* dryocrassin ABBA treatment. The mean and standard error are expressed by the values and error bars as indicated, respectively, from thrice independent measurements. (a) Superoxide dismutase (SOD) activity of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (b) Peroxidase (POD) activity of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (c) Malondialdehyde (MDA) content of Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. (d) Soluble protein content in Kexin No. 13 potato slices after *D. crassirhizoma* dryocrassin ABBA treatment. CK, Kexin No. 13 potato slices treated by distilled water (DW); Treatment, Kexin No. 13 potato slices treated by 200 μL of 2 mg/mL *D. crassirhizoma* dryocrassin ABBA.

the high expression of *StLTPa1*, *StLTPa7*, *StLTPb1* and *StLTPb3* after treatment, the significant change of resistance-related transcripts could be important for potato responses to pathogen.

Discussion

*D*ryocrassin ABBA against potato dry rot pathogen

This study was conducted to evaluate the efficacy of a *D. crassirhizoma* extract, *D. crassirhizoma* dryocrassin ABBA, to prevent or lessen potato dry rot caused by *F. solani* var. *coeruleum*. Several studies have been conducted that demonstrate antifungal activities of many kinds of plant extracts against *Fusarium* pathogens. One study demonstrated inhibition of extracts from *Azadirachta indica*, *Atropa belladonna*, *Calotropis procera*, *Ocimum bacillicum*, *Eucalyptus amygdalina*, *Ailanthus excelsa* and *Lantana camara* against *F. oxysporum* (Bansal and Gupta 2000). In other studies, *Allium sativum*, *Allium cepa*, *Mentha arvensis* (Taskeen-Un-Nisa et al. 2011) and *P. granatum* (Elsherbiny et al. 2016) extracts inhibited mycelial growth and spore germination of *F. oxysporum* and *F. sambucinum*. Moreover, another study showed inhibition of *Z. bungeanum* oil extract against *F. sulphureum* (Li and Xue 2014). *Dryopteris crassirhizoma*, classified as a traditional Chinese herb, has been directly used for medical applications such as prevention and treatment of cancer (Kapadia et al. 1996; Chang et al. 2010) and contains the major constituent dryocrassin ABBA. Against this historical backdrop, *D. crassirhizoma* extract could be considered a good candidate to test as a new tool for control of potato dry rot. Notably, the spore germination and mycelial growth results reported here suggest that dryocrassin ABBA strongly inhibits spore germination and mycelial growth of *F. solani* var. *coeruleum*, in agreement with the results mentioned above.

SEM results described in this work suggest that dryocrassin ABBA exerts a strong inhibitory effect on hyphal development. Moreover, these results are in agreement with previous reports, including Li et al. (2009a,b), Li and Xue (2014) and Elsherbiny et al.

