

Article

High-Vigor Maize Seeds Resist *Fusarium graminearum* Infection through Stronger Ca²⁺ Signaling

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Abstract: Seeds with high vigor have strong resistance to various adverse environmental conditions. However, little is known about how seed vigor affects the resistance of seeds to biotic stress. In this study, newly harvested seeds that had high vigor and seeds with low vigor, achieved via an artificially accelerated aging treatment, were used in the germination test after inoculation with *Fusarium graminearum* for 24 h. The results showed that high-vigor seed-related germination and seedling growth were not significantly affected by *F. graminearum* infection, while those related to low-vigor seeds were significantly inhibited. Analysis of transgenic maize seeds expressing the luminescent Ca²⁺ probe encoded by *aequorin* indicated that the concentration of free Ca²⁺ in the cytoplasm and nucleus of the embryo cells of high-vigor seeds was significantly higher than that of the low-vigor seeds. Through an experiment with Ca²⁺ inhibitor treatment and exogenous Ca²⁺ application, we further confirmed that Ca²⁺ played an important role in seed germination and seedling growth. Interestingly, in the presence of *F. graminearum*, the Ca²⁺ required for seed germination and seedling growth mainly came from the vacuolar calcium pool, while in the absence of *F. graminearum*, the required Ca²⁺ mainly came from the apoplastic calcium store. This study helps understand how high-vigor seeds resist disease and provides theoretical support for the wide application of high-vigor seeds in agricultural production.

Keywords: seed vigor; *Fusarium graminearum*; disease resistance; Ca²⁺; aequorin



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1. Introduction

Ca²⁺ is a key signaling molecule in eukaryotes. It regulates the response of plants to various environmental signals and controls developmental processes in plants [1,2]. Ca²⁺ is rapidly released from the intracellular and apoplastic calcium pools to form pulsed Ca²⁺ signals in cytoplasm and some organelles including nuclei, mitochondria and chloroplasts, in response to various biotic and abiotic stimuli [3]. After Ca²⁺ signals are perceived by the sensor proteins of Ca²⁺, the downstream genes are regulated to respond to the stimuli.

Higher levels of cytoplasmic Ca²⁺ promote seed germination [4]. Ca²⁺ is involved in the upregulation of gibberellin to promote seed germination of *Nigella sativa* [5] and participates in regulating the early growth of the plumular axis during the germination of mung bean seeds [6]. Treatment with exogenous calcium significantly increased the levels of auxin, gibberellin, and cytokinin during seed germination, thereby promoting seed germination [7,8]. In addition, appropriate concentrations of exogenous Ca²⁺ improve resistance to *Botryosphaeria dothidea* in pear [9] and alleviates the damage resulting from abiotic stress caused by high temperature, drought, freezing, salinity, and heavy metal [10–14].

Seed vigor is an important indicator for evaluating seed quality. High-vigor seeds generate evenly emerging and fast-growing seedlings that are robust and resistant to biotic

and abiotic stress [15]. The level of seed vigor is gradually formed during seed development, reaches a peak at physiological maturity, and then decreases as the seed enters an irreversible aging process [15]. Oxidative damage caused by reactive oxygen species (ROS) is considered the main cause of seed aging [16]. During the long-term storage of seeds, as one of the byproducts of aerobic respiration, gradually accumulated ROS results in DNA/RNA damage, protein carbonylation, and lipid peroxidation [16]. ROS degrade long-chain fatty acids by attacking polyunsaturated fatty acids in membrane phospholipids, leading to the loss of membrane integrity [16] and the compromised function of transmembrane proteins, such as transporters and signaling receptors [17,18]. During seed imbibition, phospholipids in cell membranes were rebuilt, and the function of transmembrane proteins was restored [19]. A higher degree of seed aging causes a higher degree of cell membrane damage, and as a result, it is more difficult to repair the membranes and restore the function of the transmembrane proteins [19].

Living in complex environments, plants have evolved a sophisticated innate immune system that senses and responds to pathogenic infection. This system includes two aspects: pathogen-associated molecular patterns (PAMP)/microbe-associated molecular patterns (MAMP)-induced immune response (PTI) and effector-induced immune response (ETI) [20]. Initially, plant pattern-recognition receptors (PRRs) on cell membranes recognize microbial conserved PAMPs/MAMPs or plant-originated damage-associated molecular patterns (DAMPs) and activate PTI [20]. Plants trigger a series of cellular and physiological changes, including the rapid increase of cytoplasmic Ca^{2+} content, high levels of extracellular ROS, and the increase of mitogen-activated protein kinase (MAPK) activity, and then start the synthesis of plant hormones, such as salicylic acid, jasmonic acid, and ethylene [21,22]. About 30 s to 2 min after perceiving MAMPs/DAMPs, extracellular Ca^{2+} begins to flow into the cytoplasm, and about 4 to 6 min later, cytoplasmic Ca^{2+} content reaches its peak [23].

Fusarium graminearum is a pathogenic fungus that is highly harmful to agricultural production and causes head scab in wheat and barley, and ear and stalk rot in maize, resulting in serious losses due to yield and quality reduction as well as to the contamination of trichothecene mycotoxins, which threaten the health of humans and animals [24]. The main aim of this present study was to determine how vigor level of seeds affected their resistance to *F. graminearum*. In this study, we found that high-vigor seeds were more resistant to *F. graminearum* infection. Using the luminescent Ca^{2+} probe *aequorin* transgenic maize seeds as materials, the study found that the concentration of free Ca^{2+} in cytoplasm and nuclei of the embryo cells of the high-vigor seeds was significantly higher than that of the low-vigor seeds. Furthermore, in the presence of *F. graminearum*, the Ca^{2+} required for seed germination and seedling growth mainly came from the vacuole, while when there was no *F. graminearum* infection, the required Ca^{2+} was mainly from the apoplasmic calcium store.

2. Materials and Methods

2.1. Plant Materials

Maize hybrid “Xianyu 335” and transgenic maize expressing the luminescent Ca^{2+} probe *aequorin* were used in this study. The vector pBIN-YA (B) was used to express the 35S promoter-driven *YFP-aequorin* and was obtained from The Arabidopsis Information Resource (TAIR, www.arabidopsis.org, accessed on 2 November 2018). Transgenic maize seeds expressing the luminescent probe were used to detect free Ca^{2+} in the cytoplasm and nuclei [25]. The transformation of maize was conducted by the Center for Life Science and Technology of China National Seed Group (Wuhan, China). The seeds used in this study were generated through strict manual pollination.

2.2. Measurements and Methods

2.2.1. Acquisition of Homozygous Transgenic Seeds

Seeds of the T_1 generation were sown, and DNA was extracted from leaves at the seedling stage. Primers pBIN-YA-F (5'-ATGACGCACAATCCCCTATCC-3') and

pBIN-YA-R (5'-AGTTCACCTTGATGCCGTTTC-3') were designed for PCR amplification according to the 35S promoter and YFP sequence of pBIN-YA (B) vector [25]. The plants without amplification bands were removed and the remaining plants were self-pollinated. Twenty seeds were randomly selected from each harvested T₂ generation ear and germinated on a paper bed at 25 °C. Four days later, the germinated seeds were irradiated with LUYOR-3260 fluorescent protein excitation light source (Shanghai Luyang Instrument, Shanghai, China). If all the seeds emitted fluorescence, this indicated that all the seeds on the ear were homozygous. Homozygous seeds were used for further related assays.

