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Research article

Hormonal and enzymatic responses of maize seedlings to chilling stress as affected by triazoles seed treatments

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ABSTRACT

Triazole fungicides have been used for seed treatment to control soilborne diseases of maize, but seedlings coming from triazole-coated seed show serious phytotoxicity under chilling stress. To understand this phytotoxic impact, maize seed was treated with four triazoles fungicides and the corresponding seedlings were analysed on growth and gene expression. We found that maize seed coated with difenoconazole and tebuconazole exhibited either no or increased effects on germination and growth of maize at 25 °C, regardless of chemical concentrations. When maize seedlings were subjected to chilling treatment, however, their growth was significantly inhibited, and the inhibition was positively correlated with the rate of triazole application. Mesocotyl length decreased by 32.19–44.73% by difenoconazole, and 23.53–32.08% by tebuconazolet at rates of 1:50 and 1:25, respectively. However, myclobutanil did not have any effects at any temperatures. The contents of the gibberellin GA12 and abscisic acid in maize seedlings developed from difenoconazole- or tebuconazole-coated seed were significantly increased under chilling stress. The expression of two key catabolic enzyme genes, *GA2ox3* and *GA2ox4*, was significantly up-regulated immediately following chilling stress and 2 days after recovery at 25 °C in the seedlings treated with difenoconazole or tebuconazole. This imbalance in phytohormones may explain why difenoconazole- or tebuconazole-coated seed more likely results in the phytotoxicity of maize seedlings under a low temperature condition during seed emergence and seedling growth. Since myclobutanil did not have this negative effect, it can be applied for seed coating in areas where temperatures are low during early seedling growth.

1. Introduction

Maize (*Zea mays* L.) is the third most important staple crop in the world (Kresovic et al., 2014; Rajasekar et al., 2016). One challenge of maize production is that this crop is susceptible to chilling stress (Miedema, 1982). With the global climate change, sudden and unexpected chilling stress are anticipated after seed planting, which has negative impacts on seed germination and plant growth of maize (Hola et al., 2003; Janowiak et al., 2003). More importantly, soilborne pathogens causing seed rot and damping off are a big threat to seed germination and seedling growth (Wilson and Mohan, 1992; Mao et al., 1997; Smit et al., 1997; Koike, 2016; Katan, 2017; Navi et al., 2019).

To better control maize diseases, fungicide have been used for seed treatments (Sundin et al., 1999). Triazoles provide a broad spectrum of fungicidal activity and have therefore been widely used as seed coating in agriculture, horticulture, and forestry (Fletcher et al., 1986; Gilley and Fletcher, 1997). In addition, triazole fungicides can regulate plant growth (Fletcher et al., 1986). Triazoles are commonly used to coat maize seeds to ensure the production, but this often results in unexpected phytotoxicity, especially under low temperature conditions (Yang et al., 2014, 2016; Wang et al., 2009). Negative effects include low seed germination and suppressed seedling growth, especially reduction in shoot elongation (Child et al., 1993; Montfort et al., 1996). However, whether all triazoles fungicides share the same characteristics still remains unknown.

The adverse regulatory effects of triazoles appear to be that triazole fungicides cause shift of phytohormone balance in plant tissues and inhibit the biosynthesis of gibberellins (GAs), which leads to a transient rise in abscisic acid (ABA) content in plants (Fletcher et al., 2000; Izumi et al., 1985; Yang et al., 2016). GAs control many aspects of plant growth, including seed germination and seedling development (Yamauchi et al., 2004; Achard et al., 2008; Santner et al., 2009; Kuryata et al., 2017). The biosynthesis of GAs in plants can be divided into seven steps, and the process involves three *ent*-copalyl diphosphate

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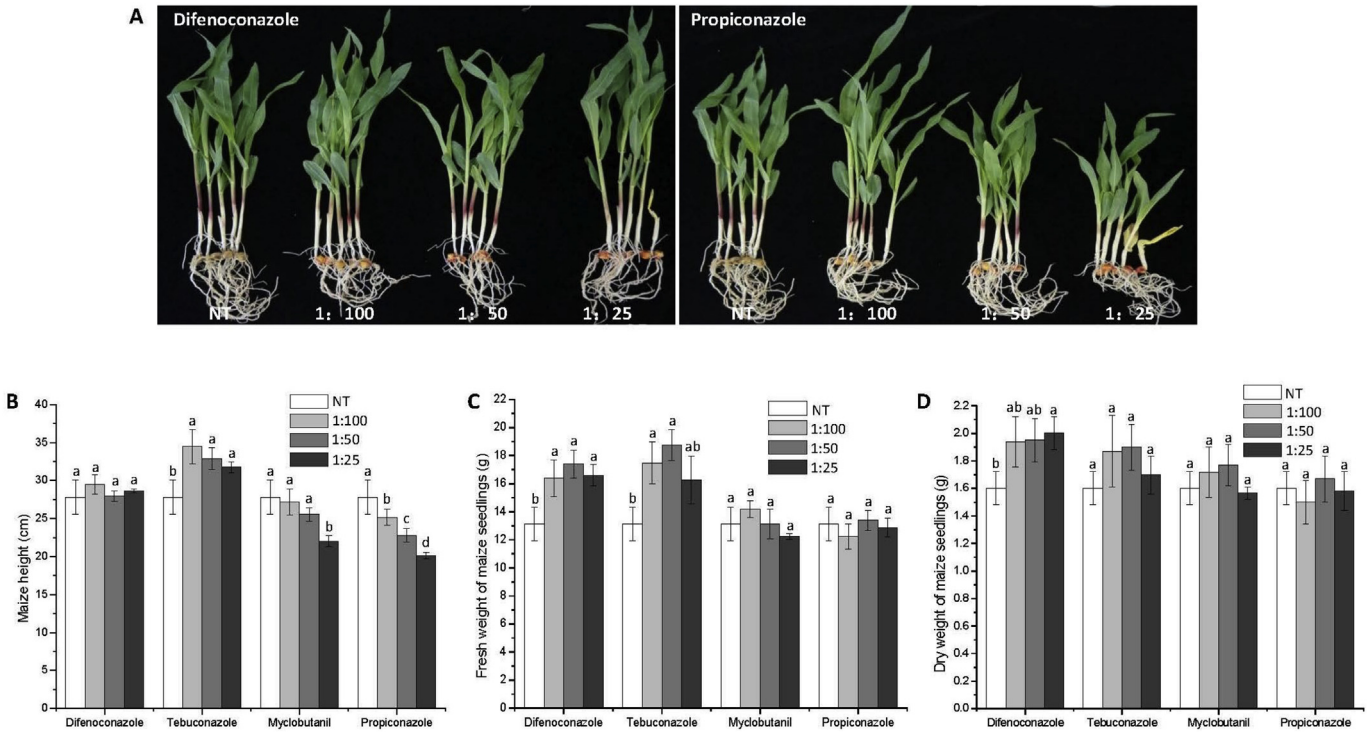

