Ethylenediaminetetraacetic Acid Disodium Salt Acts as an Antifungal Candidate Molecule against *Fusarium graminearum* by Inhibiting DON Biosynthesis and Chitin Synthase Activity

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**Abstract:** *Fusarium* fungi are the cause of an array of devastating diseases affecting yield losses and accumulating mycotoxins. Fungicides can be exploited against *Fusarium* and deoxynivalenol (DON) production. However, *Fusarium* resistance to common chemicals has become a therapeutic challenge worldwide, which indicates that new control agents carrying different mechanisms of action are desperately needed. Here, we found that a nonantibiotic drug, ethylenediaminetetraacetic acid disodium salt (EDTANa2), exhibited various antifungal activities against *Fusarium* species and DON biosynthesis. The infection of wheat seeding caused by *F. graminearum* was suppressed over 90% at 4 mM EDTANa2. A similar control effect was observed in field tests. Mycotoxin production assays showed DON production was significantly inhibited, 47% lower than the control, by 0.4 mM EDTANa2. In vitro experiments revealed a timely inhibition of H2O2 production as quickly as 4 h after amending cultures with EDTANa2 and the expression of several TRI genes significantly decreased. Chitin synthases of *Fusarium* were Mn2+-containing enzymes that were strongly inhibited by Mn2+ deficiency. EDTANa2 inhibited chitin synthesis and destroyed the cell wall and cytomembrane integrity of *Fusarium*, mainly via the chelation of Mn2+ by EDTANa2, and thus led to Mn deficiency in *Fusarium* cells. Taken together, these findings uncover the potential of EDTANa2 as a fungicide candidate to manage Fusarium head blight (FHB) and DON in agricultural production.

**Keywords:** *Fusarium*; EDTANa2; deoxynivalenol; chitin synthases; manganese ion

**Key Contribution:** EDTANa2 is identified as a fungicide candidate molecule to control the growth and DON production of *Fusarium graminearum*. The antifungal activity of EDTANa2 mainly results from Mn deficiency in cells.

1. Introduction

*Fusarium* is a globally important genus of fungal pathogens, responsible for many devastating diseases of plants and various serious diseases of humans [1,2]. *Fusarium* species are widely present in soil, plants and other organic substrates and have widespread distributions [3]. Species such as *Fusarium graminearum*, *Fusarium oxysporum* and *Fusarium verticilloides* can infect many crop plants, vegetables and flowers [2,4–8]. One of the major diseases caused by *Fusarium* is Fusarium head blight (FHB), which is becoming more and more serious recently and causing concern. FHB results in yield loss and damaging of cereal grains [9,10]. Additionally, *Fusarium* spp. produce various types of mycotoxins, including deoxynivalenol (DON) and acetyl-deoxy-nivalenol (3-ADON and 15-ADON), that suppress humoral and cellular immunity and are thus highly detrimental to human and animal health [11,12]. DON is a mycotoxin virulence factor that promotes growth of the *F. graminearum* fungus in wheat floral tissues [13].
Practices used to control FHB and DON include rotation with nonhost crops and tillage [14], planting of resistant cultivars [15], and application of fungicides [16]. Essentially, current protective measures against *Fusarium* species mainly rely on fungicides, such as benzimidazole, triazole, demethylation inhibitor and quinone outside inhibitor. However, some of these can lead to the enhancement of DON biosynthesis in the infected wheat [17,18]. Therefore, new molecules are needed to control FHB and inhibit DON biosynthesis.

In addition, *Fusarium* isolates are susceptible to mutations that lead to phenotypes of tolerance towards common antifungal drugs [19,20]. Resistance to fungicides allows pathogens to survive fungicide treatment. The time taken for a new resistant mutant to reach a population size that is unlikely to die out by chance is called “emergence time”. Prolonging emergence time would delay loss of control [21]. To date, the drawbacks of both scientific control strategies and the use of effective fungicides need to be addressed [8,22,23]. Undoubtedly, the processes of discovery and development of new antifungal drugs in the pharmaceutical industry are not only laborious and time consuming but also costly [24]. This is particularly true, as fungicides with novel modes of action are only rarely found, and resistance to single-target fungicides may occur within few years [25]. Some nonantibiotic drugs were recently reported to exhibit some antimicrobial activity against bacteria and *Candida albicans* [26,27].

