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ORIGINAL ARTICLE

Differences in immunity between pathogen-resistant and -susceptible yam cultivars reveal insights into disease prevention underlying ethylene supplementation

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Abstract

Leaf necrosis induced by fungal pathogens is one of the most devastating diseases of yam. The objectives of this study were to examine the production of defensive phytohormones and perform a comparative transcriptome analysis between two yam cultivars with different resistance levels against *Botrytis cinerea* inoculation. After inoculation with *B. cinerea*, the endogenous ethylene level was found to have accumulated to a higher level in the MH1 resistant cultivar. Meanwhile, expression profiles identified differential defense regulation of ethylene pathway in MH1 (versus susceptible cv. MH3) in response to *B. cinerea*. A number of ethylene-synthesis and -responsive genes were expressed at higher levels in MH1 than in MH3 after inoculation. Furthermore, ethylene supplementation in MH3 plants by ethephon spraying indeed enhanced the resistance against *B. cinerea* and *Colletotrichum alatae*, and elevated the expression of *DaEIN2* and *DaERF96*. Our work improves understanding of defense mechanism and highlights the function of ethylene potentially utilized for yam protection against diseases.

Keywords Yam · *Bortrytis cinerea* · Disease resistance · Ethylene · Ethephon · *Colletotrichum alatae*

Abbreviations

B. cinerea	*Botrytis cinerea*
Chl	Chlorophyll
EIL	Ethylene-insensitive 3 like
ERF	Ethylene-responsive transcription factor
ET	Ethylene
JA	Jasmonic acid
MH	Minghuai
RPKM	Reads per kilobase per million mapped reads
SA	Salicylic acid

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Introduction

Pathogen infections are one of many biotic stresses that negatively impact crop productivity. In Asia and Africa, the yam (*Dioscorea alata* L.) is an important food, medicinal, and economic crop, and is rich in dietary fiber, anthocyanin, and vitamin C (Polycarp et al. 2012). Yam is easy to grow, and is rarely attacked by insect herbivory in the field, whereas the tuber and leaves are susceptible to many diseases caused by fungal infections, including anthracnose, *Fusarium* wilt, gray mold, and southern blight (Winch et al. 1984; Egesi et al. 2007; Cooper 2012). These diseases reduce efficiency of the photosynthetic function (Winch et al. 1984), resulting in heavy losses of yam productivity (Egesi et al. 2007). In the past few decades, many chemical fungicides, such as dithianon, pyraclostrobin, thiabendazole, and difenoconazole, were generally applied to cope with pathogenic stresses of yam in the field (Shuai et al. 2013). However, chemical fungicides are not the ideal ways to control the diseases of yam due to food and environmental safety (Okigbo 2003). Recently, great efforts have been made to reduce the fungicide application in crop cultivation. The plant breeding strategy

with high immunity was proposed as a goal to cope with the disease problem (Popoola et al. 2013). In addition, some plant resistance inducers, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), brassinolide, and cinnamic acid, and biofungicides were used as substitute for chemical fungicides to control pathogen infection in plants (Hua et al. 2018; Li et al. 2020a, b). Several biofungicides have been intensively investigated in terms of disease managements against root rot and leaf spot diseases in yam (Okigbo 2003; Soares et al. 2006; Ijato 2019). Nevertheless, due to the lack of basis on hormone-mediated defense in yam, the resistance inducers have been rarely characterized.

JA, SA, and ET are important phytohormones that are responsible for inducing defense responses against pathogen infection (Zhao et al. 2012; Broekgaarden et al. 2015; Amorim et al. 2017). To date, several studies have shown the regulatory mechanisms of JA, SA, and ET in plant defense systems. Many types of transcription factor (TF) genes that respond to defensive phytohormones have been reported that function in the susceptibility or resistance to necrotic disease caused by *Botrytis cinerea* in Arabidopsis. WRKY TFs (*WRKY28/51*), NAC TFs (*ANAC019/055*), bHLH TFs (*MYC2/3/4*), EIN3/EIL TFs, and AP2/ERF TFs (*ERF1/4/5/96, ORA59, and RAP2.2*) are synergistically regulated by JA and ET in Arabidopsis upon *B. cinerea* infection. These JA/ET-induced TFs are associated with the expression of the genes encoding PDF1.2, PR, NPR, and chitinase to resulting in proper plant responses against pathogen attack (Bu et al. 2008; Wild et al. 2012; Windram et al. 2012; Zhao et al. 2012; Zhu and Zhu 2013). Moreover, several WRKY transcription factors, such as *WRKY8*, *WRKY38, WRKY53, WRKY66* and *WRKY70*, were induced by SA that regulated the expression of *NPR1* against necrotic pathogen (Wang et al. 2006; Chen et al. 2010; Ishihama and Yoshioka 2012).

The antagonistic and synergistic regulations between JA and ET are able to fine-tune the defense against invaders in plants. Previous studies indicated that JAZ proteins directly interact with and repress EIN3/EIL, while JA induces JAZ degradation to de-repress EIN3/EIL. Subsequently, the EIN3/EIL1 interacts with and represses the transactivation of MYC2 to attenuate JA-induced resistance to disease. Similarly, MYC2 also interacts with EIN3 and EIL1 to inhibit ET-regulated responses (Song et al. 2014; Yang et al. 2015). Li et al. (2020a, b) also reported that EIN3/EILs transcription factors repressed multiple target genes involved in SA biosynthesis, transport, and signaling to balance vitality and immunity at lower temperature when immunity was enhanced. These studies suggest that the study on dynamic accumulation of plant hormones is vital for the defense of plant during the pathogenic invasion.

Hormone analysis and profiling the expression of defense-related TF are able to examine the plant immunity. Many plants have been dissected to profile the expression of defense regulators by wide-ranging analyses of transcriptional maps of plant–pathogen interactions. Consistent with their expressions in response to infection, most genes play critical roles in reprogramming defense signaling in plants (Soto-Suárez et al. 2017; Xing et al. 2017). In this study, two yam cultivars with different resistances were selected to dissect the molecular basis of the reaction to a pathogen stress. Differences in phytohormone accumulation and gene expression patterns mediated by *B. cinerea* between the resistant and susceptible yam cultivars were discussed, and a strategy was proposed to improve the resistance against necrotic pathogen by ethylene supplement. From an agricultural perspective, identifying and studying the transcriptional regulation of defense responses provide important molecular events for unveiling the defensive mechanism in resistant yams. In addition, supplementation of a hormone elicitor to enhance the immunity confers a new strategy for disease protection in yam.