Fig. 1. The effect of triazoles in maize seed treatment on the growth of corresponding seedlings after 21 days at 25°C. Seed was coated with the formulated triazoles at 1:100, 1:50, or 1:25 (formulation:seed, w:w), and tap water was used as a non-treated control. (A) Maize seedlings developed from seed treated with either difenoconazole or propiconazole. (B–D) Effects of triazoles fungicides on maize (B) seedling height, (C) fresh weight, and (D) dry weight. For B–D, each bar represents means (± SE) of six replicates; means with different letters are significantly different ($P \leq 0.05$).

synthases (CPSs), four *ent*-kaurene synthases (KSs), two *ent*-kaurene oxidases (KOs), one *ent*-kaurenoic acid oxidase (KAO), five GA 20-oxidases (GA20oxs), and two GA 3-oxidase (GA3ox) (Song et al., 2011). KO and CPS catalyze the first two committed steps in GA biosynthesis; and overexpression of AtCPS and AtKS in Arabidopsis confers increased ent-Kaurene production (Fleet et al., 2003). The transcript level of *ZmKO2* is inhibited by paclobutrazol during maize seed germination (Song et al., 2011). GA20ox involved in the last steps of gibberellin biosynthesis, and independent mutations in GA20ox1 leads to *Arabidopsis* semidwarfs (Barboza et al., 2013). The pool of active GAs and its precursor GA20 can be depleted by the action of GA 2-oxidase (GA2ox), which converts those GAs into inactive forms (Busov et al., 2003; Hedden and Phillips, 2000; Schomburg et al., 2003). GA2ox plays an important role in plant height, and silencing GA2ox can increase tobacco growth and fiber production (Dayan et al., 2010). Overexpression of the rice *GA2ox* genes causes a dwarf phenotype and a delay in reproductive development (Sakamoto et al., 2001). The transcript levels of *ZmGA2ox3* are upregulated by paclobutrazol treatment (Song et al., 2011). *ZmGA2ox4* and *ZmGA2ox5* exhibit an upward trend with increasing dose of microencapsulated tebuconazole (Yang et al., 2016). Because GA-mediated development is regulated in part by changes in the cellular concentration of bioactive GAs, the concentrations of bioactive GAs must be carefully modulated, perhaps by integrating various endogenous and external signals (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). Till now, the molecular mechanism on phytohormone change in maize seedling triggered by triazoles is not clear.

In this study, we would examine four triazole fungicides, including difenoconazole, tebuconazole, myclobutanil, and propiconazole for maize seed treatment. These are the most widely used triazole fungicides in agriculture. Although all four are used as foliar spray, only the first three are used as seed coating (http://www.icama.org.cn/fwb/index.jhtm). The objectives of this study were to determine the effect of these four triazole fungicides on seedling growth at different temperature; and investigate the mechanism of triazoles responsible to phytotoxicity under chilling stress.

2. Materials and methods

2.1. Chemicals and maize seed

Chemicals included epoxiconazole (97.8%; Fengdeng Pesticide Co., Jiangsu, China), difenoconazole (98%; Yulong Chemical Industrial Co., Hangzhou, China), myclobutanil (96%; Gengyun Chemical Co., Jiangsu, China), and propiconazole (98%; Qizhoulvse Chemical Industry Co., Jiangsu, China). These chemicals were stored at 4 °C in the dark. Seed of maize (Zhengdan 958) was purchased from Beijing Sinong Seed Co., Ltd.

2.2. Seed coating

The seed coating formulations were prepared by Beinongdaxueyuan Seed Coating Applied Chemistry Research and Development Center (Beijing, China). The chemicals was added to an aqueous solution including 2% dispersants, 3% wetting agent and 1.5% pigmant, and the solution was continuously stirred until the chemicals was completely suspended (Ren et al., 2019; Marín et al., 2016). The concentration of difenoconazole, tebuconazole, myclobutanil, and propiconazole in the formulations was 1.0%, 1.2%, 1.5%, and 0.2% (w/w), respectively. Based on the registration information in the China Pesticide Information Network, maize seed was treated with each formulation at a normal rate of 1:100 (formulation: seed, w/w) and at two higher rates: 1:50 and 1:25. Control seed was treated with the same amount of tap water. The fungicide-coated seed was air dried.

2.3. Greenhouse experiment 1 at ambient temperature

On day 0, the treated maize seed was sown in a plastic trays (20

seeds tray^{-1}) containing a mixture of peat and vermiculite (2:1, v: v). The trays were maintained in a greenhouse at 25 ± 2 °C, 80% relative humidity, and a 12-h photoperiod. The height, dry weight, and fresh weight of maize seedlings in these trays were measured on day 21. Each treatment had six replicated trays, and the experiment was conducted twice.