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent targeting divalent cations and has been previously used in oil fields to increase oil production and inhibit scale formation [28,29]. EDTA has also been shown to possess antimicrobial activities against bacteria and *C. albicans* because it can limit the availability of essential cations. The chelation of cations causes a separation of lipopolysaccharides from the outer membrane of microbial cells and thus increases the membrane permeability and subsequently leads to cell death in bacteria [30–35]. Generally considered safe, EDTA has been used intensively in the food and therapeutic industry [33,36,37]. Here, we demonstrated that EDTANa$_2$ had antifungal activity against *Fusarium graminearum* and DON biosynthesis. Our study contributes to the understanding of the mechanisms underlying EDTANa$_2$ control of FHB and provides a fungicide candidate molecule against *Fusarium graminearum* and its mycotoxin biosynthesis.

### 2. Results

#### 2.1. Ethylenediaminetetraacetic Acid Disodium Salt Exhibits Various Antifungal Activities against *Fusarium* Species

Mycelial growth was inhibited with 0.15 mM ethylenediaminetetraacetic acid or ethylenediaminetetraacetic acid sodium salt (Figure 1A). The effectiveness of ethylenediaminetetraacetic acid sodium salt was affected by its number of sodium ions. For all tested reagents, EDTANa$_2$ had the best antifungal activity in terms of growth inhibition as well as cell swelling effects, closely followed by EDTANa$_3$. The antifungal activity of EDTANa$_4$ was similar to that of EDTA. Moreover, the mycelial growth and morphology with the 0.3, 0.45, 0.6 mM sodium ion treatments showed no difference from the control group (only the treatment with 0.6 mM sodium ion is shown in Figure 1A), indicating that the sodium ions had no effect on *Fusarium* growth at a concentration less than 0.6 mM.

To test the antifungal activity of EDTANa$_2$, thirteen isolates of *Fusarium*, including *F. acuminatum*, *F. asiaticum*, *F. avenaceum*, *F. concentricum*, *F. culmorum*, *F. equiseti*, *F. fujikuroi*, *F. graminearum*, *F. lateritium*, *F. oxysporum*, *F. proliferatum*, *F. solani* and *F. verticillioides* were inoculated into 96-well microtiter plates. The minimum inhibitory concentrations (MICs) of EDTANa$_2$ on the test pathogens varied from 9.37 to 18.75 mM (Table S1). The lowest MIC value was observed for *F. asiaticum*, *F. avenaceum*, *F. equiseti*, *F. fujikuroi* and *F. proliferatum*, while the other *Fusarium* isolates (except *F. lateritium*) had MIC values of 18.75 mM. The MIC value of EDTANa$_2$ to *F. lateritium* was 200 mM. Surprisingly, EDTANa$_2$ did not show any antifungal activity against *F. lateritium* but promoted its growth in the range of 4.69–150 mM.
2.2. The Control Effect and Phytotoxicity Test of Ethylenediaminetetraacetic Acid Disodium Salt

For *F. graminearum* PH-1 strain, a linear regression of the percentage inhibition related to the control of mycelial growth versus the log$_{10}$ transformation for each of EDTANa$_2$ concentration was obtained. The median effective concentration (EC$_{50}$) was calculated for each strain using a linear equation. The EC$_{50}$ value of EDTANa$_2$ for *F. graminearum* PH-1 strain was 0.29 mM (107.88 mg L$^{-1}$) (Figure 1B).

2.2. The Control Effect and Phytotoxicity Test of Ethylenediaminetetraacetic Acid Disodium Salt

The EDTANa$_2$ for control of seedling blight in wheat was effective, reducing disease severity by 59%, 79%, 92% at 1 mM, 2 mM and both 4 mM and 8 mM, relative to the inoculated control, respectively (Figure 2A). No significant different effect was observed among 1 mM to 8 mM EDTANa$_2$, indicating that the control effect was stable within that range under controlled conditions. To ascertain whether the EDTANa$_2$ molecule could inhibit *Fusarium* infection under natural conditions, a crop phytopathogen, *F. graminearum*, was chosen for pathogenicity assays by spray inoculation experiments. The disease incidences were recorded 21 days post inoculation (dpi). The field experiment was conducted for two years (2018 and 2019) and produced similar results to the previously described experiments. As shown in Figure 2B, EDTANa$_2$ significantly reduced *Fusarium* head blight in the field. After spray treatment with 7 g ha$^{-1}$ EDTANa$_2$, *F. graminearum* caused 52% and 45% spikelets infection at 21 dpi, 45% and 49% reduction compared to the sterile water control in 2018 and 2019, respectively. When the dosage of EDTANa$_2$ increased to 70 g ha$^{-1}$,
the incidence of disease decreased to 12% and 8%, 87% and 91% reduction compared to the sterile water control in 2018 and 2019, respectively. In 2019, the incidence of disease treated with 70 g ha\(^{-1}\) EDTANa\(_2\) had a 70% reduction compared to the 140 g ha\(^{-1}\) carbendazim treatment (Figure 2B). To further analyze the influence of EDTANa\(_2\) treatments on spikelet morphology (Figure 2A), we sprayed a series of EDTANa\(_2\) concentrations (7 g ha\(^{-1}\) to 4000 g ha\(^{-1}\)) onto wheat spikelets, and the results showed that there was little change in spikelets morphology following spray application of 7 g ha\(^{-1}\) to 1600 g ha\(^{-1}\) EDTANa\(_2\). However, when the dosage of EDTANa\(_2\) increased to 2000 g ha\(^{-1}\), phytotoxicity was observed (Figure 2C). These results suggested that EDTANa\(_2\) could be used as a safe antifungal agent at low concentration.