Materials and methods

Plant materials

Two yam (*D. alata*) cultivars Minghuai 1 (MH1) and Minghuai 3 (MH3) were bred by the Institute of Dryland Crops, Sanming Academy of Agricultural Sciences, Fujian, China. Minghuai 1 (MH1) is a purple-flesh yam cultivar with strong tolerance to biotic and abiotic stresses, and has been used in food processing, anthocyanin extraction, and phytoremediation (Chen et al. 2019). Minghuai 3 (MH3) is a white-flesh yam cultivar for food consumption but is more susceptible to biotic stress. Seedlings of 5 cm in length from tissue culture were transferred to plastic pots (7-cm diameter) containing soil and vermiculite (v/v = 2:1), and plants were grown in a growth chamber (BIC-400, Fujian, China) under 100 μmol m^{-2} s^{-1} of light with a 16-h photoperiod at 30/25 °C (light/dark) temperatures and 70–80% relative humidity. Plants were watered twice a week, and fertilized (14:14:14, N:P:K) once a month. The third set of expendable leaves counted from the terminal bud of MH1 and MH3 were used for all experiments. Leaf samples were collected and rapidly frozen in liquid nitrogen. After that, frozen samples were stored at − 80 °C.

Assay of resistance to *Botrytis cinerea* and *Colletotrichum alata*

Botrytis cinerea was grown on potato dextrose agar (PDA) medium at 25 °C for 14 days. After that, a 6-mm agar disc

with *B. cinerea* was placed on the third leaves of 45-day-old MH1 and MH3 plants. The resistance levels of MH1 and MH3 were observed and compared at 48 h after inoculation.

For the resistance assay of MH3 in the presence of ethephon, 30-day-old leaves of MH3 were sprayed with freshly prepared ethephon (Sigma, Ronkonkoma, NY, USA) solutions at 0 (CK), 0.1, 1, and 10 mM. After 24 h of spraying, 5×10^5 spores/ml of *B. cinerea* was placed on the leaves. Pictures of leaves and the sizes of disease lesions were recorded at 72 h post-inoculation (hpi). Treated leaves of MH3 were then ground in liquid nitrogen and assayed for chlorophyll (Chl) a and b as previously described by Minocha et al. (2009).

Colletotrichum alatae was isolated form leaf anthracnose of MH3, and subsequently identified by using ITS sequences. *Colletotrichum alatae* was grown on PDA medium at 25 °C for 28 days. For the resistance assay, 30-day-old leaves of MH3 were sprayed with 0 mM (CK) and 1 mM freshly prepared ethephon. After 24 h of spraying, 1×10^6 spores/ml of *C. alatae* was dropped on the leaves.

Phytohormones quantification

Phytohormones quantification were measured using JA (MyBioSource, San Diego, CA, USA), SA (MyBioSource, San Diego, CA, USA), and ET (Tsz Biosciences,CA, USA) detection kits described by Lin et al. (2019), Gao et al. (2017). In general, sample powder (0.2 g) ground in liquid nitrogen was mixed with 1 ml 1× phosphate-buffered saline (PBS, pH 7.4), and then homogenized by vortexing. The mixtures were centrifuged at 4 °C for 15 min to remove the pellet, and 10 µl of supernatants were then mixed with 40 µl diluted solution and 100 µl HRP-conjugate of JA, SA, and ET to each well, respectively. After incubation for 60 min at 4 °C, the wells were washed by 1× Wash Solution. Subsequently, the chromogen solution A and B were added to each well. The levels of phytohormones were measured with an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) at an optical density of 450 nm.

Comparative transcriptome analysis

Leaf samples inoculated with or without 5×10^5 spores/ml of *B. cinerea* for 48 h were used to generate complementary cDNA libraries, and subsequently analyzed with the HiSeq 4000 Sequencing System. After trimming adapter sequences and filtering low-quality reads, the clean reads were used to De novo assemble contigs of yam transcriptomic datasets. In total, 135, 431, 904 raw reads of these cDNA libraries were subsequently recorded. After discarding reads of low quality, the clean pair reads were then mapped to unigenes by de novo assembly, and the distribution of contig lengths of mapped contigs is listed in Supplementary Fig. S1. The contigs were further annotated against GenBank using the BLASTn and BLASTx. Expression levels of mapped genes were calculated by the value of the reads per kilobase per million mapped reads (RPKM). Transcriptional changes of ET-related genes between the MH1 and MH3 cultivars were surveyed (ratio > 1.5 or < 0.7 as thresholds for up- and down-regulation). Three biological replicates for each cDNA library were performed and the raw sequences have been deposited in the NCBI short read archive (SRA) under accession PRJNA544814.

RNA extraction, cDNA synthesis, and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Yam leaves were ground to a powder in liquid nitrogen. Total RNA from 20 mg of leaf powder was extracted using the Trizol reagent (Life Technologies, Carlsbad, CA, USA), and then concentrations and quality of total RNA were determined with an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) at 260 and 280 nm. First-strand cDNA was synthesized from 1 µg of total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Japan). cDNA was diluted 1:5 and then amplified with gene-specific primers and SYBR® Green Real-Time PCR Master Mixes (Takara Bio, Otsu, Japan) for the real-time qPCR analysis in the ABI QuantStudio 3 system (Applied Biosciences, Waltham, MA, USA), which quantified relative changes in gene expressions by $2^{-\triangle\triangle Ct}$ method. Gene-specific primers for the RT-qPCR were designed and are listed in Table S1. To normalize the total amount of cDNA in each reaction, the *DaActin1* gene was co-amplified as the internal control. The amplification steps were as follows: one cycle of 94 °C for 3 min for initial denaturation, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s. A melting-curve analysis was conducted to confirm the amplicon quality of the RT-qPCR.

Effect of ethephon on gene expressions of the MH3 cultivar

Thirty-day-old leaves of MH3 were sprayed with freshly prepared ethephon at 0 mM (CK) and 1 mM. Expressions of ET-responsive genes were determined by RT-qPCR at 24 h after spraying.

Statistical analysis

All measurements in this study are presented as the mean value with the standard deviation (SD) of at least three

independent replicates, as stated in each figure legend. Statistical significance was analyzed by a two-tailed Student's *t*-test at significant *p* values of < 0.05 or < 0.01 using Excel 2018 software.

Results

MH1, a resistant yam cultivar against *B. cinerea*

Leaves of MH1 and MH3 were inoculated with *B. cinerea* to determine whether MH1 exhibited stronger resistance to necrosis disease compared to the susceptible MH3 cultivar. Figure 1a shows that the vicinity of the infected region on the leaf of MH3 had turned brown at 48 hpi, indicating that early necrotic symptoms had occurred. In contrast, no obvious necrotic symptoms were observed on MH1 leaves after inoculation, suggesting that the difference in immunity between MH1 and MH3 could be a key to studying defense mechanisms against necrotic pathogens in yams. Furthermore, the in planta growth of *B. cinerea* were measured at 24 and 48 hpi by RT-qPCR. At 24 hpi, the expression of *BcActin* was detected in MH3, but not in MH1 (Fig. 1b). Consistent with the disease symptom observed on leaves surface at 48 hpi, significantly lower fungal hyphae were detected in MH1 (0.1) compared to those in MH3 (3.6), indicating that MH1 had better resistance to *B. cinerea* infection than MH3.