2.4. Greenhouse experiment 2 with chilling treatment

Trays containing sown seed were kept in the same greenhouse as experiment 1 for 4 days before they were moved to a growth chamber at 10 °C/4 °C (light/dark). Two days later, these trays were then returned to 25 °C, and seedling height and mesocotyl length were measured on day 8. In addition, samples were collected on day 6 (after the chilling treatment) and on day 8 (after 2 days of recovery at 25 °C as described in Fig. 1). These samples were frozen in liquid nitrogen, preserved at −80 °C, and used to assess phytohormone levels or gene expression levels as described in the following sections. Each treatment was represented by six replicate trays, and the experiment was conducted twice.

2.5. Extraction of hormones in experiment 2

The extraction was performed as described previously with some modifications (Barker, 2006). A 0.5 g quantity of sample tissue was placed in a glass mortar containing 2.0 g of C18 and was disrupted in pre-cooled hexane using a pestle. The resulting fine powder was placed in 8 mL of 80% methanol at 4 °C for 5 h. The suspension was then passed through one layer of 0.22 μm Miracloth and dried in a vacuum (SpeedVac Plus; Savant) at room temperature.

2.6. Chromatographic and mass spectrometric analyses

The GA and ABA contents prepared in the experiment 2 were assayed byultra high performance liquid chromatography-mass spectrumetry (UHPLC-MS)/MS analysis, which was accomplished with a Thermo UltiMate3000 HPLC-TSQ Quantum Ultra MS/MS system (Thermo Fisher Scientific, USA) using a Thermo Syncronis C18 capillary column (100 mm × 2.1 mm, 1.7 μm). Chromatographic separation was carried out with a mobile phase consisting of acetonitrile (phase A) and 0.1% formic/water (phase B) at a flow rate of 0.5 ml min^{-1}. The gradient elution program was as follows: A (50%)-B (50%) (3.5 min), A (50%–90%)-B (50%–10%) (2.5 min), A (90%)-B (10%) (2 min), A (90%–50%)-B (10%–50%) (0.1 min), and A (50%)-B (50%) (1.9 min). Injection volume was 5 μl, and the column temperature was 30 °C. Mass spectrometry analysis was operated using electrospray ionization (ESI) in negative ion mode with multiple reaction monitoring (MRM). The optimized ESI condition was: flow rate 0.3 m/min, collision gas pressure 1.5 mTorr, capillary temperature 300 °C, vaporizer temperature 300 °C, sheath gas pressure 35 Arb, Aux gas pressure 10 Arb, and spray voltage 2500 v. High-purity nitrogen was used as the ESI nebulizing gas. For GA_{12}, the precursor ion was 331.2 m/z, the product ion was 313.4 m/z, and the collision energy was 30 eV; For ABA, the precursor ion was 263.2 m/z, the product ion was 153.3 m/z, and the collision energy was 14 eV.

2.7. RNA extraction and reverse transcription

Total RNA was extracted using the SV Total RNA Isolation kit (Promega, Beijing, China). The amount and quality of the total RNA were checked by electrophoresis on a 1% agarose gel. The concentration of RNA was measured with a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). A 2-μg quantity of RNA was used to synthesize cDNA in a 20 μl reaction using the PrimeScrip RT reagent Kit with gDNA Eraser (Takara, Beijing, China) following the manufacturer's protocol. Aliquots of 2 μl of the obtained cDNA were subjected to quantitative real-time PCR analysis (qRT-PCR) analysis.

2.8. SYBR green qRT-PCR assay

Primers specific to GA and ABA metabolic genes were used for quantitative real-time PCR analysis, and 18S rRNA gene was amplified as an endogenous control (Table S1). RT-PCR was performed using an ABI 7500 sequence detection system (Applied Biosystems, USA). Amplifications were conducted using the SYBR Premix Dimer Eraser kit (Takara) in a 20-μl volume following the manufacturer's protocol. Thermal cycling settings included 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 34 s; and a final dissociation stage at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s, and 60 °C for 15 s. Relative quantities of products were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three independent assays were performed. All tests were carried out with at least three replicates.

2.9. Statistical analysis

Data were analysed using DPS software ver. 7.05 (Zhejiang University, Hangzhou, China). Means were separated using a one-way ANOVA with Duncan's multiple range test. The values are mean ± SD of the repeated samples in each experiment. P values < 0.05 were considered significant.

3. Results

3.1. Effect of triazoles seed treatment on maize seedlings at ambient temperature

With triazoles-treated maize seed grown for 21 days at 25 °C, the height of emerged seedlings was affected differently depending on the fungicide. Difenoconazole did not affect seedling height; but tebuconazole increased, and propiconazole and myclobutanil decreased seedling height, with the effect being negatively correlated with rate of application for tebuconazole and positively correlated for both propiconazole and myclobutanil (Fig. 1A and B). Seedling fresh weight was generally increased by difenoconazole and tebuconazole but was unaffected by myclobutanil or propiconazole (Fig. 1C). Seedling dry weight tended to be increased 21.06–25.01% by difenoconazole but was not significantly affected by the other three triazoles seed treatments (Fig. 1D, $P < 0.05$).