**Figure 2.** Control effect and phytotoxicity test of EDTANa\(_2\). (A) Control effect of EDTANa\(_2\) on wheat seedling blight in laboratory. The test was designed with two random replications for each race of plant. Ten seedlings per treatment were inoculated with macroconidia suspension 24 h after EDTANa\(_2\) spray. The lesions of diseased leaves were measured and photographed on sixth day post inoculation. Different letters represent a significant difference at \(p < 0.05\). A linear regression equation of the percentage control effect for each EDTANa\(_2\) concentration was developed using the SPSS 20.0 (IBM, Chicago, IL, USA) statistical package \((y = 4.67 + 3.329x, R = 0.9476)\). (B) Control effect of EDTANa\(_2\) on Fusarium head blight in the field. Wheat spikelets (cultivar Huaimai33) were sprayed with water, 40% carbendazim (140 g ha\(^{-1}\)), or EDTANa\(_2\). Twenty-four hours later, the spikelets were inoculated via a spray inoculation experiment with a conidial suspension. Each combination of fungicide treatment and fungus was represented by 30 heads. After 21 days, the percentages of infected spikelets were determined, and representative heads were photographed. Values are means ± SD. Different letters represent a significant difference at \(p < 0.05\). (C) Phytotoxicity test of EDTANa\(_2\) in the field. Wheat spikelets (cultivar Huaimai33) were sprayed with EDTANa\(_2\) and photographed at 21 days post inoculation. The data are an average ± standard error from 30 randomly selected heads. The experiment was replicated three times.
2.3. EDTANa$_2$ Decreases DON Biosynthesis and TRI Gene Expression of Fusarium Graminearum In Vitro

Because the mycotoxin DON is a virulence factor, we investigated the mycotoxin biosynthesis potential of strains under EDTANa$_2$ treatment. To verify the ability of the EDTANa$_2$ to limit toxin production, DON amounts were measured using a competitive ELISA-approach. As shown in Figure 3A, the DON production in TBI media was significantly inhibited by 0.4 mM and 0.8 mM EDTANa$_2$, about 47% and 57.3% lower than the control group, respectively.

Figure 3. Effect of EDTANa$_2$ on production of deoxynivalenol (DON) (A), H$_2$O$_2$ concentrations (B) and TRI genes expression (C). DON content was determined using a competitive ELISA approach 7 d after start of the experiments. The experiment was repeated three times. H$_2$O$_2$ was measured at 4 h and 12 h and calculated based on a standard curve included in each experiment. TRI gene expression was assayed by qRT-PCR. Hyphae were harvested from 2-day-old TBI cultures (1 day after EDTANa$_2$ adding). Data are represented as the means ± SD of three biological replicates (significant differences at * $p < 0.05$).
As several lines of evidence in the literature corroborate an important role for H$_2$O$_2$ in induction of toxin production, the accumulation of H$_2$O$_2$ upon EDTANa$_2$ application was monitored using an in vitro assay. It showed that adding 0.4 mM and 0.8 mM EDTANa$_2$ resulted in a decreased H$_2$O$_2$ content in the medium compared to the control as fast as 4 h after the start of the assay (Figure 3B). This indicated that EDTANa$_2$ decreased the intracellular oxygen content within a short time after adding to the medium, which may reduce the activation of oxygen to toxin synthesis.