Differences in the dynamic accumulation of defensive phytohormones between MH1 and MH3

To investigate accumulated levels of defense-related phytohormones in response to *B. cinerea* inoculation, levels of endogenous JA, SA, and ET were quantified in MH1 and MH3 cultivars at 0, 24 and 48 hpi. The JA content was significantly higher in MH1 (1122.1 pg g^{-1} FW) compared to MH3 (662.5 pg g^{-1} FW) at 24 hpi, whereas it did not significantly differ between MH1 and MH3 at 0 and 48 hpi (Fig. 2a). In contrast to JA, there was no difference in SA accumulation between MH1 and MH3 at 0 and 24 hpi, whereas less SA had accumulated in MH3 (8.2 µg g^{-1} FW) compared to MH1 (13.7 µg g^{-1} FW) at 48 hpi (Fig. 2b). Furthermore, the pronounced accumulations of ET were found in both MH1 and MH3 after inoculation (Fig. 2c). Although the basal level of ET was no significant difference between MH1 and MH3, the level of ET in MH1 was approximately 2.2-fold and 2.0-fold higher than in MH3 at 24 and 48 hpi, respectively.

Transcriptome profiles reveal distinct differences in ET to resistance between MH1 and MH3

In order to ascertain the transcriptional responses of ET signaling genes in response to *B. cinerea* between MH1 and MH3, transcriptome datasets of MH1 and MH3 with inoculation were generated. Meanwhile, the transcriptomic

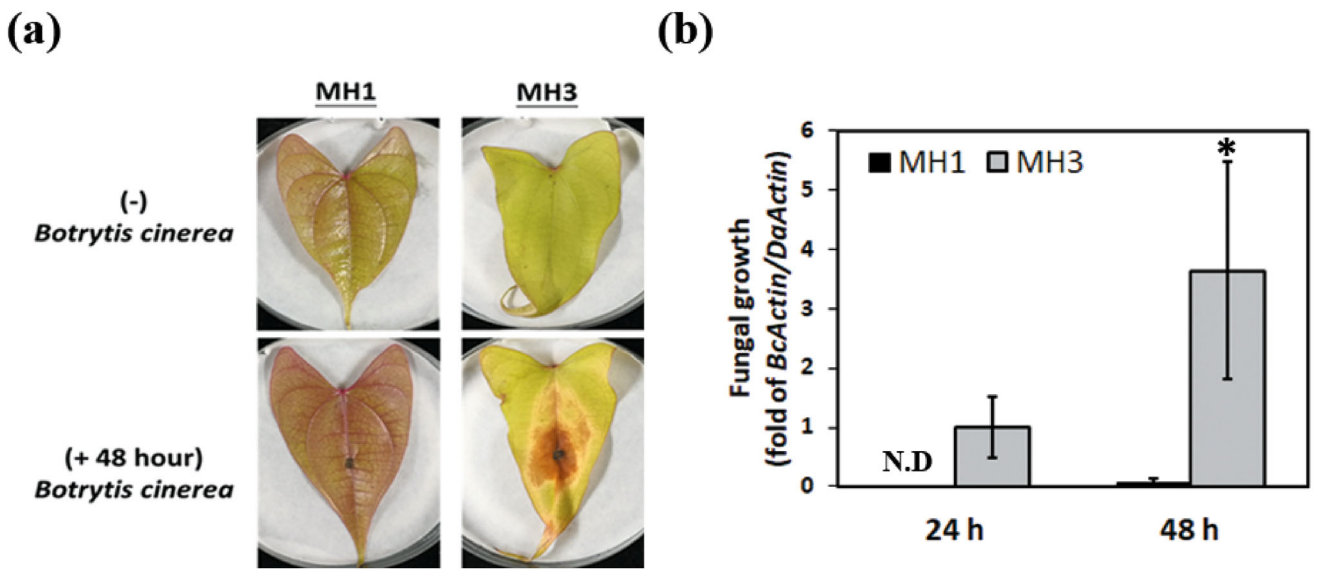

Fig. 1 Comparisons of pathogenic resistance between MH1 and MH3 yam cultivars using *Botrytis cinerea* inoculation. **a** Phenotypic differences in disease symptom between MH1 and MH3 were observed after *B. cinerea* inoculation for 48 h. **b** In planta growth of *B. cinerea* in MH1 and MH3 plants. The hyphae of *B. cinerea* were determined by the expressions of *B. cinerea BcActin* and *Dioscorea alata DaActin*. Relative fungal growth was measured by ratios of *BcActin/DtActin*. The error bar indicates the standard deviation (SD) from four independent replicates. An asterisk indicates a significant differences between MH1 and MH3 as determined by Student's *t*-test (*$p < 0.05$). *ND* non detectable

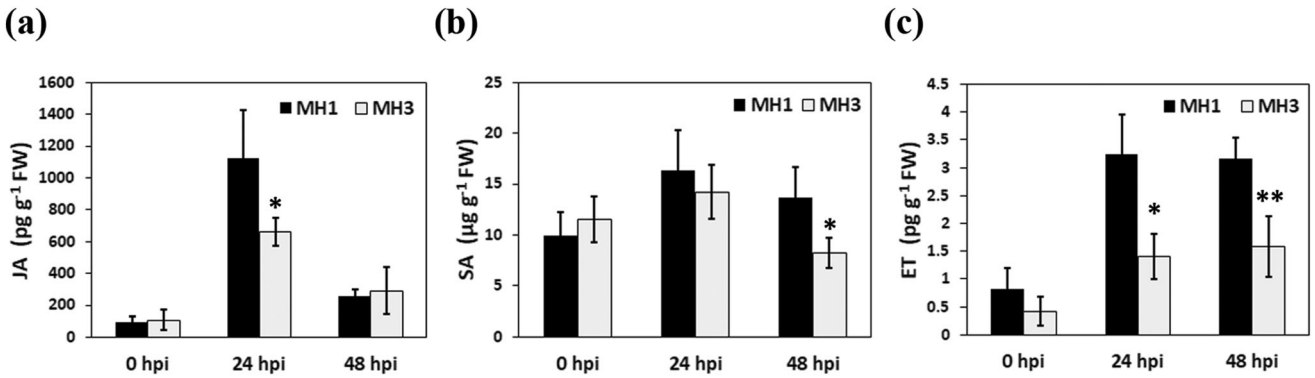

Fig. 2 Hormone accumulations in MH1 and MH3 after *B. cinerea* inoculation. Levels of JA (**a**), SA (**b**), and ET (**c**) were determined in MH1 and MH3 leaves after *B. cinerea* inoculation for 0, 24, and 48 hpi. The error bar indicates the standard deviation (SD) from four independent replicates. An asterisk indicates a significant difference between MH1 and MH3 as determined by Student's *t*-test (*$p < 0.05$; **$p < 0.01$)

profile of MH1 without inoculation with *B. cinerea* was also established to compare the changes in MH1. The comparative transcriptome showed that the genes involved in ET signaling were found to be critical for defense against *B. cinerea* between MH1 and MH3 (Supplementary Table S2). Compared to MH3, expression patterns of several ET synthesis-related genes, such as *S-adenosyl-methionine synthase* (SAMS) and most ET synthesis-related genes were expressed at higher levels in MH1 at 48 hpi (Fig. 3a), suggesting that *B. cinerea*-induced ET synthesis may determine the defensive ability in yams. Furthermore, *SAMS and 1-aminocyclopropane-1-carboxylate oxidase* (ACO) in MH1 were highly induced at 48 hpi. To further study whether ET signaling transduction also differed between MH1 and MH3, expressions of an ET receptor and transmembrane protein genes were surveyed. In particular, expression levels of most *EIN2* genes were lower in MH3 than in MH1 at 48 hpi (Supplementary Fig. S2), implying that both the synthetic regulation and signal transduction of ET significantly differed between MH1 and MH3.