3.2. Effect of triazole seed treatments on maize seedlings subjected to chilling

In the case of chilling, seedling height was decreased by all four fungicides and negatively correlated with rate of application (Fig. 2A and B). Seedling height was significantly reduced by all triazole treatments but was most reduced by propiconazole with the highest inhibition rate reached 61.93% and was least reduced by myclobutanil with the inhibition rate between 11.43 and 23.75% as the coating rate increasing (Fig. 2B, $P < 0.05$). Mesocotyl length significantly decreased 32.19–44.73% of difenoconazole, 23.53–32.08% of tebuconazole, and 12.76–23.75% of propiconazol at the rates 1:50 and 1:25, respectively (Fig. 2C, $P < 0.05$). Mesocotyl length also decreased as the rate of myclobutanil increased but the differences between means were not statistically significant (Fig. 2C, $P < 0.05$). Overall, the growth of maize seedling subjected to chilling stress was less suppressed by myclobutanil than by difenoconazole, tebuconazole, or propiconazole. These results indicated that chilling stress could aggravate phytotoxicity when using the triazole fungicides, especially difenoconazole and tebuconazole, as seed treatments.

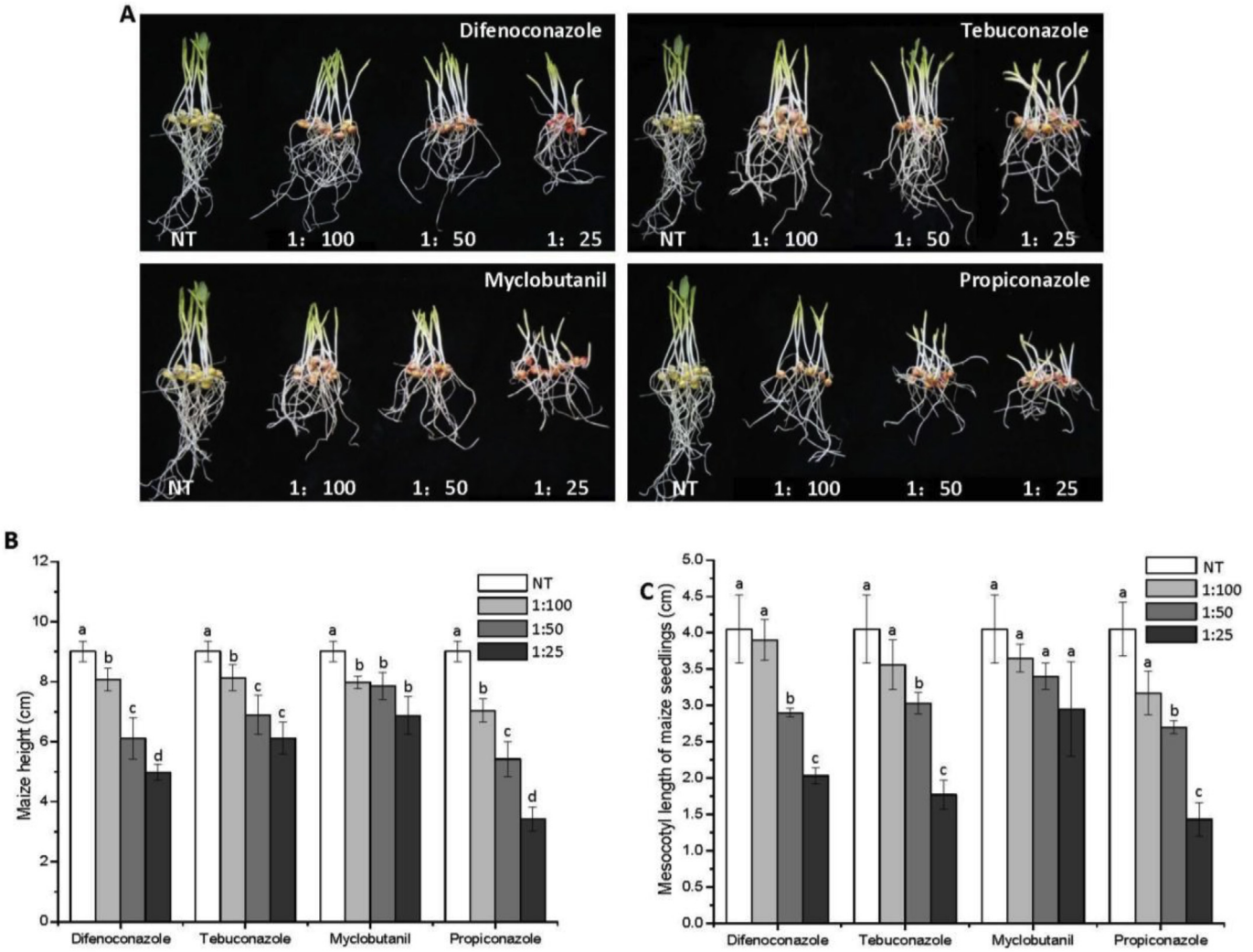

Fig. 2. The effect of triazoles in maize seed treatment on the growth of seedlings under chilling stress. Seed was coated with the formulated triazoles at 1:100, 1:50, or 1:25 (formulation:seed, w:w), and tap water was used as a non-treated control. Seedlings were grown at 25 °C for 4 days, at 10 °C/4 °C (light/dark) for 2 days, and then at 25 °C for 2 additional days. (A) Seedlings on day 8. (B–D) Effect of triazole fungicides on (B) seedling height and (C) mesocotyl length. For B and C, each bar represents means (± SE) of six replicates; means with different letters are significantly different ($P \leq 0.05$).