To further reveal the expression profiles of individual genes and their coordination in the trichothecene biosynthesis pathway, we measured the expression of several TRI genes after treating with EDTANa$_2$. We found four genes in the trichothecene biosynthesis pathway were significantly down-regulated compared with the control group (Figure 3C). After treating with 0.4 mM EDTANa$_2$ at 12 h, the expression of the TRI6 and TRI10 genes, which have been identified as positive transcription factor genes for trichothecene biosynthesis in *F. graminearum*, decreased by 2- and 2.13-times; The TRI12 gene, which is associated with trichothecene accumulation and resistance in *F. graminearum*, decreased by 3.45-times compared with the control group; The expression of the TRI11 gene also decreased by 1.56-times. The inhibitory effect of 0.8 mM EDTANa$_2$ on the TRI genes expression was consistent with that of 0.4 mM EDTANa$_2$. It should be mentioned that the Tri101 gene encoding a trichothecene 3-O-acetyltransferase showed a remarkable up-regulation, with 2.24- and 3.02-fold more transcripts after treating with 0.4 mM and 0.8 mM EDTANa$_2$, respectively (Figure 3C). These in vitro results suggested that EDTANa$_2$ can indeed inhibit DON biosynthesis and may be useful for reducing DON contamination in grains caused by *F. graminearum*.

2.4. Ethylenediaminetetraacetic Acid Disodium Salt Affected Cell Wall Formation and Cell Permeability

The inhibition of *Fusarium* growth by EDTANa$_2$ was observed, so we analyzed the cell wall formation and cell permeability of cells grown in the presence of EDTANa$_2$. The mycelia treated by EDTANa$_2$ were sensitive to the preparation condition of scanning electron microscopy (SEM), and were destroyed and lysed with SEM observation (Figure 4A). As shown in Figure 4B, the ultra-structures of the untreated cells exhibited normal electron-dense layers and patterns under transmission electron microscopy. In contrast, the cells cultured in 0.075 mM EDTANa$_2$ had a clearly altered cell wall electron density, thickness and ultrastructure. The most notable visual alteration was a thicker cell wall in the treated cells, which topped 573 nm compared with that of the control cell, which had an average of 142 nm (the biggest thickness was 153 nm) (n = 20). Furthermore, the number of layers in the treated cell wall was reduced, and the electron density declined, whereas three layers were easily recognized in the normal cell wall. In filamentous fungi, chitin, a β-1,4-linked polysaccharide of N-acetylglucosamine, is a key structural component of the cell wall [38]. To further analyze the effect of EDTA and EDTANa$_2$ on cell wall formation, we measured the chitin content of *F. graminearum* cell walls after EDTA or EDTANa$_2$ treatment. The results illustrated that the EDTA and EDTANa$_2$ treatment groups produced only 33.43% and 25.23% chitin relative to the hyphal dry weight, values 35.35% and 51.2% lower than those of the control group, respectively (Figure 4C). It is noteworthy that EDTANa$_2$ was more effective than EDTA, which concurred with our above results.
Figure 4. Effects of EDTANa2 on Fusarium cell wall formation and cell permeability. (A) Scanning electron microscopy of hyphae of Fusarium graminearum treated with or without EDTANa2. (B) Transmission electron microscopy of hyphae of F. graminearum treated with or without EDTANa2. The right panel is an enlarged view of the area in the black box in the left panel. CW, cell wall; EL, external electron-dense layer; ML, middle electron-dense layer; IL, internal electron-dense layer; PM, plasma membrane. Chitin content (C) and relative conductivity assay (D) of F. graminearum treated with 0.3 mM NaCl, 0.15 mM EDTA or EDTANa2. The experiment was performed in triplicate. Different letters represent a significant difference at \( p < 0.05 \).

In most cases, the mode of action of antimicrobials against pathogens depends on the destruction of the fungal cell membrane and the resulting increase in cell permeability. The
change in electrical conductivity reflects the change in the cell membrane permeability of *Fusarium*. Additionally, our data showed that the relative conductivity of hypha was significantly increased by 11% and 15% after 0.15 mM EDTA and 0.15 mM EDTANa₂ treatment compared with that of the control group, respectively (Figure 4D). There was no difference between 0.6 mM NaCl treatment and the control group. Therefore, EDTA and EDTANa₂, especially EDTANa₂, indeed affected both cell wall formation and cell permeability and resulted in a decreasing resistance to the external environment in *F. graminearum*.

2.5. The Inhibitory Effects of Ethylenediaminetetraacetic Acid Disodium Salt against Fusarium are Ameliorated by Mn²⁺ but not Mg²⁺ and Ca²⁺

In order to find out the mechanism of inhibiting effect of EDTANa₂, we examined the effect of the saturation of EDTANa₂ (0.15 mM) by the addition of excess cations (0.15 mM to 1.2 mM). As shown in Figure 5A, the adding of Mn²⁺ increased the biomass of mycelia, but the adding of Mg²⁺ and Ca²⁺ had no significant effect on mycelia biomass. In addition, when an additional 0.3 mM MgCl₂, 0.3 mM CaCl₂ or 0.15 mM FeCl₃ was added into the media as sources of divalent ions to bind 0.15 mM EDTANa₂, mycelial growth was not resumed. 0.15 mM EDTANa₂ plus 0.3 mM Mn²⁺, however, rescued mycelial growth and morphology, which was similar to that in control wells not given EDTANa₂ (Figure 5B). That indicated that the inhibiting effect of EDTANa₂ was related to its function of chelation.