Moreover, ET-related transcription factor families were also surveyed in this study. In the comparative transcriptome analysis, we noted that only *DaEIL3* expression in MH1 was higher than that in MH3 after *B. cinerea* infection (Fig. 3b), suggesting that the DaEIL3 transcription factor may have an important function in resistance compared to other EIN3/EIL genes in MH1. Furthermore, several pathogen defense-related ERF genes, including *DaERF1* (TR25722_c0_g1_i1 and TR38245_c0_g1_i1), *DaERF2* (TR15217_c0_g1_i1), and *ERF96-like 1*(TR52730_c0_g1_i1), were upregulated in MH1 after *B. cinerea* inoculation, and were expressed at higher levels in MH1 than in MH3, suggesting that significant defense by yams against pathogens may be initiated through ET regulation.

To validate the transcriptome results of ET genes, expressions of selected genes, *ACO* (TR24075_c0_g1_i7), *ERF1* (TR38245_c0_g1_i1), *EIL3* (TR17182_c0_g2_i2), and *ERF96-like 1* (TR52730_c0_g1_i1), were analyzed by an RT-qPCR. Consistent with the transcriptomic profiles, expressions of the selected ET-related genes showed significantly higher levels in MH1 (set as 1.0-fold) compared with those in MH3 (ranging from 0.08 to 0.4-fold) at 48 hpi (Fig. 4). The agreement of the data between hormone analysis and expression profiling suggests that the difference in immunity to pathogen between MH1 and MH3 may be controlled by ET.

Ethephon improves leaf resistance against necrotic diseases in the MH3 cultivar

Botrytis cinerea inoculation induced higher accumulation levels of ET in MH1 than in MH3. Therefore, we investigated whether a deficiency of ET was closely related with resistance against *B. cinerea* in MH3. Leaves of MH3 pretreated with 0 (CK), 0.1, 1, or 10 mM of ethephon were inoculated with *B. cinerea*. The leaves with CK treatment exhibited severe necrotic symptoms at 72 hpi. In contrast, all of the ethephon-supplied plants presented lower necrosis levels compared to CK plants (Fig. 5a). Among them, plants treated with 1 mM ethephon showed significant function of improving resistance against *B. cinerea* compared to CK plants. Approximately 47%, 64%, and 53% reductions in the average lesion diameter were obtained in leaves treated with 0.1, 1, and 10 mM ethephon, respectively (Fig. 5b). Meanwhile, the chlorophyll content was taken as another indicator of the immunity of yam against *B. cinerea*. Compared to CK treatment (0.39 mg g^{-1} FW), leaves sprayed with 1 mM ethephon presented significantly higher levels of total chlorophyll

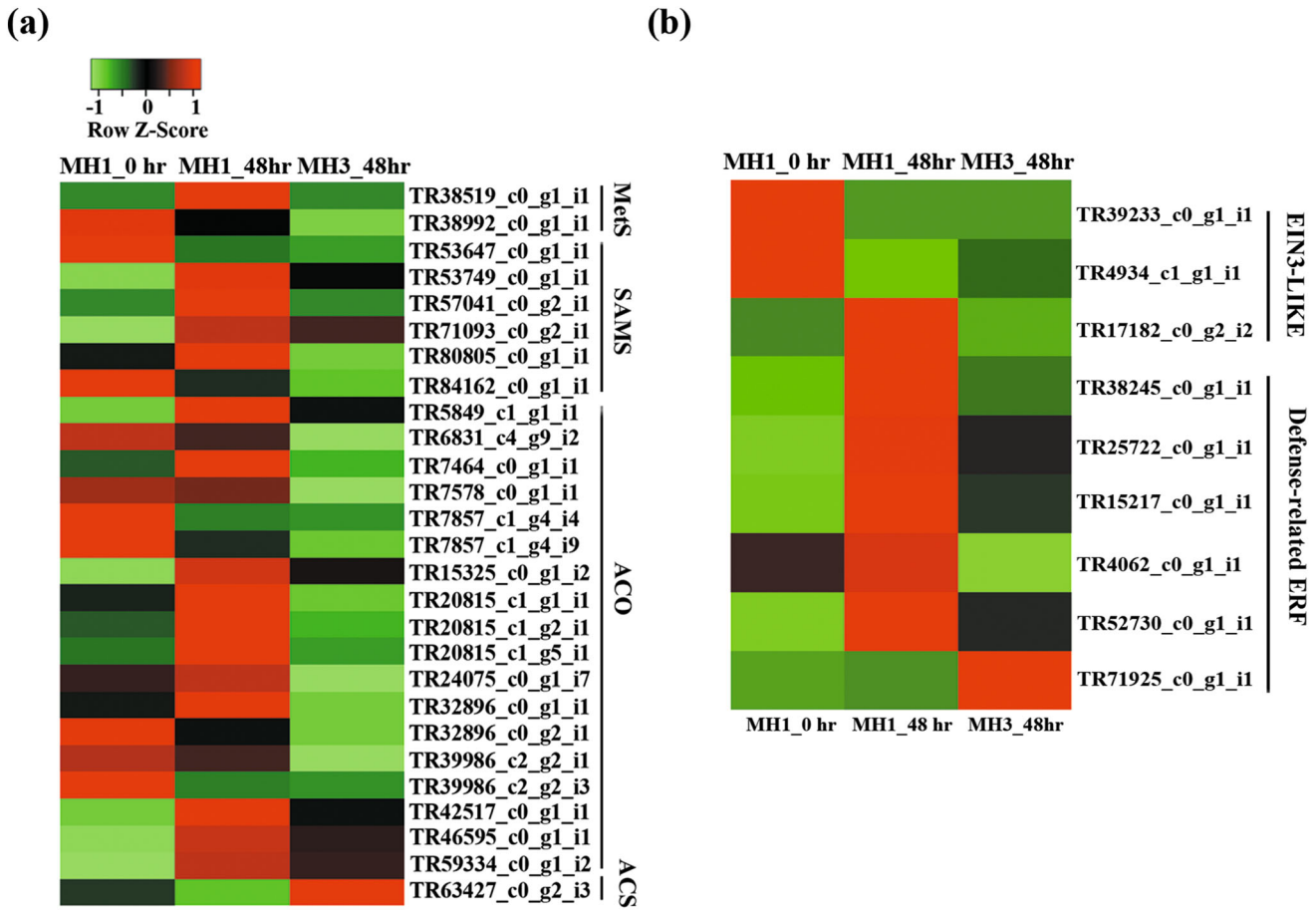

Fig. 3 Differential transcript levels of ET signaling genes in MH1 and MH3. The basal and induced (48 hpi) expressions of ET synthesis genes (**a**) and ET-responsive transcription factor genes (**b**) between MH1 and MH3 were profiled and presented. Red and green boxes showed the up- and down-regulated genes normalized by the Z-score