2.2.2. Subcellular Localization Assays

The protoplast isolation and transient expression were performed as described by Tu et al. [26]. Mesophyll protoplasts were isolated using the 11 d old seedling leaves under dark conditions. The pBIN-YA (B) vector was transfected into protoplasts using the PEG-calcium solution (0.4 g/mL PEG4000, 0.8 M mannitol, and 0.1 M CaCl₂). After being washed and resuspended with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES), mesophyll protoplasts were incubated under dark conditions for 12–18 h. The YFP fluorescence was examined and imaged with a Leica TCS SP8 confocal microscope (Leica Microsystems, Mannheim, Germany).

2.2.3. Determination of Free Ca²⁺ Concentration in Embryo Cells

Undamaged transgenic maize seeds were randomly selected and placed in an embryo-upward position on five layers of germination paper saturated with deionized water in a petri dish. Subsequently, the seeds were covered with three sheets of water-saturated germination paper. The seeds were allowed to imbibe water for different durations at 25 °C. Excessive water on the seeds was then removed, and the embryos were carefully isolated with a scalpel blade and forceps. Fifteen embryos were prepared from each imbibition duration treatment, and the embryos were used to determine the free Ca²⁺ concentration.

The seed embryos were individually placed in the wells of a black opaque microplate, and each well included one embryo. After the addition of 200 µL coelenterazine solution (10 mmol/L) to each well, the microplate was covered with a lid to completely block out light and incubated on an 80 rpm shaker at 25 °C for 8 h.

After incubation, the coelenterazine solution in each well was removed and replaced with 200 µL *F. graminearum* suspension. Then, the fluorescence intensity (L_{stimuli}) was immediately measured with a microplate reader. After the measurement, the fungal suspension in each well was removed and replaced with 200 µL discharge buffer (2 M CaCl₂ in 20% ethanol). The fluorescence intensity (L_{rest}) was measured immediately.

$$L_{\max} = L_{\text{stimuli}} + L_{\text{rest}}$$

$$k = L_{\text{stimuli}} / L_{\max}$$

$$\text{pCa} = 0.332588 (-\log k) + 5.5593$$

The pCa value was multiplied by 10⁶ to obtain the concentration of free Ca²⁺ in µM [27].

2.2.4. Aging Treatment in Maize Seeds

For the seed aging treatment, mesh bags and the inside of a seed aging chamber were sterilized using 75% ethanol. Randomly selected undamaged maize seeds were placed in a mesh bag and then placed on a plate in a compartment of the seed aging chamber. The seeds were evenly spread out without overlapping. The seeds were treated continuously for 4 d under the conditions of 95% relative humidity and 45 °C.

2.2.5. Treatment of Maize Seeds with Ca²⁺ Channel Inhibitors

Maize seeds were placed in ethylene glycol-bis(beta-aminoethyl ether)-N-tetraacetic acid (EGTA, 5 mmol/L) solution to chelate extracellular free Ca²⁺, LaCl₃ solution (3 mmol/L)

to block the cell membrane Ca^{2+} channel, RR solution (Ruthenium Red, 0.5 mmol/L) to inhibit the release of vacuolar Ca^{2+} , and distilled water (control) for 12 h at 25 °C.

2.2.6. Treatments of Maize Seeds with Exogenous Ca^{2+}

Maize seeds were soaked in CaCl_2 solution (10 and 20 mmol/L), CaSO_4 solution (10 and 20 mmol/L), and distilled water (control) for 12 h at 25 °C.

2.2.7. Standard Germination Test of Maize Seeds

One kilogram of quartz sand (about 0.35 mm in diameter) was mixed well with 170 mL of deionized water. In a germination box (17 cm × 12 cm × 7 cm) with 800 g sand, scraped flat, 50 seeds were then placed in the sand with the embryo in an upward position. Subsequently, more sand was added to cover the seeds until a total of 1400 g sand was in the germination box. Each treatment included four replicates. The performance during seed germination and the calculation of related indices were carried out according to the previous description [28].

2.2.8. Inoculation of Maize Seeds with *F. graminearum*

In an ultra-clean laminar flow hood, a *F. graminearum* spore suspension ($1.5\text{--}2 \times 10^5$ spores/mL) was prepared. Four layers of germination paper saturated with the spore suspension were placed in a sterile petri dish. Maize seeds were evenly placed without overlapping on the germination paper with the embryos in an upward position, and then covered with 3 sheets of germination paper saturated with the spore suspension. The petri dish was placed in an incubator at 25 °C for 24 h.

2.2.9. Determination of Enzyme Activity

Maize seeds with high and low vigor were treated with a Ca^{2+} inhibitors and exogenous Ca^{2+} , respectively, for 12 h and then co-cultured with *F. graminearum* for 24 h. Subsequently, the seeds were sampled at 0, 24, and 48 h after the end of the inoculation period. Enzyme assay kits produced by Cominbio (Suzhou, China) were used to determine the activity of chitinase, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Each treatment included three biological replicates, and each sample had three technical replicates. The operation followed the instructions provided by the kit producer.

2.2.10. Statistical Analysis

The results are expressed as means ± SD. Data were tested for significance using one-way ANOVA with the IBM SPSS Statistics 19.0 software (SPSS, Chicago, IL, USA).

3. Results

3.1. High-Vigor Seeds Had Higher Resistance to *F. graminearum*

To clarify the resistance of seeds differing in vigor to *F. graminearum*, we used the newly harvested high-vigor seeds of maize hybrid “Xianyu 335” and low-vigor seeds obtained through an artificially accelerated aging treatment to perform germination tests after they were co-cultured with *F. graminearum*. The results showed that *F. graminearum* infection inhibited seed germination and seedling growth. The germination energy, germination index, germination percentage, and vigor index of the low-vigor seeds inoculated with *F. graminearum* were significantly decreased (Figure 1). High-vigor seeds related to germination and seedling growth were inhibited to a certain extent, but the inhibition effect was not significant (Figure 1), indicating that high-vigor seeds had higher resistance to *F. graminearum* at the germination stage and during seedling growth.

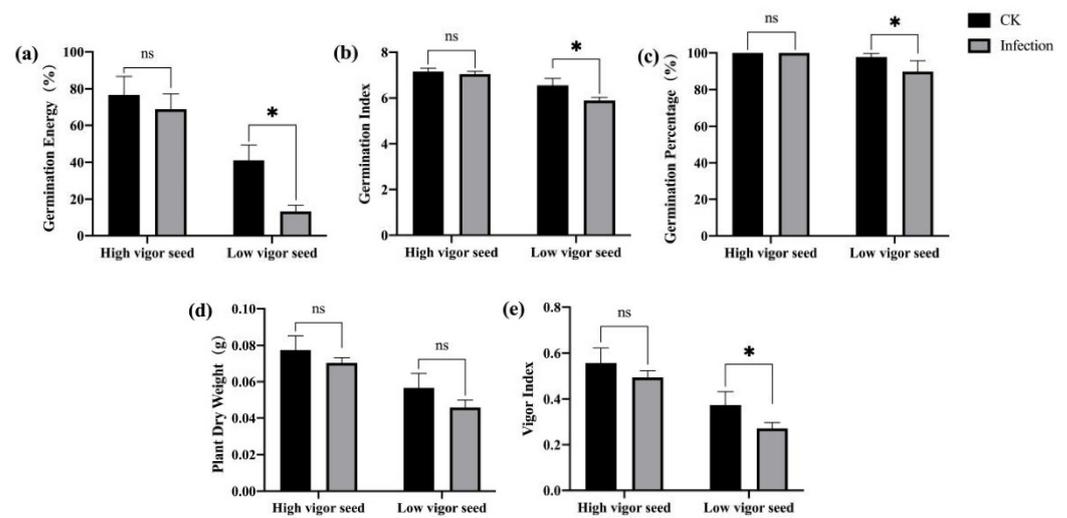


Figure 1. Effects of *F. graminearum* infection on seed germination and seedling growth of maize with different vigor levels. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. * indicates $p < 0.05$ (Student's *t*-test), and ns represents not significant.