![Figure 5](image)

Figure 5. (A) EDTANa₂ saturation with Mg²⁺, Ca²⁺ and Mn²⁺ induces the resumption of mycelial growth. The mycelial biomass was measured after 52 h of incubation as from MgCl₂, CaCl₂ or MnCl₂ adding. Mycelial biomass was expressed as absorbance. (B) Effects of different cations on EDTANa₂ activity against *Fusarium graminearum*. The right panel is an enlarged view of the area in the black box in the left panel. The arrows indicate conglobate structures. The same amounts of cations were added to the medium as controls. The experiment was repeated three times with the same patterns. (C) Concentration of selected trace elements in fungi treated with 0.15 mM EDTANa₂ or 0.15 mM EDTANa₂ plus 0.3 mM additional cations. Mycelial biomass and trace element assays were performed using three biological replicates for each group.

We further measured the trace element content of *Fusarium* hyphae in different treatments. As shown in Figure 5C, when EDTA and EDTANa₂ were added to the medium, the Mg and Mn elements decreased as expected. However, the contents of Ca and Fe elements were not significantly changed after the addition of EDTA and EDTANa₂. When an additional Mg²⁺ or Ca²⁺ was added into the medium containing EDTANa₂, all the trace el-
elements, except Mn, increased or showed no difference compared with the untreated group. The Mn was always in a deficient state in the mycelia treated by EDTA and EDTANa₂, unless Mn²⁺ was added into the medium. These results suggested that intracellular Mn²⁺ was chelated by EDTANa₂, resulting in cell wall and cell membrane defects.

2.6. Chitin Synthases are More Active in F. graminearum When Mn²⁺ Is Used as a Cofactor

To further study the mechanism of the EDTANa₂ control effect, chitin synthases were extracted for enzymatic analysis. Unlike most chitin synthases in other fungi that used Mg²⁺ as the cofactor, chitin synthases in F. graminearum were more active when Mn²⁺ was used at the active site. The chitin synthases with 5 mM Mn²⁺ had a 26% higher activity than that with 5 mM Mg²⁺, indicating that chitin synthases in F. graminearum may use Mn²⁺ as the cofactor. The addition of 0.25 mM EDTANa₂ into the reaction systems could decrease enzymatic activity by 40% and 44% after chelation of Mn²⁺ and Mg²⁺, respectively. However, chitin synthase in the Mn²⁺ reaction system still had a 33% higher activity than that in the Mg²⁺ reaction system. When the EDTANa₂ concentration was increased to 1 mM, the activity of chitin synthase in the Mn²⁺ and Mg²⁺ reaction systems decreased to 0.09 and 0.11 nmol GlcNAc h⁻¹ mg⁻¹, respectively (Figure 6A). Consistent with this, the metal ion addition experiment showed that the chitin content returned to normal levels after the addition of 0.15 mM Mn²⁺ to SNA culture medium (containing 0.15 mM EDTANa₂). While wells adding Mg²⁺ or Ca²⁺ produced equivalent chitin to wells adding EDTANa₂ only, a 35–50% reduction was observed compared to the control group (Figure 6B).

![Figure 6](image)

**Figure 6.** Chitin synthase activity and chitin content assay of *Fusarium graminearum*. (A) Chitin synthase activity was measured following (¹⁴C) GlcNAc incorporation into the filter-retainable polymer in the presence of Mg²⁺ or Mn²⁺. Data are expressed as nmol of GlcNAc incorporated per hour per mg of protein. (B) Mycelia were treated with EDTANa₂ or EDTANa₂ with additional cations. EDTANa₂ (0.15 mM) was saturated by adding Mg²⁺, Ca²⁺ or Mn²⁺ (0.45 mM) to SNA media. Data are represented as the means ± SD of three biological replicates (* indicates significant differences at *p* < 0.05 compared to CK).