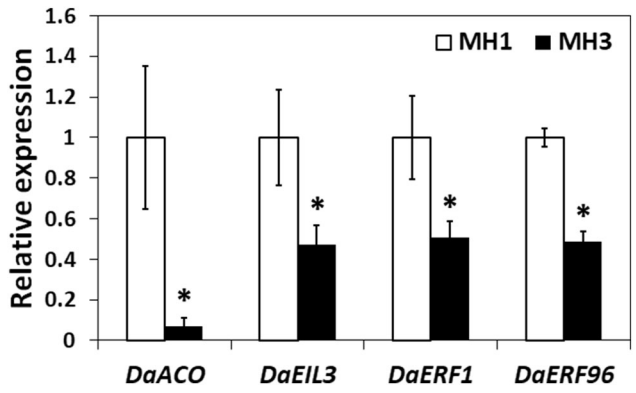

Fig. 4 Expression analysis of ET signaling genes between MH1 and MH3 upon *Botrytis cinerea* inoculation. Expressions of selected ET-related genes, *ACO*, *EIL3*, *ERF1*, and *ERF96-like 1*, were monitored in MH1 and MH3 at 48 hpi with *B. cinerea* by RT-qPCR. *DaActin1* was used as an internal reference gene. Fold changes of expression levels were calibrated by MH1. The error bar indicates the standard deviation (SD) from four independent replicates. An asterisk indicates a significant differences between MH1 and MH3 as determined by Student's *t* test ($*p < 0.05$)

(0.52 mg g^{-1} FW) at 72 hpi (Fig. 5c). Fungal hyphae in ethephon-treated plants were also examined at 72 hpi by RT-qPCR. In consistence with the results of disease symptom and chlorophyll content, the expression of *BcActin* gene in ethephon-treated plants was approximately 10-fold lower than that in CK-treated plants, suggesting that ethephon significantly improved the resistance of yam to *B. cinerea* (Fig. 5d).

Anthracnose caused by *C. alatae* is another severe necrotic disease of yam. Here, the resistance against *C. alatae* in ethephon-pretreated leaves was assayed. The MH3 leaves with CK treatment exhibited severe necrotic symptoms caused by *C. alatae*. In contrast, the leaves treated with 1 mM ethephon showed obvious function of improving resistance against *C. alatae* (Fig. 5e), suggesting that ET supplementation activates the immunity of yam leaves in response to necrotic pathogen.