3.2. *F. graminearum*-Infected High-Vigor Maize Seeds Had Stronger Ca^{2+} Signals during Imbibition

In a previous study, the flow rate of Ca^{2+} flowing from the extracellular matrix to the embryo cells showed a significant positive correlation with maize seed vigor during imbibition [29]. To determine how the vigor of maize seeds affected their resistance to *F. graminearum*, we transformed maize with the expression vector pBIN-YA (B) and obtained transgenic plants expressing the *YFP-aequorin* gene [25]. Homozygous transgenic seeds were obtained for subsequent analysis (Figure 2). The aequorin protein was used as a Ca^{2+} probe to detect the Ca^{2+} concentration in cytoplasmic and nuclear (Figure 3) prior to and after *F. graminearum* infection during seed imbibition.

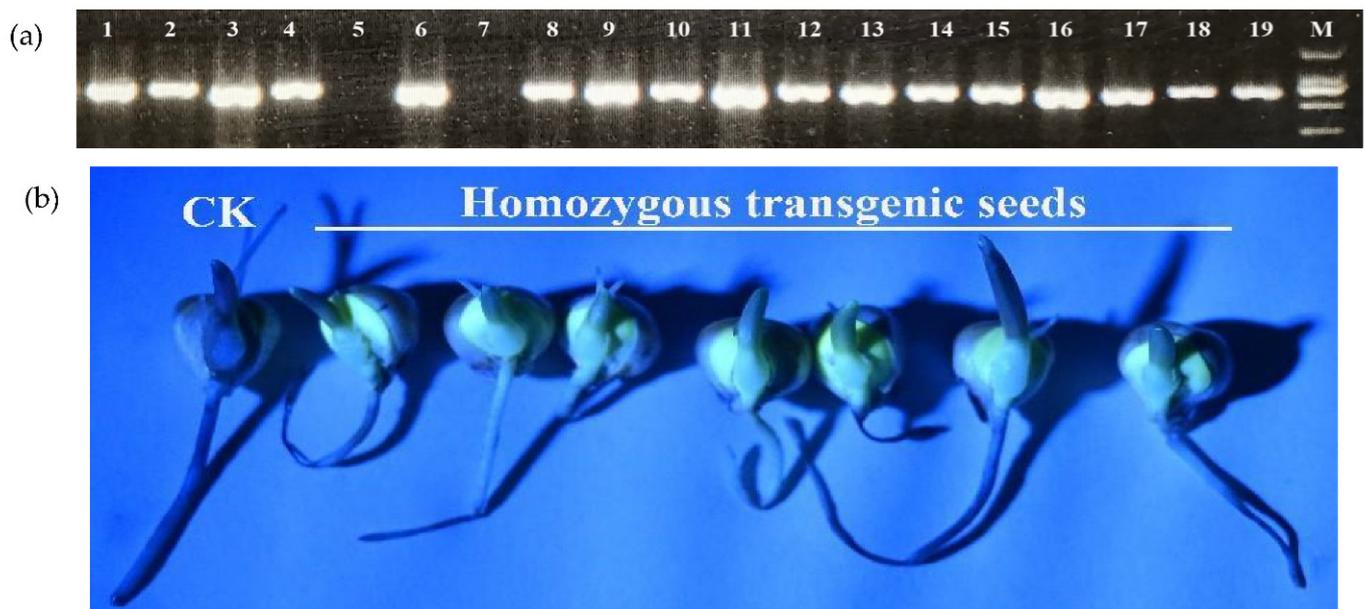


Figure 2. Acquisition of homozygous transgenic seeds by PCR and fluorescence detection. (a) PCR products of T_1 generation plants (lane 1–18; lane 5 and 7 are of negative plants), and pBIN-YA (B) plasmid (lane 19). M: DL 2000 marker; (b) fluorescence detection.

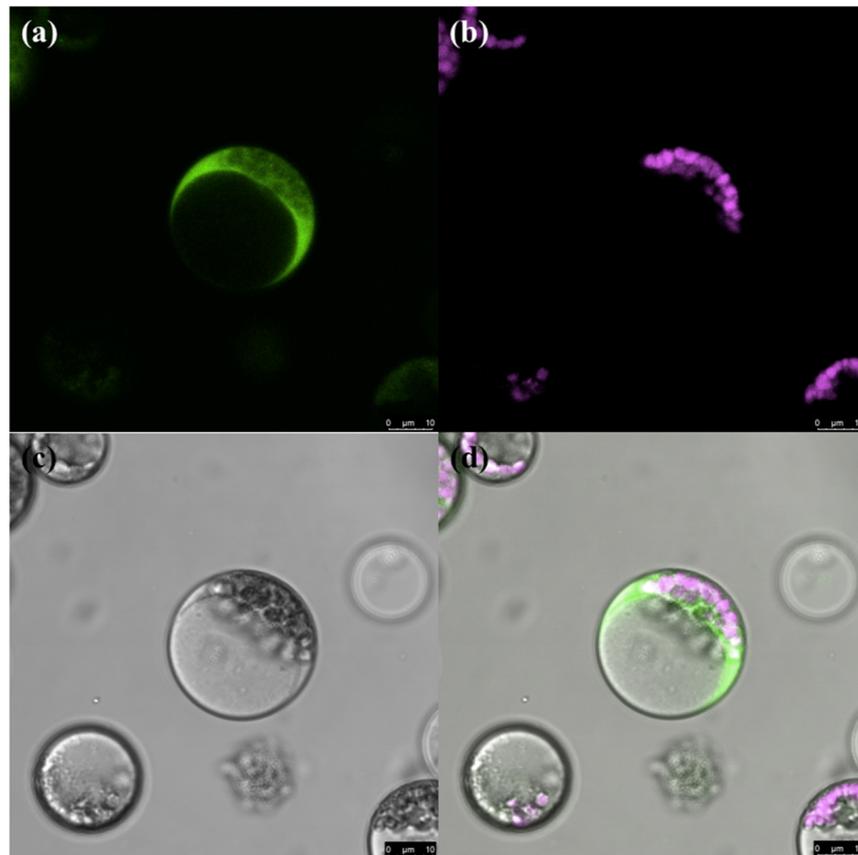


Figure 3. Subcellular localization of YFP-aequorin in maize protoplast. (a) YFP-aequorin, (b) chlorophyll, (c) bright field, (d) merged image.

The embryos that were isolated from the seeds with different vigor levels and imbibed for different durations were used to determine the concentration of free Ca^{2+} in the cytoplasm and nuclei. The free Ca^{2+} in the embryo cells of high-vigor seeds was significantly higher than that in low-vigor seeds (Figure 4a). Exposure of the embryos to *F. graminearum* resulted in a rapid increase in the free Ca^{2+} concentration in the embryo cells of seeds with high and low vigor. The free Ca^{2+} concentration in the embryo cells of the high-vigor seeds was higher than that of the low-vigor seeds, and the differences reached a significant level for most imbibition durations (Figure 4b).