3.3. The content of GA12 and ABA in maize seedlings

To understand the mechanism of interaction between different triazoles and chilling stress, GA contents and the expression of GA related genes were assessed. According to linear regression, the contents of GA12 and ABA detected in standards were strongly related ($R^2 > 0.98$) to the known contents in those standards; this indicated that the UHPLC-MS/MS analysis was accurate for quantification of GA12 and ABA. Triazole seed treatments affected the GA12 and ABA contents in maize seedlings subjected to 2 days of chilling stress (CS2d) (Fig. 3A and B). GA12 contents were 7.28, 4.22, and 3.68 fold in difenoconazole, tebuconazole, and myclobutanil treatments than in controls at the coating rate of 1:100, respectively (Fig. 3A). The increase in GA12 content was proportional to the concentrations of these triazoles in the formulations, and the GA12 contents were 12.00, 9.78, and 7.60 fold in difenoconazole, tebuconazole, and myclobutanil treatments than in controls at the coating rate of 1:25, respectively (Fig. 3A). When these seedlings were returned to 25 °C for 2 days (RT2d), GA12 contents returned to the control level. After the 2 days of chilling, the GA12 contents were lower in the propiconazole treatment than in the control, and the contents were lower yet when the seedlings in the propiconazole treatment were returned to 25 °C for 2 days (Fig. 3A). The ABA content increased 8.67, 2.85, and 11.55 fold in difenoconazole, tebuconazole, myclobutanil, or propiconazole treatment at the coating rate of 1:100 following 2 days of chilling, respectively; and had no direct relationship with coating rates (Fig. 4B). The ABA contents tended to drop to the control level when the seedlings were returned to 25 °C for 2 days (Fig. 3B).

3.4. The expression levels of five key GA synthase genes in maize seedlings

Following subjected to chilling for 2 days (CS2d), the expression levels of five key GA synthase genes (*CPS2, KO2, GA20ox1, GA20ox4,* and *GA3ox1*) tended to be slightly lower in the difenoconazole and tebuconazole treatments than in the control but these differences were not statistically significant (Fig. 4A, B, $P < 0.05$). After 2 days of recovery at 25 °C (RT2d), the expression level of *GA20ox1* was increased 2.22–9.76 fold and 2.87–5.85 fold relative to the control in the difenoconazole and tebuconazole treatments with coating rates increasing, respectively (Fig. 4A and B). Following chilling, the expression levels of *CPS2*, *GA20ox1*, *GA20ox4*, and *GA3ox1* were decreased in the myclobutanil treatment; the expression level was particularly low for *GA3ox1* with 0.01–0.10 fold compared to the control. After 2 days of recovery at 25 °C, *CPS2*, *GA20ox4*, and *GA3ox1* were still down-regulated while *GA20ox1* was 2.76–6.33 fold up-regulated in the myclobutanil treatment (Fig. 4C). The response to chilling in the propiconazole treatment

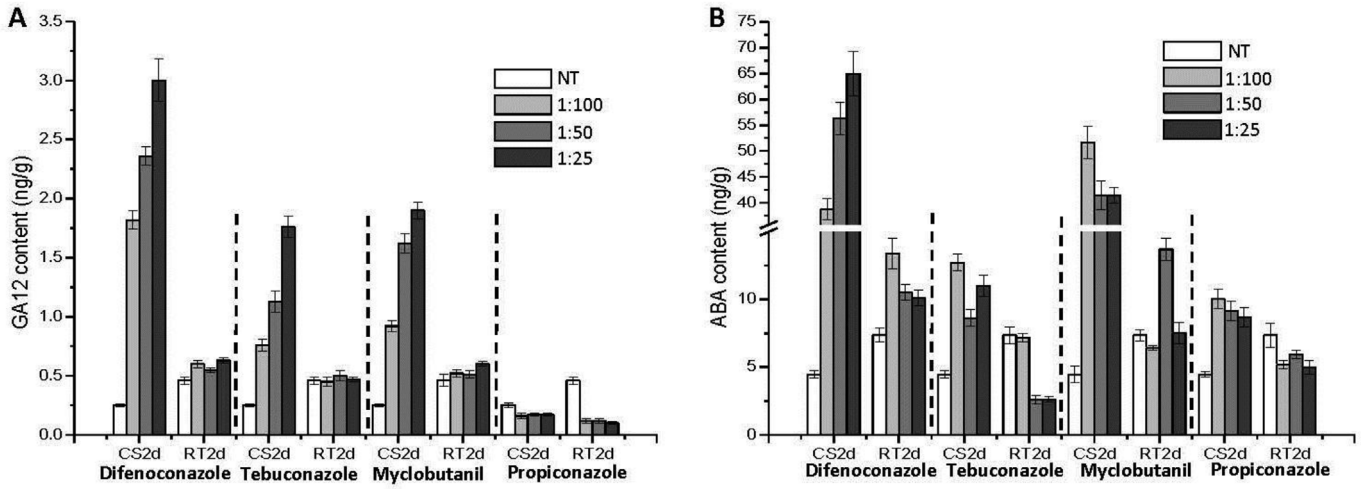

Fig. 3. Effect of triazoles in seed treatment on the content of GA12 and ABA in maize seedlings after 2 days of chilling and after 2 days of recovery at 25°C. Seed was coated with the formulated triazoles at 1:100, 1:50, or 1:25 (formulation:seed, w:w), and tap water was used as a non-treated control. Seedlings were grown at 25 °C for 4 days, at 10 °C/4 °C (light/dark) for 2 days, and then at 25 °C for 2 additional days. (A) GA12 contents and (B) ABA contents were determined immediately after the 2 days of chilling stress (CS2d) or after 2 additional days at 25 °C (RT2d). Each bar represents means (± SE) of six replicates.

differed among the five synthase genes. The expression of *CPS2*, *KO2*, and *GA20ox4* was unaffected by chilling, but the expression of *GA20ox1* and *GA3ox1* was highly increased. After 2 days of recovery at 25 °C, the expression of *GA20ox1* and *GA3ox1* in the propiconazole treatment was significantly decreased, and the expression of *GA3ox1* was similar to that of the control (Fig. 4D).