3. Discussion

The genus *Fusarium*, which is pathogenic and toxic to plants and humans, is one of the most economically damaging fungal genera. *Fusarium* colonizes a wide range of environments, and control of *Fusarium* floral infections remains problematic. Some fungicides, including triazoles, benzimidazoles and strobilurins, are moderately effective, but reports of fungicide failure resulting from resistance have increased since the late 1960s [39,40]. To make matters worse, the development of new fungicides is much slower than the appearance of fungicide resistance in *Fusarium* populations [41,42]. Here, a
nonantibiotic drug, EDTANa₂, exhibited novel antifungal activity against F. graminearum and DON production. Through combined analyses of morphology, DON content and TRI gene expression detection, chitin detection, cell permeability, transmission electron microscopy and field experiments, we demonstrated that EDTANa₂ destroyed cell wall and cytomembrane integrity and inhibited TRI gene expression in F. graminearum, and the antifungal effect of EDTANa₂ relied on Mn²⁺-chelating abilities.

EDTA is considered as an ion chelator, and it has been recommended as an antimi-
crobial agent against bacteria and C. albicans [43–45]. The EC₅₀ value of EDTANa₂ for F. graminearum is 107.88 mg L⁻¹, which is higher than that of carbendazim (about 0.5 mg L⁻¹). The field test, however, shows that 70 g ha⁻¹ EDTANa₂ decreased the incidence of disease by 70% compared to 140 g ha⁻¹ carbendazim treatment. As DON is an important virulence factor in wheat, previous studies suggest that significant decreases in FHB incidence in field situations are possible with proper DON inhibited fungicide applications [46]. In our study, the DON production was significantly inhibited by EDTANa₂, which may increase the antifungal effects on FHB.

The fungal cell wall protects the cell against osmotic pressure and other environmental stresses and is considered the carbohydrate armor of the fungal cell [47]. After EDTANa₂ treatment, all layers across the cell wall were affected, and the chitin content was decreased in Fusarium. In addition, the membrane permeability increased significantly when Fusarium was cultured in medium containing EDTANa₂. This suggests that the fungal cells became more sensitive to changes in environmental stresses with EDTANa₂ treatment.

Our next question was which divalent cation was chelated by EDTANa₂ and then caused chitin content reduction and cell wall defects in Fusarium. There have been reports that calcium-binding agents inhibit Cryptococcus neoformans and C. albicans by disrupting the assembly of the polysaccharide capsule through Mg²⁺ and Ca²⁺ chelation [31,48,49]. However, the results in our paper showed that EDTANa₂ chelated Mn²⁺ and resulted in a reduction of chitin synthesis. In fungi, chitin is synthesized by chitin synthase, whose activities are known to depend upon the presence of a divalent cation [50,51]. Chitin synthases (CHSs) in Fusarium were previously classified into seven categories [52]. Different chitin synthases are distinct in their responses to the divalent cation, for example, Chs2 and Chs3 are stimulated, while Chs1 is inhibited by Co²⁺ in Saccharomyces cerevisiae [53]. On the basis of the data obtained in our study, it may be logical to assume that Mn²⁺ is essential for the main chitin synthase of Fusarium. EDTA had a fungistatic effect on F. fujikuroi growth, a pathogen causing bakanae disease, and its action was largely suppressed by Mn²⁺ and slightly by Ca²⁺ [54]. Combining with our findings, we can speculate that this kind of chelating agent, such as EDTA and EDTANa₂ inhibits Fusarium spp. mainly because of Mn²⁺ deficiency.

In summary, EDTANa₂ inhibits DON production and disrupts the cell wall and cell membrane functionality of Fusarium, an effect that appears to mainly result from Mn²⁺ chelation. The results of our study provided new material and candidate compound against Fusarium in crop protection.

4. Materials and Methods

4.1. Fungi, Plants, and Culture Conditions

Fusarium graminearum strains PH-1, F. asiaticum strain 2021, F. acuminatum, F. avenaceum, F. concentricum, F. culmorum, F. equiseti, F. fujikuroi, F. lateritium, F. oxysporum, F. proliferatum, F. solani and F. verticillioides (Figure S1, Table S2) used in this study were stored in our laboratory [55]. The wheat variety Huaimai33 was maintained in our laboratory. Carboxymethyl cellulose (CMC) broth [36] and SNA medium (0.1% KH₂PO₄, 0.1% KNO₃, 0.05% MgSO₄.7H₂O, 0.05% KCl, 0.02% glucose, and 0.02% sucrose) were used for conidia production and assessments of mycelial growth, respectively. EDTA (99%) and inorganic metal salts were purchased from Sigma-Aldrich (St. Louis, MO, USA).
4.2. Control Effect Measurement on Wheat Seedling Blight

The test was evaluated under controlled conditions using a completely randomized design with two replications for each treatment. Ten seedlings per treatment were inoculated on the fully expanded primary leaves 8 d after Huaimai33 planting. The EDTANa2 was sprayed on leaves at different concentration from 0.5 to 8 mM. After 24 h, leaves were punctured and inoculated with three microliter of macroconidia suspension (1 × 10^6 spores mL^-1). The lesions of diseased leaves were measured and photographed at 6th day post inoculation. Duncan’s multiple comparison test (SPSS20.0, IBM, Chicago, IL, USA) with a significant difference set as P < 0.05 was used to compare sample means. Mean values and standard deviations were reported. The differences between means with P less than 0.05 were considered statistically significant. The control effect was characterized by linear regression analysis (R = 0.94) using the SPSS statistical package.