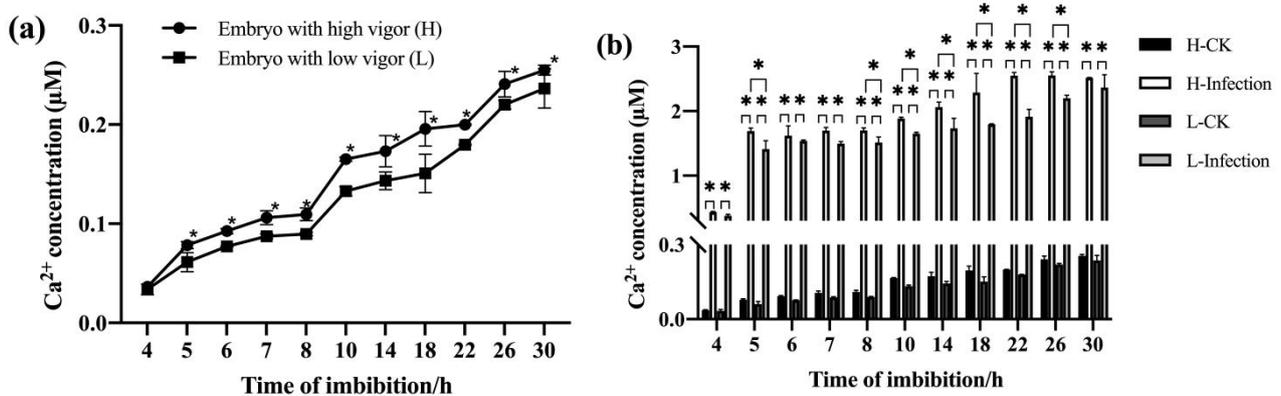


Figure 4. Ca^{2+} concentration in the cytoplasm and nucleus of embryo cells during imbibition of maize seeds with different vigor levels. (a) Ca^{2+} concentration without *F. graminearum* infection, (b) Ca^{2+} concentration before and after *F. graminearum* infection. * indicates $p < 0.05$ (Student's *t*-test), and ns represents not significant.

3.3. Effects of the Inhibition of Ca^{2+} Entry into the Cytoplasm and Exogenous Ca^{2+} Treatments on the Resistance of Maize Seeds to *F. graminearum*

3.3.1. Inhibiting the Entry of Ca^{2+} into the Cytoplasm Reduced the Resistance of Maize Seeds to *F. graminearum*

To clarify the effects of inhibiting the entry of Ca^{2+} into the cytoplasm on the disease resistance of maize seeds, high- and low-vigor maize seeds were treated with cell membrane Ca^{2+} channel inhibitor LaCl_3 , extracellular Ca^{2+} chelator EGTA, and vacuolar Ca^{2+} channel inhibitor RR. After *F. graminearum* infection, a standard germination test was used to estimate indices related to seed germination and seedling growth. For high-vigor seeds, Ca^{2+} channel inhibitor/chelator treatments inhibited germination and seedling growth. LaCl_3 treatment significantly reduced germination energy, germination index, germination percentage, and vigor index. EGTA treatment significantly reduced the vigor index and average dry weight of a single seedling. RR treatment significantly reduced the germination energy, germination index, germination percentage, average dry weight of a single seedling, and vigor index. Among the treatments, the RR treatment had the largest effect (Figure 5). Similar results were obtained for low-vigor seeds after treatments with Ca^{2+} channel inhibitors/chelators (Figure 6).

To further examine the effects of LaCl_3 , EGTA, and RR treatments on the germination and seedling growth of maize seeds with different vigor levels, a standard germination test was conducted on maize seeds treated with three inhibitors but not inoculated with *F. graminearum*. The three inhibitors also significantly inhibited seed germination and seedling growth (Figures 7 and 8). However, after inoculation with *F. graminearum*, RR treatment had the highest effect in the case of *F. graminearum* infection (Figures 5 and 6), while LaCl_3 had the highest effect in the absence of *F. graminearum* infection (Figure 7). This indicates that the Ca^{2+} required for germination was mainly from the vacuolar calcium pool when the seeds were treated with the inhibitors and exposed to *F. graminearum*, while the Ca^{2+} required for germination was mainly from the apoplastic calcium store when the seeds were treated only with the inhibitors.

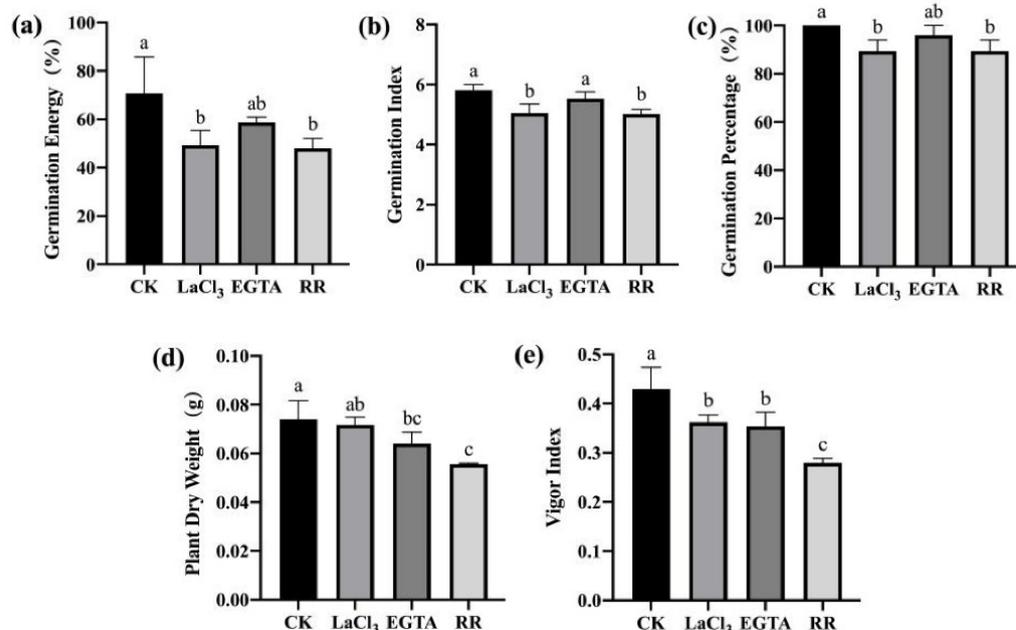


Figure 5. Ca^{2+} chelating agent/channel inhibitor treatments reduced *F. graminearum* resistance in high-vigor maize seeds. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*-tetraacetic acid; RR, Ruthenium Red; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

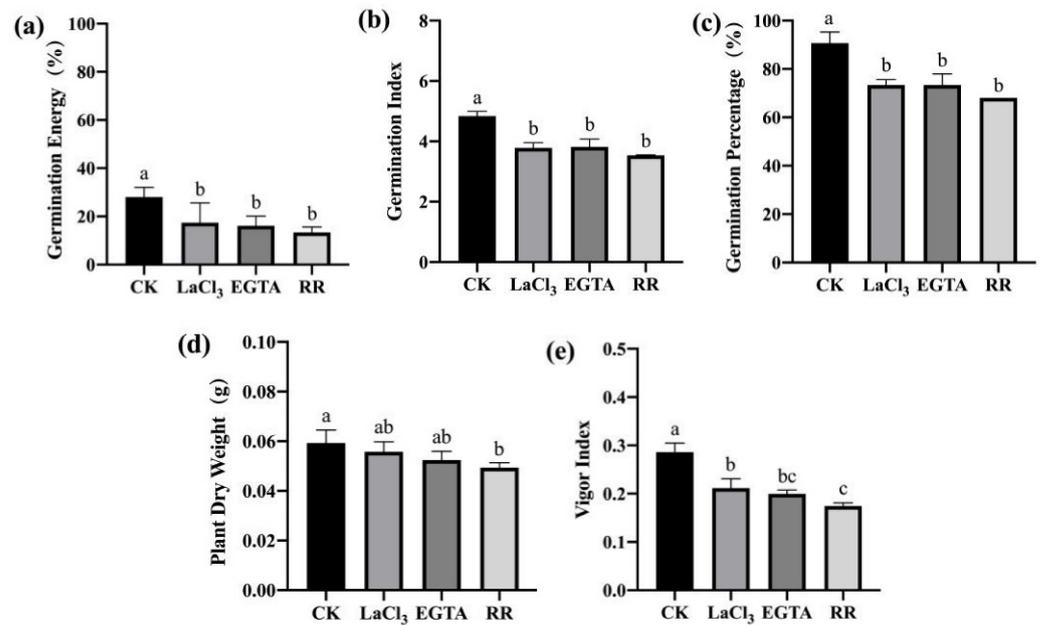


Figure 6. Ca^{2+} chelating agent/channel inhibitor treatments reduced *F. graminearum* resistance in low-vigor maize seeds. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*-tetraacetic acid; RR, Ruthenium Red; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