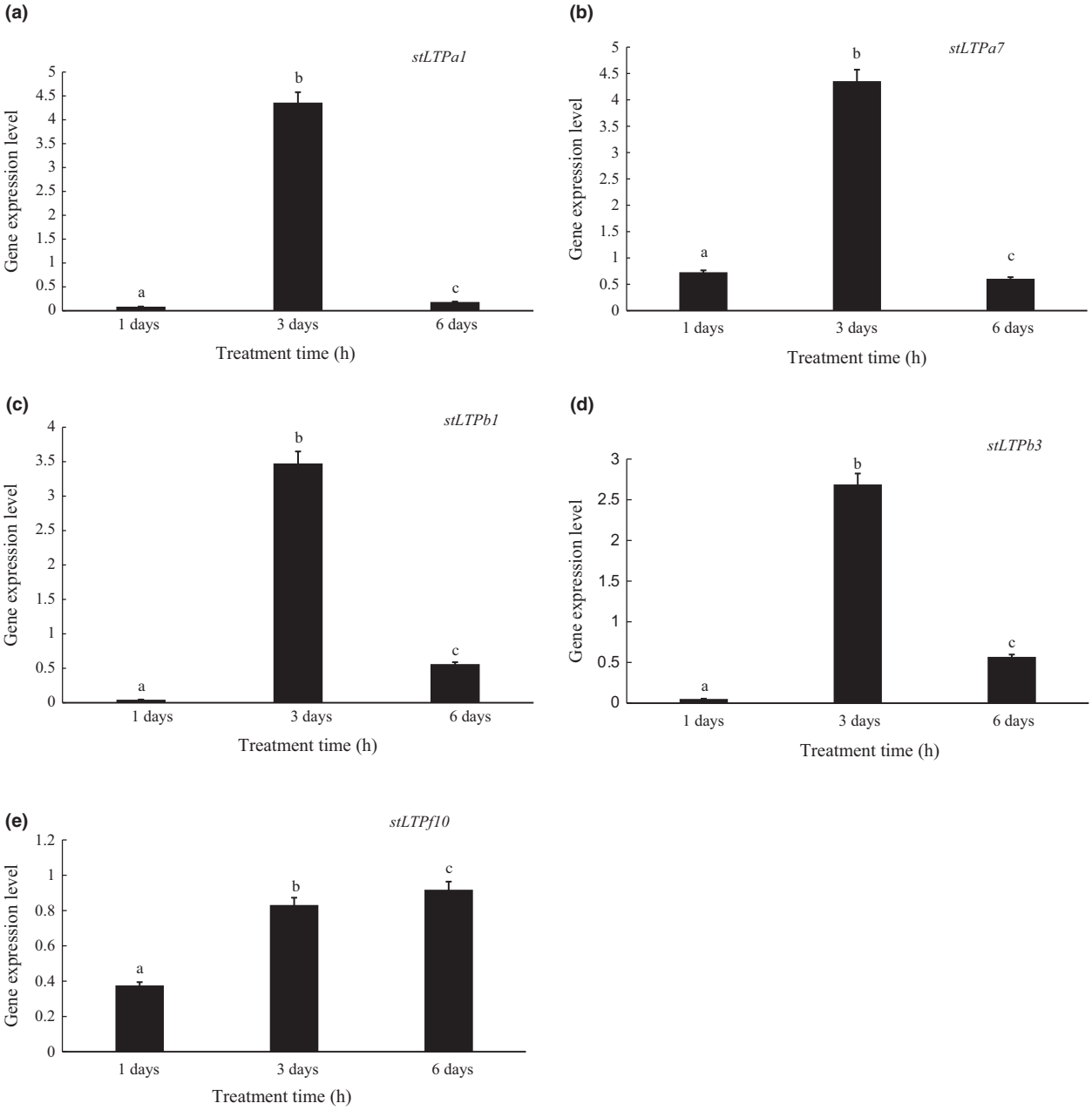

Fig. 5 Real-time PCR analyses of resistance-related genes on dry rot of potato slices Kexin No. 13 treated with *Dryopteris crassirhizoma* dryocrassin ABBA after 1-, 3- and 6-day incubation at 25°C. The expression level of *StLTPa1* (a), *StLTPa7* (b), *StLTPb1* (c), *StLTPb3* (d) and *StLTPf10* (e) is showed. The mean and standard error are expressed by the values and error bars as indicated, respectively, from thrice independent measurements. Significant differences are shown by dissimilar letters ($P < 0.05$, one-way ANOVA, followed by Duncan's test).

(2016); these previous studies demonstrated that treatment with sodium silicate, chitosan, the essential oil of *Z. bungeanum* and *P. granatum* peel extract could each inhibit hyphal development of *F. sulphureum*. Therefore, inhibition of hyphal development may turn out to be an important strategy for control of fungal pathogens.