3.5. Expression of catabolic enzyme genes in maize seedlings

Two key catabolic enzyme genes in maize seedlings were analysed on their expressions. Following chilling treatment (CS2d), the expression of *GA2ox3* in seedlings treated with difenoconazole, tebuconazole, or propiconazole increased 3.35 to 4.95, 1.83 to 3.06, and 7.28–8.90 folds as chemical concentration increased (Fig. 5A). Following 2 days of recovery at 25 °C (RT2d), *GA2ox3* expression remained to be up-regulated, and it also showed the highest level with 6.45–9.01 fold change under propiconazole treatment (Fig. 5A). In the case of myclobutanil, *GA2ox3* expression did not significantly change following chilling stress but significantly increased following the 2 days of recovery at 25 °C (Fig. 5A). No matter following chilling or after the 2 days of recovery at 25 °C, the expression level of *GA2ox4* was increased in all four triazole treatments with the coating concentration increasing, but the increase was smaller with myclobutanil treatment than with difenoconazole, tebuconazole, or propiconazole. It up-regulated 1.93–4.02 -fold at CS2d, and 2.64–3.30 fold at RT2d when coating with myclobutanil compared to the control (Fig. 5B).

4. Discussion

Triazole fungicides have been used for seed treatment to control soilborne diseases of maize, but seedlings coming from triazole-coated seed show serious phytotoxicity under chilling stress (Gilley and Fletcher, 1997; Zhang et al., 2007). In the present study, the four test triazoles fungicides showed differences on maize seedling growth. The growth of maize seedlings developed from triazoles-coated seed were affected at 25 °C but reduced when subjected to chilling stress, particularly in the seedlings with difenoconazole and tebuconazole treatments. The level of reduction was positively correlated with rate of application of these triazoles. The results were in agreement with a previous study indicating that low temperature significantly suppress maize growth when the seeds are coated with tebuconazole and difenoconazole compounds (Wang et al., 2009). However, seedling growth were affected with or without chilling stress when maize seeds were coated with propiconazole; this may be the reason that propiconazole is not commonly used as seed treatment. Interestingly, myclobutanil treatment showed difference with these three above triazoles, and it did not affect seedling growth of maize under chilling stress. Therefore, we hypothesis that the phytotoxicity expressed in maize seedlings in northern China could result from an interaction between triazole treatment and chilling stress.

GAs are phytohormones that highly affect plant development, and geranylgeranyl diphosphate (GGPP) is the common precursor for the biosynthesis of GA and ABA (Seo and Koshiba, 2002). We have demonstrated that the contents of GA12 and ABA in maize seedlings were significantly increased with either difenoconazole, tebuconazole or chilling treatment. GA12 is the substrate for the biosynthesis of GA9 and GA20. GA12 accumulation indicates that the biosynthesis of downstream active GAs is inhibited (Schomburg et al., 2003). The accumulation of GA12 lead to a transient increase in ABA content when the seedlings grew from difenoconazole or tebuconazole-coated seeds under chilling stress. This result was consistent with the previous report that azoles can cause ABA imbalance in relation to GA metabolism (Kitahata et al., 2005), and shift the balance of GAs in plants (Grossmann, 1990; Fletcher et al., 2000). Elevated ABA levels have been associated with an increased tolerance to cold stress (Lee et al., 1993; Xin and Li, 1993). However, the metabolic pathways of phytohormones are very complicated, so possibly more hormones are involved in this process.

We also have detected expression level of major genes involved in GA biosynthesis. Among the five key GA synthase genes, only some of them responded to expression chilling stress. Interestingly, the expression of *GA2ox3* and *GA2ox4*, which encode key catabolic enzymes, was significantly up-regulated when seedlings from either difenoconazole- or tebuconazole-coated seed under chilling stress. GA2ox converts active GAs and precursors into inactive forms, and it plays an important role in plant growth (Busov et al., 2003). A previous study indicates that the triazole paclobutrazol affects not only GA synthesis but also the activity of catabolic enzymes, which led to an increased rate of GA decomposition and to dwarf plants (Rieu et al., 2008). Dwarfed plants are also regulated by *KO*, *KAO*, *GA20ox*, and *GA3ox* as well as by the overexpression of *GA2ox* (Davidson et al., 2003; Schomburg et al., 2003). Overexpression of *GA2ox* genes in rice causes a dwarf phenotype and a delayed reproductive development (Sakamoto et al., 2001). Yang et al. (2016) report that the expression of *GA2ox* shows upward trends