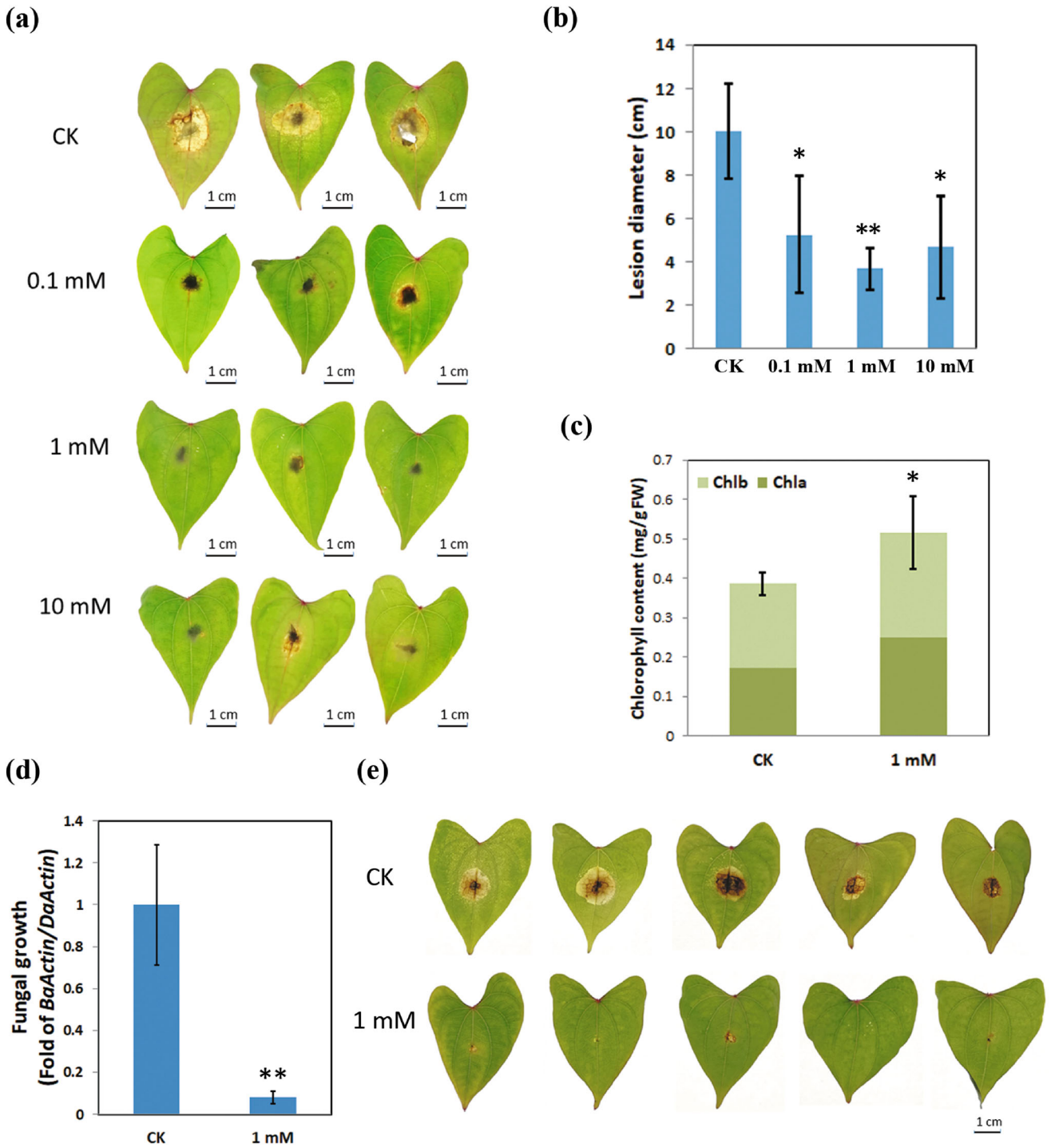

Fig. 5 Effect of ethephon on resistance against pathogen in MH3. Leaves of MH3 pretreated with 0 (CK), 0.1, 1, and 10 mM ethephon were inoculated with *B. cinerea* and *C. alatae* spores. **a** Disease symptoms caused by *B. cinerea* were shown in MH3. **b** Leaf necrosis development by *B. cinerea* (72 hpi) was evaluated by measuring the necrosis diameter. **c** Chlorophyll (Chl) a and b in MH3 were examined after 72 hpi with *B. cinerea*. **d** Fungal growth of *B. cinerea* in MH3. The hyphae of *B. cinerea* were determined by the expressions of the *B. cinerea BcActin* and the *Dioscorea alata DaActin*. Relative fungal growth was measured by ratios of *BcActin*/*DtActin*. **e** Disease symptoms caused by *C. alatae* (7 days post inoculation) were shown in MH3. The error bar indicates the standard deviation (SD) from three independent replicates. An asterisk indicates a significant differences between the CK and ethephon treatment as determined by Student's *t* test (*$p < 0.05$; **$p < 0.01$)

Expression analysis of ET signaling genes involved in defensive functions of ET

Ethephon spraying effectively enhanced the resistance of the MH3 cultivar (Fig. 5). To further understand the regulatory mechanism of ET in MH3, the expressions of ET-signaling genes induced by *B. cinerea* in MH1 (Fig. 3) were monitored in ethephon-treated MH1 and MH3 plants. The relative RNA levels of *DaEIN2* (TR69160_c1_g2_i4) were significantly increased in both cultivars after ethephon treatment (Fig. 6). Likewise, the relative RNA levels of *DaERF96-like* (TR52730_c0_g1_i1) in MH1 and MH3 were 12.7-fold and 6.3-fold increase in response to ethephon, respectively. Thus, the expressions of *DaEIN2* and *DaERF96-like* were induced by the presence of 1 mM ethephon, and the dramatically increased level of *DaERF96* implied its important function in ET-mediated resistance of yam. However, the transcription of *DaERF1* (TR38245_c0_g1_i1) and *DaEIL3* (TR17182_c0_g2_i2) showed no significant difference after treatment with ethephon in both MH1 and MH3. In addition, a slight increase of *EIN4* (TR73119_c0_g1_i1) was found in MH3 after ethephon treatment, but the increase was not significant in MH1.

Discussion

The molecular mechanisms underlying pathogen attacks are poorly understood in *Dioscorea alata*. In the study, the variations in ET synthesis were highly related to resistance between the resistant (MH1) and susceptible (MH3) cultivars in the defense against *B. cinerea*. ET often plays a critical role in biotic stress defense, particularly for defense against necrotrophic fungi (Broekgaarden et al. 2015). Conversion of SAM to ACC by ACS and oxidation of ACC by ACO are two important steps in the formation of ET. Apart from ACS and ACO, SAM synthase (SAMS), which functions in conversion of methionine into SAM, is also considered to be a critical enzyme gene in ethylene synthesis (Alexander and Grierson 2002; Zheng et al. 2020). In MH1, most of *SAMS* and *ACO* genes were up-regulated after inoculation with *B. cinerea* (Fig. 3a), which was reflected in the increase of ET (Fig. 2). Simultaneously, the contrasting levels of resistance due to ET accumulation between MH1 and MH3 could be explained by low expression levels of the ET-synthesis genes *MetS, SAMS*, and *ACO* in MH3 (Fig. 3a). As a consequence, the downstream ET- responsive regulator genes were expressed differentially between MH1 and MH3 (Figs. 3b and 4).

In ET-mediated signaling, EIN3/EIL transcription factors functions as a master regulator that determine the expressions of *ERF1* transcription factor leading to activation of defense genes, including *PR*s and *PDF1.2*, in plants (Alexander and Grierson 2002; Broekgaarden et al. 2015; Huang et al. 2015). Thus, the increased transcription of the EIN3/EIL transcription factor by ET effectively helps the plant resist pathogen attacks. Interestingly, *DaEIL3* and *DaERF1* were significantly up-regulated upon *B. cinerea* infection and was abundantly expressed compared to that in MH3 (Figs. 3 and 4), whereas the expression of *DaEIL3* and *DaERF1* in both plants could not be triggered by ethephon (Fig. 6). Notably, ethephon dramatically triggered *DaERF96-like* expression compared to other ET-related genes in yam (Fig. 6), which is consistent with the induced expression of *DaERF96-like* in MH1 upon inoculation with *B. cinerea* (Fig. 3). These suggest that ET induced by *B. cinerea* may mainly activate the expression of *DaERF96-like* for defense in yam. The DaERF96-like gene belongs to a small ERF gene family containing ERF95, − 96, − 97, and − 98. Catinot et al. (2015) reported that overexpression of *AtERF96* gene in

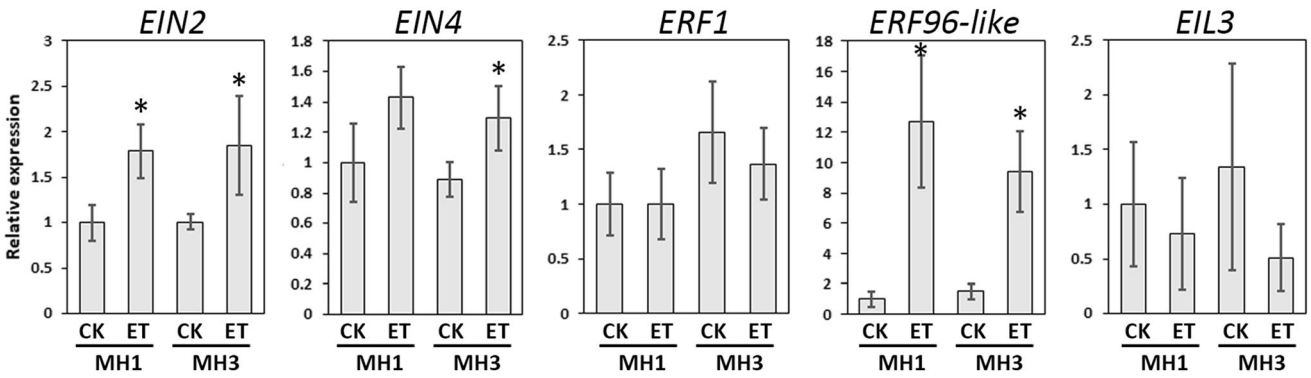

Fig. 6 Effect of ethephon on expressions of ethylene (ET)-responsive genes in MH3. Expression levels of *EIN2*, *EIN4*, *EIL3*, *ERF1*, and *ERF96-like* were monitored by an RT-qPCR analysis in MH1 and MH3 pretreated with 0 mM (CK) and 1 mM ethephon (ETH) for 24 h. *DaActin1* was used as an internal reference gene. The error bar indicates the standard deviation (SD) from four independent replicates. An asterisk indicates a significant differences between the CK and ethephon treatment as determined by Student's *t*-test (*$p < 0.05$)

Arabidopsis enhanced the resistance to *B. cinerea* and *Pectobacterium carotovorum* via activating downstream defensive genes of JA/ET signaling. However, the intact functions of other small ERF genes have rarely been characterized in plants. Thus, further study of the function of the DaERF96-like gene is important for understanding yam resistance to biotic and abiotic stresses, especially for pathogen infections.