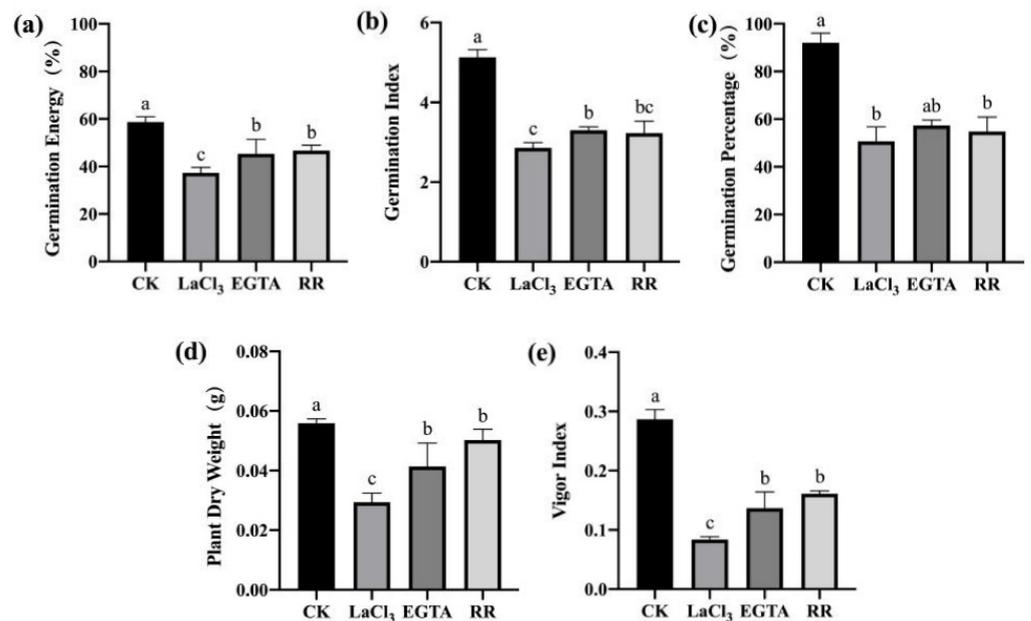


Figure 7. Ca^{2+} chelating agent/channel inhibitor treatments inhibited seed germination and seedling growth of high-vigor maize seeds. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*-tetraacetic acid; RR, Ruthenium Red; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

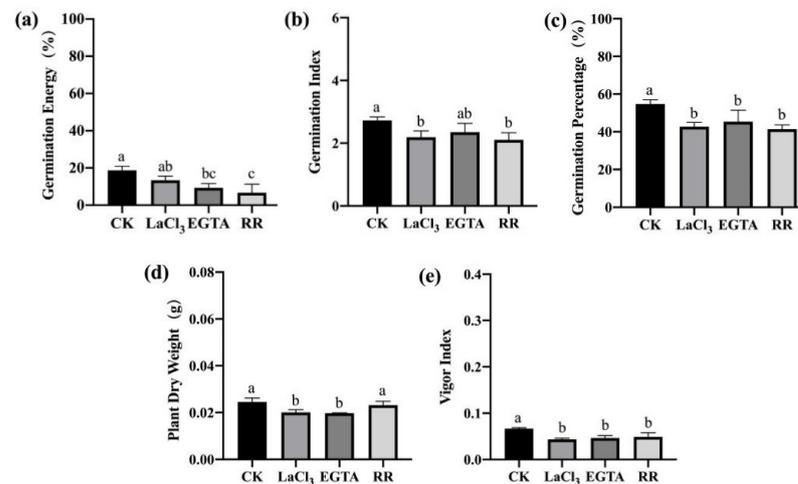


Figure 8. Ca²⁺ chelating agent/channel inhibitor treatments inhibited seed germination and seedling growth of low-vigor maize seeds. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-N-tetraacetic acid; RR, Ruthenium Red; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

3.3.2. Exogenous Ca²⁺ Treatments Enhanced the Resistance of Maize Seeds to *F. graminearum*

To clarify the effect of exogenous Ca²⁺ application on the disease resistance of maize seeds, we used CaCl₂ solution (10 and 20 mmol/L) and CaSO₄ solution (10 and 20 mmol/L) to treat maize seeds. After *F. graminearum* infection, a standard germination test was performed to estimate the indices related to seed germination and seedling growth. Meanwhile, non-inoculated maize seeds treated with exogenous Ca²⁺ were also germinated. For both high- and low-vigor seeds, exogenous Ca²⁺ treatment promoted germination and seedling growth; the germination index, germination percentage, and vigor index of the seeds and the dry weight of seedlings were significantly higher than those of the control (Figures 9 and 10). The results indicate that exogenous Ca²⁺ treatment significantly improves the resistance of maize seeds to *F. graminearum*.

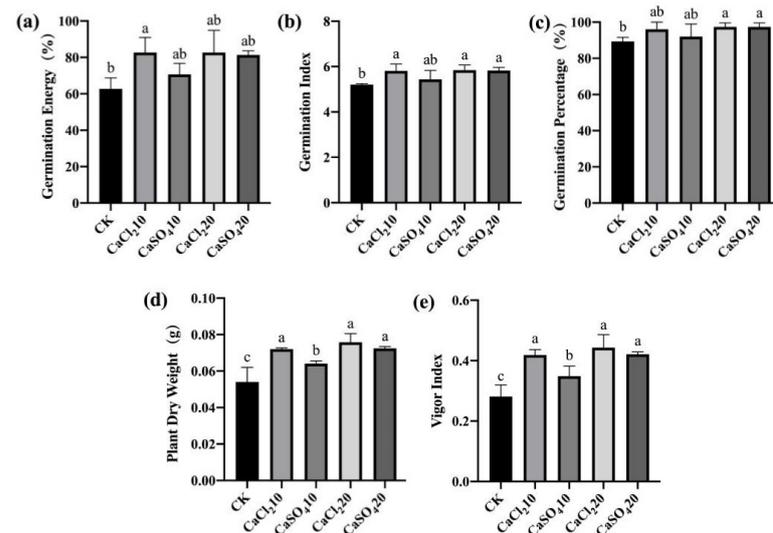


Figure 9. Exogenous Ca²⁺ enhanced the resistance of high-vigor maize seeds to *F. graminearum*. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. CaCl₂10/20, 10/20 mmol/L CaCl₂ solution; CaSO₄10/20, 10/20 mmol/L CaSO₄ solution; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