4.3. Control Effect Measurement on Fusarium Head Blight and Phytotoxicity Field Test

The field study was conducted for two years (2018 and 2019) at the same location with different randomizations for each year (Table 1). Wheat (Triticum aestivum L. cv. Huaimai33) was grown on the experimental farm of Nanjing Agricultural University. At Zadok’s growth stage (ZGS) 65, while more than half of the wheat spikes were in bloom, field plots were arranged in a randomized block, which was designed with three 3 plots (each plot was 4 × 5 m). The treatments were as follows: (1) a control consisting of water; (2) 140 g ha^-1 carbendazim; (3) 7 g ha^-1 EDTANa2; (4) 70 g ha^-1 EDTANa2. 7–4000 g ha^-1 EDTANa2 were used for phytotoxicity assays. For floral spray inoculations, each plant was sprayed with 0.5 mL of 1 × 10^4 spores mL^-1 F. graminearum strain PH-1 conidia 24 h after EDTANa2 spray treatment. Pathogenicity assays were performed 14 or 21 days after EDTANa2 spray treatment as described previously [57]. The influence of EDTANa2 on wheat was tested by assessing browning spikelets. Thirty wheat heads were randomly selected to calculate the browning spikelets ratio for each concentration of EDTANa2. The browning spikelets ratio was defined as follows: browning spikelet (%) = browning spikelets/total spikelets. The experiment was replicated three times.

### Table 1. Climate conditions in the field study.

| Year | Experimental Date | Rainy Period | Temperature °C (°C) |
|------|-------------------|--------------|---------------------|
|      |                   |              | \(T_{av}\) | \(T_{max}\) | \(T_{min}\) |
| 2018 | 20 Apr.–4 May.    | 21 Apr.–23 Apr., 29 Apr.–1 May | 21 | 30 | 11 |
| 2019 | 20 Apr.–11 May.   | 21 Apr., 27 Apr. and 28 Apr. | 18 | 30 | 9 |

* Data represent the average of the daily mean (\(T_{av}\)), minimum (\(T_{min}\)) and maximum (\(T_{max}\)) temperatures recorded during each experimental period.

4.4. Mycelial Growth Inhibition by EDTANa2

For a fungicide-sensitivity assay in the laboratory, a three-day-old mycelial plug (5 mM in diameter) was placed in the center of a minimal medium (MM) (10 mM K2HPO4, 10 mM KH2PO4, 2.5 mM NaCl, 4 mM NH4NO3, 10 mM glucose) plate amended with EDTANa2 at 0, 0.1, 0.2, 0.4, 0.8, 1.6 mM (0, 37.2, 74.4, 148.8, 297.6, or 595.2 mg L^-1). After 4 d at 25 °C, the colony diameters in two perpendicular directions of each plate were measured and averaged. Each combination of strain and concentration was represented by three biological replicates. The median effective concentration (EC50) value was calculated with DPS software (version 7.0, DPS Inc., Cary, NC, USA).

4.5. Optical, Scanning Electron Microscopy and Transmission Electron Microscopy Observation

Morphological observation of mycelia was performed using an inverted Olympus IX71 microscope (Olympus Canada, Markham, ON, Canada). Images were captured and analyzed by Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, Maryland). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were
carried out using hyphae germinated from spores in SNA supplemented with 0.15 mM EDTANa₂ at 25 °C for 24 h. All the cultures were performed in triplicate. For SEM determination, mycelia were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) at 4 °C for 12 h. The samples were then washed with sodium phosphate buffer (0.1 M, pH 7.2) and treated with 1% osmium tetroxide in sodium phosphate buffer for 1 h, subjected to gradual dehydration in ethanol (70, 80, 90 and 100%), and dried to the critical point (CPD 030 Critical Point BALTEC Dryer, Leica Microsystems, Liechtenstein). After drying, the samples were glued on stubs using carbon tape and coated with gold (Sputter Coater BALTEC SDC 050, Leica Microsystems, Liechtenstein). For TEM determination, sections were prepared and visualized using a H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) as described by Song et al. [57].