In addition to ET-based defense, different patterns of JA and SA accumulations between MH1 and MH3 during *B. cinerea* inoculation (Fig. 2) implied their potential roles in defense capability. Yang et al. (2015) reported that antagonistic regulation of JA, SA, and ET accurately coordinated resistance in plants against pathogen invasion at different stages. The SA-mediated immunity against bacterial pathogen was suppressed by ET via EIN3/EIL transcription factors at low temperature (Li et al. 2020a, b). Other studies also showed that MYC2 interacted with and repressed EIN3/EIL to inhibit the ET-induced effects on resistance to necrotrophic pathogen (Song et al. 2014; Zhang et al. 2014). Accordingly, a significant increase of JA may function in trade-offs with ET-mediated programs in MH1 during the early inoculation compared to those in MH3. Furthermore, although MH1 presented higher SA level than MH3, biotrophic microbes activated defensive responses controlled by SA, whereas necrotrophic plant pathogens triggered defensive responses regulated by JA and ET signalings (Glazebrook 2005; Catinot et al. 2015). Thus, it would be more interesting to study the relationship between JA and ET in response to *B. cinerea* infection in yam.

Reducing the application of chemical fungicide is an immediate problem that needs to be addressed in yam cultivation. In this study, ethephon exhibited the effective function to improve the resistance against *B. cinerea* and *C. alata* in susceptible yam cultivar, MH3 (Fig. 5). Ethephon is known as an ET-releasing reagent in agriculture that acts as a potent regulator of plant growth, fruit ripeness, stress defense, and senescence (Duan et al. 2019; Yoosukyingsataporn and Detpiratmongkol 2019). To date, it is widely applied to many fruits and vegetables to enhance the harvest efficiency and improve the reproductive quality (Saltveit 1999). Although ET was reported in many studies to enhance defense against biotic stresses, which was well-observed in our study, the application of ethephon in agriculture to defend against pathogens is rare. Several benefits of using ethephon as a plant protective agent in agriculture include low environmental toxicity compared to other fungicides and easy conversion to ET (Cole et al. 2004; Ma et al. 2004). In our study, ethephon application on leaves with 0.1, 1, and 10 mM could trigger the defense program against *B. cinerea* in MH3, but the leaves sprayed with 1 mM of ethephon presented the minimum colony size of *B. cinerea* (Fig. 5). Accordingly, the application of 1 mM ethephon seems to be an appropriate solution to cope with necrotic pathogens. However, some studies indicated that an excessive concentration or frequency of ET application can result in inhibition of plant growth and promotion of pathogen growth (El-Kazzaz et al. 1983; Mutlu and Öktem 2017; Demir and ÇELİKEL 2019). Therefore, details of suitable usage amounts and frequencies of ethephon for spraying need to be further examined in yam prevention.

Conclusions

This study reveals a cultivar-dependent specificity of ET-mediated defense and provides a strategy with low toxicity for pathogen defense in yam. *Bortrytis cinerea* triggered the expressions of several ET synthesis genes, including *SAMS* and *ACO*, resulting in high level of ET accumulation in MH1. High level of ET could activate the transcriptional signaling of downstream responsive gene, such as *EIN2* and *ERF96-like 1*, to cope with the infection injury. In contrast, the lack of ET-depending defense was found in MH3 due to the low expressions of ET synthesis genes, but it could be rescued by ethephon spraying. Next, It is essential to study the effect of ethephon on physiology changes and productivity in yam cultivation is worthy of study. Moreover, further identification and characterization of the key gene in ET synthesis between MH1 and MH3 will be helpful in unveiling the distinct regulatory mechanisms against necrotic pathogens in yam.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot 53:2039–2055

Amorim A, Lidiane L, da Fonseca dos Santos R, Pacifico Bezerra Neto J, Guida-Santos M, Crovella S, Maria Benko-Iseppon A (2017) Transcription factors involved in plant resistance to pathogens. Curr Protein Pept Sci 18:335–351

Broekgaarden C, Caarls L, Vos IA, Pieterse CM, Van Wees SC (2015) Ethylene: traffic controller on hormonal crossroads to defense. Plant Physiol 169:2371–2379

Bu Q, Jiang H, Li CB, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C (2008) Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Res 18:756

Catinot J, Huang JB, Huang PY, Tseng MY, Chen YL, Gu SY, Lo WS, Wang LC, Chen YR, Zimmerli L (2015) ETHYLENE RESPONSE FACTOR 96 positively regulates A rabidopsis resistance to necrotrophic pathogens by direct binding to GCC elements of jasmonate- and ethylene-responsive defence genes. Plant Cell Environ 38:2721–2734

Chen L, Zhang L, Yu D (2010) Wounding-induced WRKY8 is involved in basal defense in Arabidopsis. MolPlant–Microbe Interact 23:558–565

Chen Z, Lu H-H, Hua S, Lin K-H, Chen N, Zhang Y, Chen S-P (2019) Cloning and overexpression of the ascorbate peroxidase gene from the yam (*Dioscorea alata*) enhances chilling and flood tolerance in transgenic Arabidopsis. J Plant Res 132:1–10

Cole RD, Anderson GL, Williams PL (2004) The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. Toxicol Appl Pharmacol 194:248–256

Cooper DC (2012) Mobile image ratiometry for the detection of *Botrytis cinerea* (Gray Mold). Nat Preced 6989:1

Demir S, ÇELİKEL FG (2019) Effects of plant growth regulators on the plant height and quantitative properties of *Narcissus tazetta*. Turk J Agric For 43(1):105–114

Duan X, Cai C, Yang Y, Chen F, Sang Z, Ma L (2019) Fall ethephon application enhances the freezing tolerance of *Magnolia wufengensis* during overwintering. Forests 10:868

Egesi C, Onyeka T, Asiedu R (2007) Severity of anthracnose and virus diseases of water yam (*Dioscorea alata* L.) in Nigeria I: effects of yam genotype and date of planting. Crop Prot 26:1259–1265

El-Kazzaz M, Sommer N, Kader A (1983) Ethylene effects on in vitro and in vivo growth of certain postharvest fruit-infecting fungi. Phytopathology 73:998–1001

Gao H, Xia X, An L, Xin X, Liang Y (2017) Reversion of hyperhydricity in pink (*Dianthus chinensis* L.) plantlets by AgNO3 and its associated mechanism during in vitro culture. Plant Sci 254:1–11