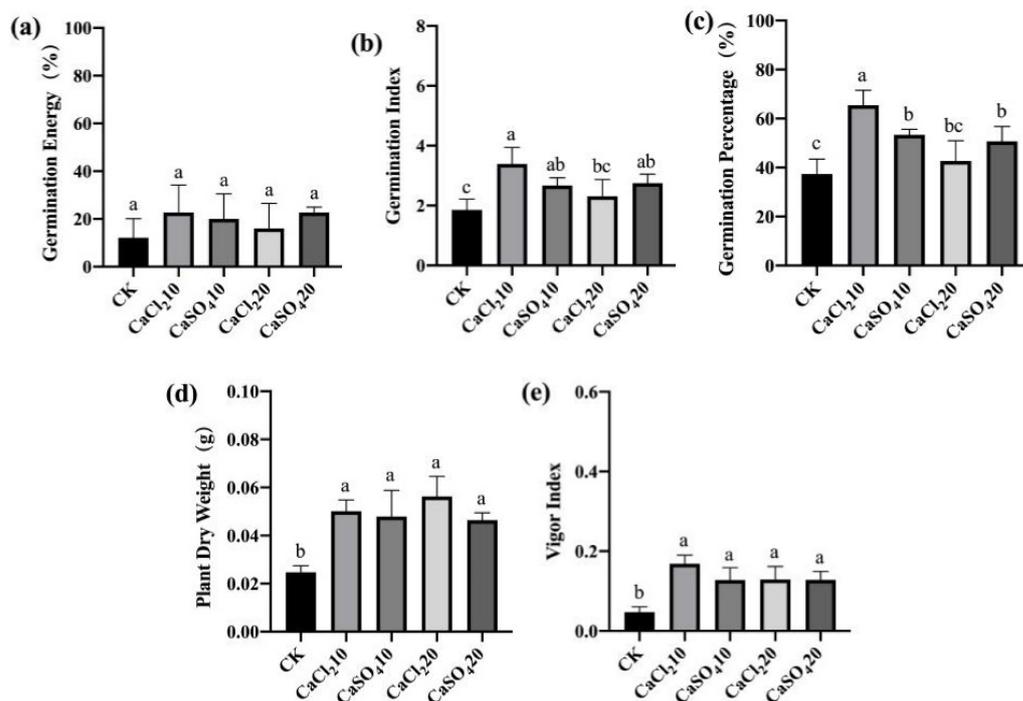


Figure 10. Exogenous Ca^{2+} enhanced the resistance of low-vigor maize seeds to *F. graminearum*. (a) germination energy (%), (b) germination index, (c) germination percentage (%), (d) dry weight of single plant (g), (e) vigor index. CaCl_2 10/20, 10/20 mmol/L CaCl_2 solution; CaSO_4 10/20, 10/20 mmol/L CaSO_4 solution; CK, control. Different letters indicate significant differences among different lines (Tukey's test).

3.4. Effects of Inhibiting Ca^{2+} Entry into the Cytoplasm and Exogenous Ca^{2+} Treatments on the Activity of Defensive Enzymes in Maize Seeds

Plants secrete a large amount of chitinase in response to pathogen infection and synthesize a large amount of SOD, POD, and CAT to cope with the oxidative stress caused by pathogen infection. The activities of these four enzymes in the seeds inoculated with *F. graminearum* and treated with Ca^{2+} channel inhibitors/chelators were determined. Ca^{2+} channel inhibitor/chelator treatment significantly reduced the activity of chitinase at 24 h after inoculation, the activity of SOD at 24 and 48 h after inoculation, and the activities of POD and CAT at all three time points after inoculation in both high- and low-vigor seeds (Figures 11 and 12). RR had the highest inhibitory effect on the activity of these enzymes, which was consistent with the previously mentioned germination data (Figures 5 and 6). Exogenous Ca^{2+} treatments increased the activity of the four enzymes mentioned above in both high- and low-vigor seeds, and the differences were significant for most of the treatments (Figures 13 and 14).

These results indicate that the inhibition of Ca^{2+} entry into the cytoplasm and the exogenous application of Ca^{2+} significantly affected the activities of chitinase, SOD, POD, and CAT in maize seeds, which then affected the resistance of the seeds to *F. graminearum* infection.

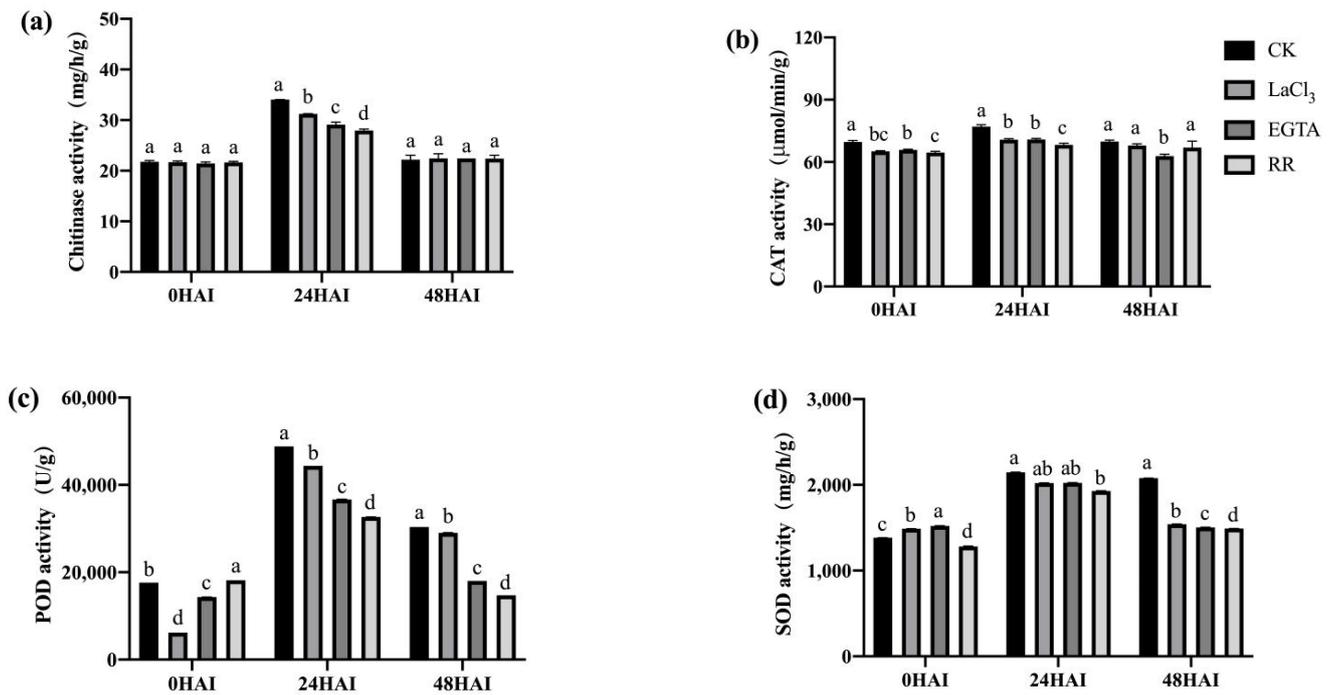


Figure 11. Ca²⁺ chelating agent/channel inhibitor treatments inhibited the activities of chitinase (a), CAT (b), POD (c), and SOD (d) in high-vigor maize seeds under *F. graminearum* infection. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*-tetraacetic acid; RR, Ruthenium Red; CK, control; HAI, hours after inoculation. Different letters indicate significant differences among different lines (Tukey's test).