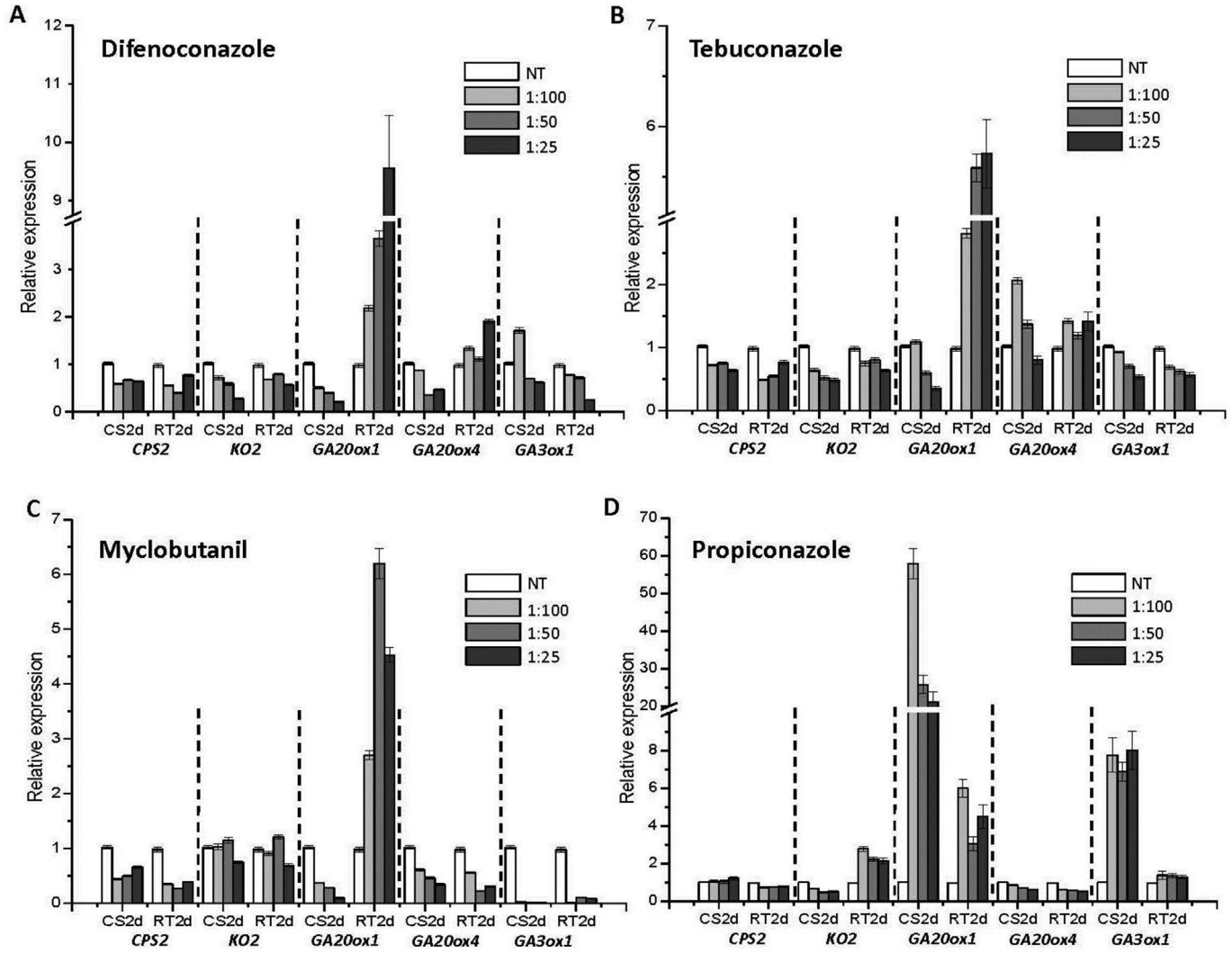

Fig. 4. Effects of triazoles in seed treatment on the expression of five key GA synthase genes (*CPS2*, *KO2*, *GA20ox1*, *GA20ox4*, and *GA3ox1*) in maize seedlings after 2 days of chilling and after 2 days of recovery at 25°C. Seed was coated with the formulated triazoles at 1:100, 1:50, or 1:25 (formulation:seed, w:w), and tap water was used as a non-treated control. Seedlings were grown at 25 °C for 4 days, at 10 °C/4 °C (light/dark) for 2 days, and then at 25 °C for 2 additional days. Expression following treatment with (A) difenoconazole, (B) tebuconazole, (C) myclobutanil, or (D) propiconazole. Expression was determined after the 2 days of chilling stress (CS2d) or after 2 additional days at 25 °C (RT2d). Each bar represents means (± SE) of six replicates.

with the increasing rate of non-microencapsulated tebuconazole seed coating on maize under chilling stress. In support of this inference, the seedlings with the myclobutanil treatment showed a relatively stable expression level of catabolic enzyme genes regardless of temperature and were tolerant of chilling stress as indicated by mesocotyl length (Fig. 2). Overall, these results suggested that the abnormal growth observed in maize seedlings that grew from difenoconazole- or tebuconazole-treated seed was mainly caused by the up-regulation of *GA2ox* genes encoding catabolic enzymes under chilling stress.

Based on the results, we established a schematic model explaining how triazoles in treating maize seed affects GA regulation and therefore the response of the seedlings under chilling stress (Fig. 6). The up-regulation of the catabolic enzyme genes *GA2ox3* and *GA2ox4* decrease the production of bioactive GAs, which leads to the accumulation of GA12 (a substrate for bioactive GAs) and to a transient increase in ABA content. A low level of GA and a high level of ABA do not support seed germination and plant growth (Kermode, 2005; Kucera et al., 2005; Razem et al., 2006). The effect of triazoles in seed treatment on maize growth is mainly reflected as the reduced mesocotyl length in this study. That is a key factor used to evaluate the safety of chemicals used as seed coating. Because treatment of maize seed with myclobutanil resulted in a relatively stable expression level of catabolic enzyme genes and a good tolerance to chilling stress, myclobutanil might be particularly better as a fungicidal seed coating in northern China and in other maize production areas where temperatures are low during germination and early seedling growth.

5. Conclusions

We have found that maize seed coated with either difenoconazole or tebuconazole under chilling stress caused significant decrease of seedling height and mesocotyl length, while myclobutanil did not show such a negative effect. The phytotoxicity associated with difenoconazole and tebuconazole was due to an imbalance of gibberellin GA12 and abscisic acid and the up-regulation of two catabolic enzyme genes under chilling stress.