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Hua L, Yong C, Zhanquan Z, Boqiang L, Guozheng Q, Shiping T (2018) Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. Food Qual Saf 2(3):111–119

Huang P-Y, Catinot J, Zimmerli L (2015) Ethylene response factors in Arabidopsis immunity. J Exp Bot 67:1231–1241

Ijato JY (2019) Fungal pathogens of yam (*Dioscorea rotundata* Poir) and their bio-control. Life Sci J 16(9):92–94. https://doi.org/10.7537/marslsj160919.11

Ishihama N, Yoshioka H (2012) Post-translational regulation of WRKY transcription factors in plant immunity. Curr Opin Plant Biol 15:431–437

Li T, Zhang J, Tang J, Liu Z, Li Y, Chen J, Zou L (2020a) Combined use of *Trichoderma atroviride* CCTCCSBW0199 and brassinolide to control *Botrytis cinerea* infection in tomato. Plant Dis. https://doi.org/10.1094/PDIS-07-19-1568-RE

Li Z, Liu H, Ding Z, Yan J, Yu H, Pan R, Hu J, Guan Y, Hua J (2020b) Low temperature enhances plant immunity via salicylic acid pathway genes that are repressed by ethylene. Plant Physiol 182:626–639

Lin HF, Xiong J, Zhou HM, Chen CM, Lin FZ, Xu XM, Oelmüller R, Xu WF, Yeh K-W (2019) Growth promotion and disease resistance induced in anthurium colonized by the beneficial root endophyte *Piriformospora indica*. BMC Plant Biol 19(1):40

Liu X, Inoue H, Hayashi N, Jiang C-J, Takatsuji H (2016) CC-NBS-LRR-type R proteins for rice blast commonly interact with specific WRKY transcription factors. Plant Mol Biol Rep 34:533–537

Ma J, Lin F, Qin W, Wang P (2004) Differential response of four cyanobacterial and green algal species to triazophos, fentin acetate, and ethephon. Bull Environ Contam Toxicol 73:890–897

Massee G (1908) Fungi exotici: VIII. Bull Miscel Inf 216–219

Minocha R, Martinez G, Lyons B, Long S (2009) Development of a standardized methodology for quantifying total chlorophyll and carotenoids from foliage of hardwood and conifer tree species. Can J For Res 39:849–861

Mutlu A, Öktem A (2017) Effect of different ethephon doses to plant height, lodging and grain yield of durum wheat (*Triticum durum* L.). Mediterr Agric Sci 30:53–58

Okigbo RN (2003) Mycoflora of tuber surface of white yam (*Dioscorea rotundata* Poir) and postharvest control of pathogens with *Bacillus* subtilis. Mycopathologia 156(2):81–85

Polycarp D, Afoakwa E, Budu A, Otoo E (2012) Characterization of chemical composition and anti-nutritional factors in seven species within the Ghanaian yam (*Dioscorea*) germplasm. Intern Food Res J19:985–992

Popoola AR, Adedibu BO, Ganiyu SA (2013) Rapid assessment of resistance of tissue-cultured water yam (*Dioscorea alata*) and white guinea yam (*Dioscorea rotundata*) to anthracnose (*Colletotrichum gloeosporioides* Penz.). Arch Phytopathol Plant Prot 46:663–669

Saltveit ME (1999) Effect of ethylene on quality of fresh fruits and vegetables. Postha Biol Technol 15:279–292

Shuai Z, Shixiong L, Taixin Y (2013) Co-toxicity and effect of mixtures of difenoconazole and pyraclostrobin against *Colletotrichum gloeosporioides* on yam. Plant Prot 39::160–163

Soares ACF, Sousa CS, Garrido MS, Perez JO, Almeida NS (2006) Soil streptomycetes with in vitro activity against the yam pathogens *Curvularia eragrostides* and *Colletotrichum gloeosporioides*. Braz J Microbiol 37(4):456–461

Song S, Huang H, Gao H, Wang J, Wu D, Liu X, Yang S, Zhai Q, Li C, Qi T (2014) Interaction between MYC2 and ETHYLENE INSENSITIVE3 modulates antagonism between jasmonate and ethylene signaling in Arabidopsis. Plant Cell 26:263–279

Soto-Suárez M, Baldrich P, Weigel D, Rubio-Somoza I, San Segundo B (2017) The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. Sci Rep 7:44898

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogen 2:e123

Wild M, Davière J-M, Cheminant S, Regnault T, Baumberger N, Heintz D, Baltz R, Genschik P, Achard P (2012) The Arabidopsis DELLA RGA-LIKE3 is a direct target of MYC2

and modulates jasmonate signaling responses. Plant Cell 24:3307–3319

Winch J, Newhook F, Jackson G, Cole J (1984) Studies of *Colletotrichum gloeosporioides* disease on yam, *Dioscorea alata*, in Solomon Islands. Plant Pathol 33:467–477

Windram O, Madhou P, McHattie S, Hill C, Hickman R, Cooke E, Jenkins DJ, Penfold CA, Baxter L, Breeze E (2012) Arabidopsis defense against Botrytis cinerea: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. Plant Cell 24:3530–3557

Xing X, Li X, Zhang M (2017) Transcriptome analysis of resistant and susceptible tobacco (*Nicotiana tabacum*) in response to root-knot nematode *Meloidogyne incognita* infection. Biochem Biophy Res Commun 482:1114–1121

Yang Y-X, Ahammed J, Wu G, Fan C, Zhou SY Y-H (2015) Crosstalk among jasmonate, salicylate and ethylene signaling pathways in plant disease and immune responses. Curr Protein Pept Sci 16:450–461

Yoosukyingsataporn S, Detpiratmongkol S (2019) Effects of ethephon on growth and yield of sweet sorghum (*Sorghum bicolor* L. Moench) at different growth stages. Iran J Plant Physiol 10(1):2987–2887

Zhang X, Zhu Z, An F, Hao D, Li P, Song J, Yi C, Guo H (2014) Jasmonate-activated MYC2 represses ETHYLENE INSENSITIVE3 activity to antagonize ethylene-promoted apical hook formation in Arabidopsis. Plant Cell 26:1105–1117

Zhao Y, Wei T, Yin KQ, Chen Z, Gu H, Qu LJ, Qin G (2012) Arabidopsis RAP2. 2 plays an important role in plant resistance to *Botrytis cinerea* and ethylene responses. New Phytol 195:450–460

Zheng H, Dong L, Han X, Jin H, Yin C, Han Y, Li B, Qin H, Zhang J, Shen Q, Zhang K, Wang D (2020) The TuMYB46L-TuACO3 module regulates ethylene biosynthesis in einkorn wheat defense to powdery mildew. New Phytol 225:2526–2541

Zhu X, Zhu J-K (2013) Double repression in jasmonate-mediated plant defense. Mol Cell 50:459–460