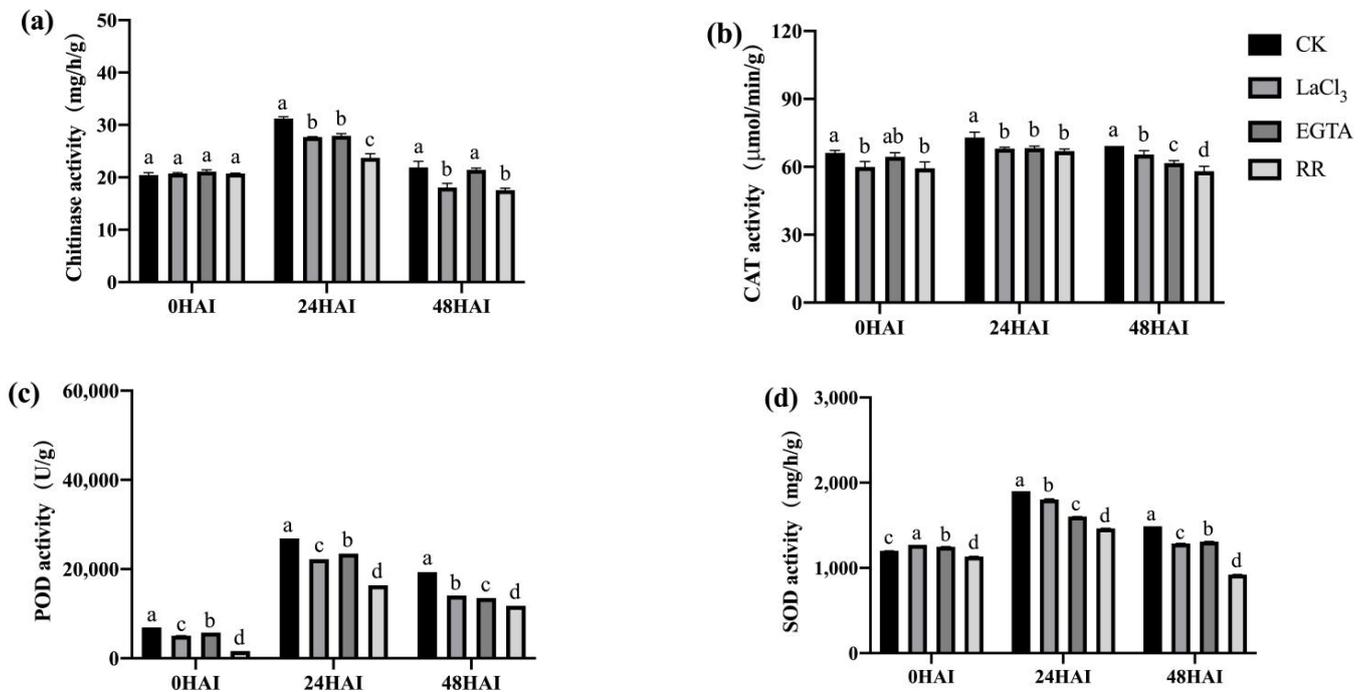


Figure 12. Ca²⁺ chelating agent/channel inhibitor treatments inhibited the activities of chitinase (a), CAT (b), POD (c), and SOD (d) in low-vigor maize seeds under *F. graminearum* infection. EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*-tetraacetic acid; RR, Ruthenium Red; CK, control; HAI, hours after inoculation. Different letters indicate significant differences among different lines (Tukey's test).

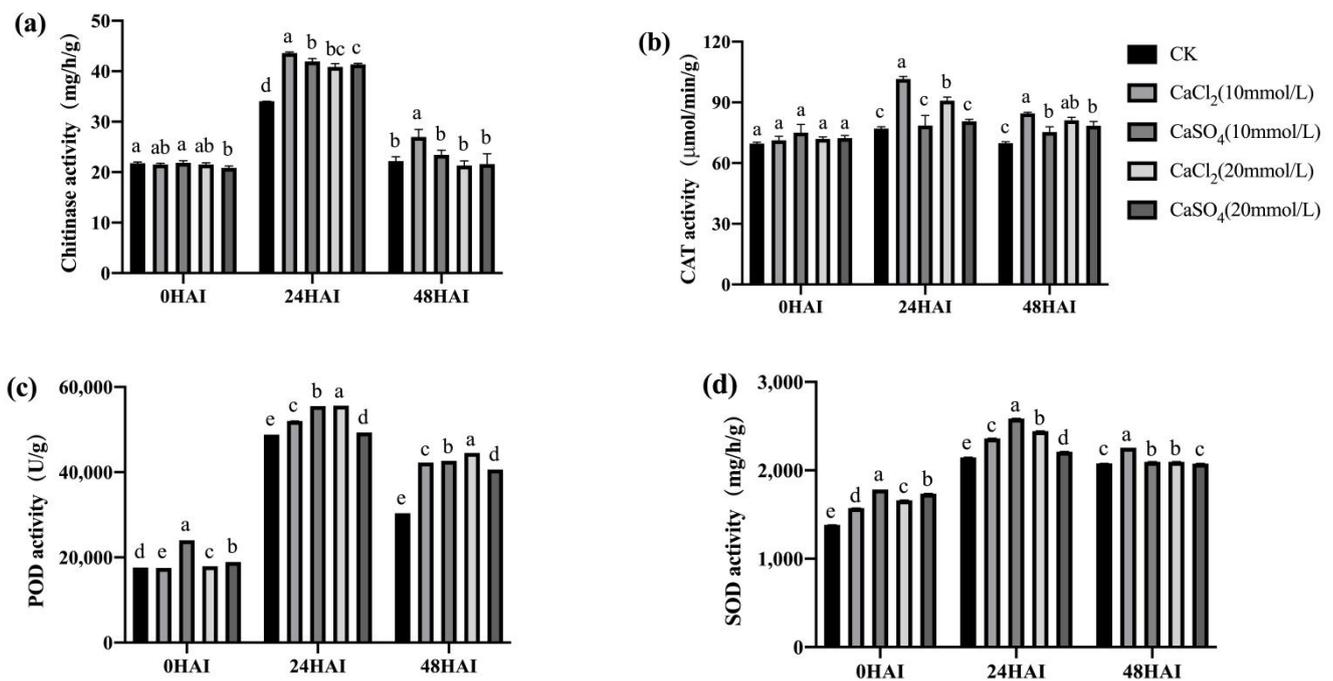


Figure 13. Exogenous Ca²⁺ enhanced the activities of chitinase (a), CAT (b), POD (c), and SOD (d) in high-vigor maize seeds under *F. graminearum* infection. HAI, hours after inoculation. Different letters indicate significant differences among different lines (Tukey's test).