Potato physiological analysis after dryocrassin ABBA treatment

After the pathogens infection, plants will produce a series of defence reactions. Some biological factors or abiotic factors also could induce the defence reaction, and these factors are called elicitor. *Dryopteris*

crassirhizoma is a Chinese herb for a long time. Dryocrassin ABBA is a kind of phloroglucinol and extracted from the whole plant, which has very effective against a board spectrum of parasitic (Lu et al. 2012), cancer (Chang et al. 2010) and virus (Ou et al. 2015). Previous studies have shown that *D. crassirhizoma* dryocrassin ABBA reduces decay incidence, caused by *F. solani* var. *coeruleum* and induced resistance against potato dry rot in potato. Besides its antifungal activity, dryocrassin ABBA also has the potential for inducing defence-related enzymes in potato. POD has great effect on plant resistance and metabolism (Lu 2002; Song et al. 2015). POD exists in peroxidase in the cell of plant and participates in the removal of hydrogen peroxide (H_2O_2) when the plants are infected. POD also produces toxic substances, such as phenol and quinone. SOD plays an important role in plant resistance and eliminate the reactive oxygen species (ROS) (Zou et al. 2008). Soluble protein content is a kind of small molecular protein and closely to plant defence response. In this study, we observed the activities of POD, SOD and soluble protein content in dryocrassin ABBA-treated potato continuously increased, and this indicated the potato resistance increased maybe connected with POD, SOD and soluble protein.

MDA is the final plant production under the adversity situation and senescence. It is used as measurement of membrane lipid peroxidation and has negatively correlated with the resistance of plants (Fenglan et al. 2015). In our research, the MDA content in dryocrassin ABBA-treated potato decreased and it demonstrated dryocrassin ABBA could induce potato increase the ability of getting rid of ROS and protect the integrity of cell membrane.

Resistance-related genes analysis after dryocrassin ABBA treatment

Plant LTPs are abundant, small, lipid binding proteins that are signalling molecules and hormones, and may be important components of direct defence against pathogens and bio stress (van Loon et al. 2006; Yeats and Rose 2008). In Solanaceae crop, *LpLtp1*, *LpLtp2* and *LpLtp3* in tomato (*Lycopersicon pennellii*) were induced by drought and abscisic acid (ABA) (Trevino and Oconnell 1998). *CALTPI*, *CALTPII* and *CALTPIII* in *Capsicum annuum* were induced by *Xanthomonas campestris* pv. *vesicatoria vesicatoria* (Jung et al. 2005). The expression level of *StLTPa1* was upregulated after affected by *F. culmorum* or *R. solanacearum* in potato tuber (Gao et al. 2009; Fu et al. 2016). *StLTPa7* was systemically upregulated by infection with *R. solanacearum* and stimulated by salicylic acid (SA), methyl jasmonate (MeJA), ABA and Ca^{2+} in potato tuber (Gang et al. 2009). It was reported that *StLTPb1* mRNA was localized in phloem cells of vascular tissues in potato leaf and stem tissues after pathogen infection. SA, MeJA and ABA could induce *StLTPb1* gene express (Gang et al. 2008). In this study, we detection five LTPs genes expression levels by real-time PCR and the results showed *StLTPa1*, *StLTPa7*, *StLTPb1* and *StLTPb3* were upregulated after dryocrassin ABBA treatment. This indicated that dryocrassin ABBA could induce these four LTPs genes express.

Conclusion

The results of this study demonstrate that *D. crassirhizoma* dryocrassin ABBA treatment can significantly inhibit spore germination and mycelial growth of *F. solani* var. *coeruleum*. This is the first report on the activity of dryocrassin ABBA for control of a postharvest fungal disease of potatoes. In addition, this dryocrassin extract can inhibit dry rot of potato tubers and slices inoculated with the pathogen. It is also effective at inducing disease resistance-related enzymes and genes increased in potatoes. This suggests that dryocrassin ABBA improves resistance of potato against *F. solani* var. *coeruleum* and is a promising natural fungicide on potatoes.

Acknowledgements

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