Author contribution

LXL and ZC contributed to the conception of the study. WQS and ZF performed plants cultivation and triazole treatment. WQS, ZBR and LPF accomplished the MSPD-HPLC-MS/MS determination. WQS, ZC and

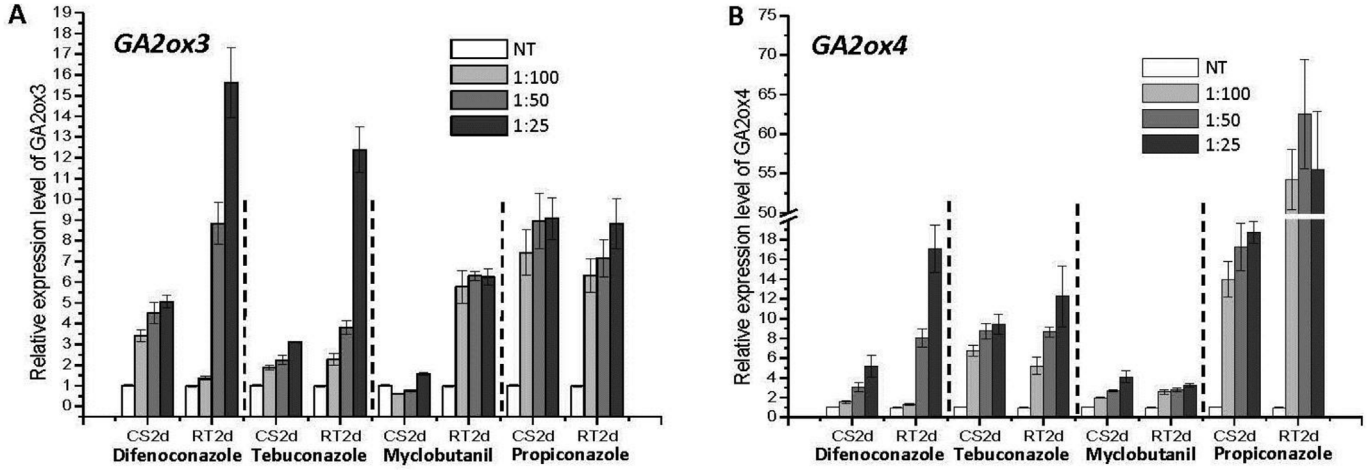

Fig. 5. Effects of triazoles in seed treatments on the expression of the catabolic enzyme genes *GA2ox3* and *GA2ox4* in maize seedlings after 2 days of chilling and after 2 days of recovery at 25°C. Seed was coated with the formulated triazoles at 1:100, 1:50, or 1:25 (formulation:seed, w:w), and tap water was used as non-treated controls. Seedlings were grown at 25 °C for 4 days, at 10 °C/4 °C (light/dark) for 2 days, and then at 25 °C for 2 additional days. Expression of (A) *GA2ox3* and (B) *GA2ox4*. Expression was determined after the 2 days of chilling stress (CS2d) or after 2 additional days at 25 °C (RT2d). Each bar represents means (± SE) of six replicates.

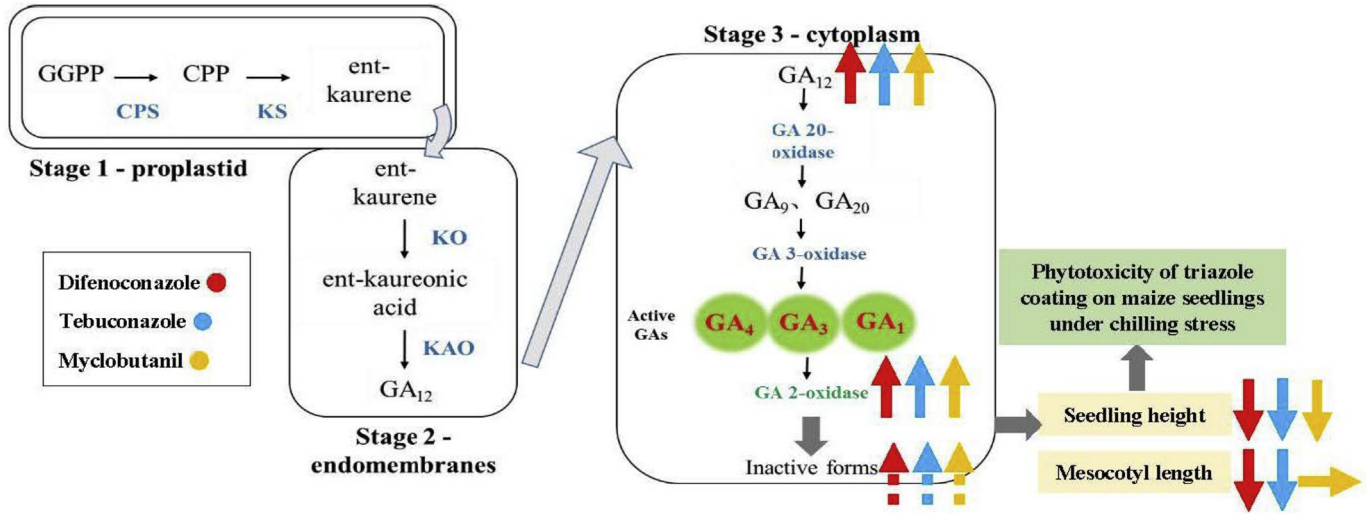

Fig. 6. A schematic model of gibberellin regulation in maize seedlings growing from triazole-treated seed under chilling stress. Colored arrows indicate three triazoles fungicides, and arrow pointing up and down indicate up- and down-regulation, respectively. A solid lined arrow means the referred type of gibberellin (GA) was detected in this study, and dotted lined arrows mean the content was extrapolation based on other published data. GGPP: geranylgeranyl diphosphate; CCPP: opalyl pyrophosphate; CPS: *ent*-copalyl diphosphate synthase; KS: *ent*-kaurene synthase; KO: *ent*-kaurene oxidase; KAO: *ent*-kaurenoic acid oxidase.

ZSL are responsible for realization the molecular part of the experiment. ZC, WQS and LXL analysed the data and ZC wrote first draft manuscript. All authors discussed the results, read and approved the manuscript.

Declaration of competing interest

The authors have declared that no competing interest exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.01.017.

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