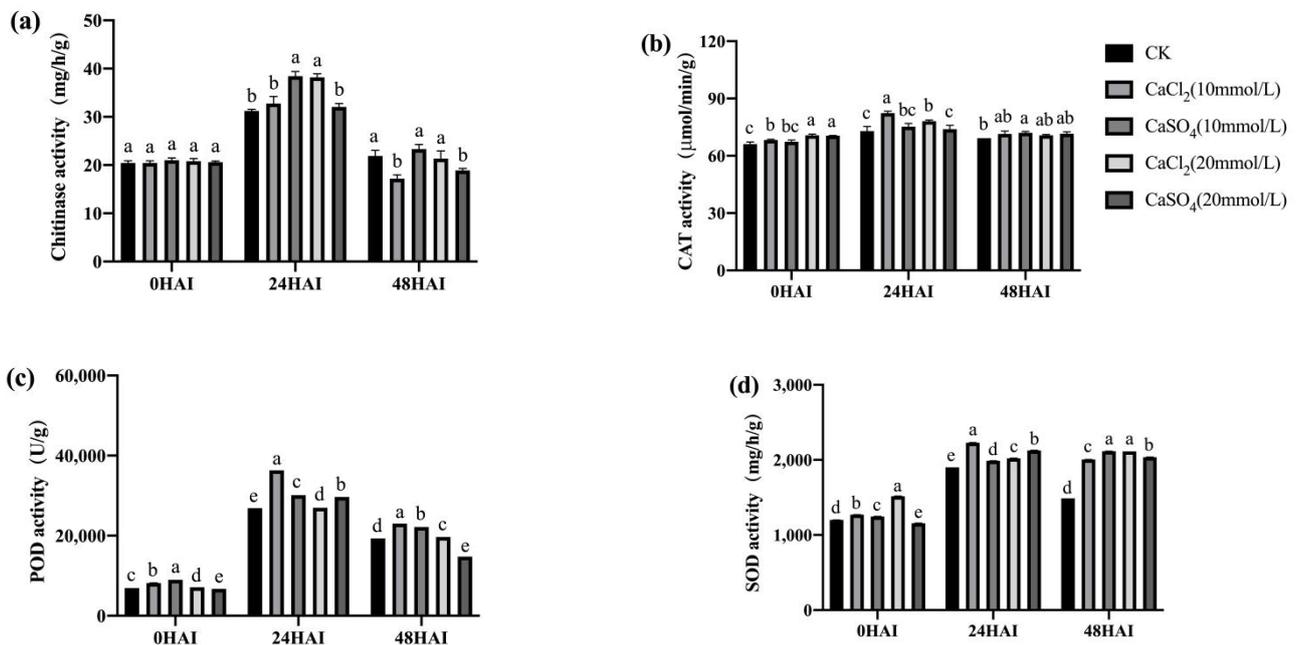


Figure 14. Exogenous Ca²⁺ enhanced the activities of chitinase (a), CAT (b), POD (c), and SOD (d) in low-vigor maize seeds under *F. graminearum* infection. HAI, hours after inoculation. Different letters indicate significant differences among different lines (Tukey's test).

4. Discussion

Seed vigor is an important indicator of seed quality. High-vigor seeds have a high level of resistance to various adverse environmental conditions. In this study, the newly harvested high-vigor seeds and seeds with low vigor caused by an artificially accelerated aging treatment were placed in a *F. graminearum* suspension to imbibe it for 24 h and then subjected to a germination test. *F. graminearum* infection did not cause distinct damage

to high-vigor seeds, while *F. graminearum* infection significantly inhibited low-vigor seed-related germination and seedling growth (Figure 1). Why are high-vigor seeds more resistant to *F. graminearum* infection?

Ca^{2+} plays a key role in promoting plant development and resistance to biotic and abiotic stress. Previous studies showed that Ca^{2+} is involved in the GA-induced synthesis of α -amylase in rice seeds [30] and that Ca^{2+} activates protease in germinated mung bean seeds [31]. Ca^{2+} is involved in regulating early axis growth during the germination of mung bean seeds [6]. Exogenous application of CaCl_2 reduced the effect of salt stress on *Phragmites karka* and *Festuca ovina* [32,33]. However, as far as we know, there is no report on the effect of Ca^{2+} on seed germination under pathogen infection. In this study, transgenic maize seeds expressing the *aequorin* gene were used to examine the concentration of free Ca^{2+} in the cytoplasm and nuclei. The results showed that the concentration of free Ca^{2+} in the embryo cells of high-vigor seeds was significantly higher than that of low-vigor seeds at different time points of imbibition (Figure 4a). After the infection of embryos with *F. graminearum*, the free Ca^{2+} concentration in the embryo cells of both the high- and low-vigor seeds increased rapidly and significantly, and the free Ca^{2+} concentration in the seed embryo cells of the high-vigor seeds was higher than that of the low-vigor seeds (Figure 4b).

To clarify the effect of Ca^{2+} on seed germination under *F. graminearum* infection, we used three Ca^{2+} antagonists (LaCl_3 , EGTA, and RR) and two exogenous calcium solutions (CaCl_2 and CaSO_4) to treat maize seeds with high and low vigor, respectively. Inhibiting the entry of extracellular or vacuolar Ca^{2+} into the cytoplasm significantly inhibited seed germination and seedling growth (Figures 5 and 6) and significantly reduced the activity of chitinase, CAT, POD, and SOD (Figures 11 and 12), while exogenous Ca^{2+} treatment promoted seed germination and seedling growth and significantly increased the activities of the four enzymes (Figures 9, 10, 13 and 14). The results indicated that Ca^{2+} played an important role in the resistance of maize seeds to *F. graminearum* infection.

Singh et al. [6] and Verma et al. [34] demonstrated that the Ca^{2+} required for the early germination of mung bean (*Vigna radiata*) seeds came from the apoplastic calcium store. Our previous study showed that during the imbibition of maize seeds, Ca^{2+} in embryo cells flowed from the extracellular calcium pool into cells, and the influx rate of Ca^{2+} in the embryos of high-vigor seeds was higher than that of low-vigor seeds [29]. In the present study, although the treatments with all the three inhibitors significantly inhibited seed germination and seedling growth, in the presence of *F. graminearum* infection, the vacuolar Ca^{2+} channel inhibitor RR showed the highest inhibitory effect (Figures 5 and 6), while in the absence of *F. graminearum* infection, the inhibitor LaCl_3 showed the highest inhibitory effect (Figure 7). The results indicated that in the case of *F. graminearum* infection, the required Ca^{2+} mainly came from the vacuolar calcium pool, and in the absence of *F. graminearum* infection, the required Ca^{2+} mainly came from the apoplastic calcium store. The vacuole and apoplast are the major calcium pools in plants. The concentrations of free Ca^{2+} were 0.2–0.5 mM, 0.33–1 mM, and 50–100 nM in the vacuole, apoplast, and cytoplasm, respectively [35]. The concentration of free Ca^{2+} in vacuoles and apoplasts is much higher than that in the cytoplasm. Therefore, when *F. graminearum* infection occurs, both the vacuolar and apoplast calcium pools can theoretically provide sufficient Ca^{2+} to form a Ca^{2+} signal to initiate the expression of downstream disease resistance genes. Then, why did Ca^{2+} mainly come from the vacuolar calcium pool during fungal infection? The answer might be that Ca^{2+} release from vacuolar is faster than Ca^{2+} influx from the apoplast upon pathogen infection. The Ca^{2+} signal responds quickly to pathogen infection. About 30 s to 2 min after the plant perceived MAMPs/DAMPs, Ca^{2+} began to flow into the cytoplasm, and in about 4 to 6 min, the Ca^{2+} content in the cytoplasm reached a peak [23]. Therefore, calcium pools need to respond quickly to pathogen infection. The hypothesis needs to be verified in further studies.

Diseases are highly harmful to agricultural production. Toxic chemicals are usually used to control diseases. In this study, we found that soaking seeds in 10 and 20 mmol/L

CaCl₂ or 10 and 20 mmol/L CaSO₄ solution significantly promoted seed germination and seedling growth (Figures 9 and 10) and improved the resistance to *F. graminearum* infection. In addition, applying exogenous CaCl₂ to plants can alleviate the damage to plants caused by stress due to abiotic factors, such as high temperature, drought, freezing, salt, and heavy metals [10–14]. Furthermore, CaCl₂ is a safe, environmentally friendly, and inexpensive chemical. It is expected that CaCl₂, as a seed treatment agent, may play a more important role in sustainable agricultural production in the future; of course, field trials are needed to confirm its practical value in agriculture.

5. Conclusions

Low-vigor seed-related germination and seedling growth were significantly inhibited by *F. graminearum* infection, while those related to high-vigor seed were not. The concentration of free Ca²⁺ in the cytoplasm and nuclei of the embryo cells of the high-vigor seeds was significantly higher than that of the low-vigor seeds prior to and after *F. graminearum* infection. Inhibiting extracellular or vacuolar Ca²⁺ into the cytoplasm significantly inhibited seed germination and seedling growth and significantly reduced the activity of chitinase, CAT, POD, and SOD. Treatments with exogenous Ca²⁺ promoted seed germination and seedling growth and significantly increased the activities of chitinase, CAT, POD, and SOD. In the presence of *F. graminearum* infection, the Ca²⁺ required for seed germination and seedling growth mainly came from the vacuolar calcium pool, while in the absence of *F. graminearum* infection, the required Ca²⁺ mainly came from the apoplasmic calcium store.

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