4.6. Measurement of H₂O₂ and DON

H₂O₂ formation in the fungicide experiments was measured 4 h and 12 h after 0.4 mM or 0.8 mM EDTANa₂ treatment using a TMB (tri-methyl-benzidin) assay [58]. H₂O₂ formation was determined by measuring the absorbance at 620 nm in duplicate in each time point and in three independent experiments. In each experiment, a standard curve of pure H₂O₂ was added in a concentration range of 0.01 mM up to 100 mM. The H₂O₂ formed in the in vitro assay was calculated based on this standard curve.

DON production in TBI cultures was assayed with a competitive ELISA-based DON detection plate kit (Wise, Zhenjiang, China) according to previous studies [59]. Ten microliter of conidia (1.5 × 10⁷/mL) were inoculated in 30 mL TBI and cultured at 28 °C for 24 h in dark, and then 0.4 mM or 0.8 mM EDTANa₂ was added and cultured for additional 6 days. The experiment was repeated three times. To assay TRI gene expression, hyphae were harvested from 2-day-old TBI cultures (1 day after EDTANa₂ adding) and used for RNA isolation. qPCR was performed as previously describe (Table S3) [57]. The tubulin gene of *F. graminearum* was used as the internal control. The results were calculated with the data from three biological replicates.

4.7. Chitin Content, Chitin Synthase Activity and Cell Membrane Permeability Measurement

Macroconidia (10⁴ mL⁻¹) were cultured in SNA, SNA amended with 0.15 mM EDTA or SNA amended with 0.15 mM EDTANa₂ for 7 days and were used for chitin determination as previously described [57]. Fresh mycelium cultured in Czapek’s medium (3 g L⁻¹ of NaNO₃, 1.31 g L⁻¹ of K₂HPO₄, 0.5 g L⁻¹ of KCl, 0.5 g L⁻¹ of MgSO₄·7H₂O, 0.01 g L⁻¹ of FeSO₄·7H₂O, 30 g L⁻¹ of sucrose, pH 7.2) for 5 d were collected and finely ground with liquid nitrogen for chitin synthase extraction. Chitin synthase activity was extracted and measured according to Song et al. [57] with some modifications. Chitin synthase activity was measured by following (¹⁴C) GlcNAc incorporation into the filter-retainable polymer in the presence of 0–1 mM EDTANa₂ plus 5 mM Mg²⁺ or Mn²⁺. To measure the cell membrane permeability, macroconidia (final concentration was 10⁵ mL⁻¹) were inoculated into SNA, SNA with 0.3 mM NaCl, SNA with 0.15 mM EDTA or SNA with 0.15 mM EDTANa₂ at 25 °C for 7 days. The conductivity was measured with a conductometer (CONS10 Eutech/Oakton, Singapore) as described previously [60]. The conductivity of mycelia boiled for 5 min represented the final conductivity. The relative conductivity was calculated as follows: relative conductivity (%) = conductivity/final conductivity × 100. Three biological replicates were tested for each treatment.

4.8. Fungicidal Activity of EDTANa₄ against Fusarium spp.

Ten microliters of spores (10⁴ mL⁻¹) of *F. graminearum* PH-1 were plated in a 96-well flat bottom culture plate containing 0.15 mM EDTA, EDTANa₂, EDTANa₃ or EDTANa₄ in 100 µL of SNA medium (pH 4.2). The plates were cultured at 25 °C for 24 h and observed using an inverted microscope. The growth and morphology of mycelia were photographed and compared among treatments using NIS-Elements AR software (version 3.2, Nikon, Tokyo, Japan). The experiment was replicated three times.
The determination of the minimum inhibitory concentration (MIC) was performed using 96-well microtiter plates. The *Fusarium spp.* (listed in ‘Fungi, plants, and culture conditions’) fungal inoculated in 96-well microtiter plates were treated with EDTANa$_2$ at different concentrations and incubated for 24 or 36 h. The lowest concentration that demonstrated no visible growth was determined as the MIC. Measurements were repeated three times.

4.9. Effect of Metal Ions on EDTANa$_2$ Activity

To assess whether cations would ameliorate the inhibitory effects of EDTANa$_2$ on mycelial growth and chitin synthesis, an additional 0.3 mM MgCl$_2$, 0.3 mM CaCl$_2$, 0.3 mM MnCl$_2$ or 0.15 mM FeCl$_3$ was added into SNA medium separately to bind the preadded 0.15 mM EDTANa$_2$, followed by inoculation at 25 °C for 24 h and subsequent micro-examination. Corresponding amounts of MgCl$_2$, CaCl$_2$, MnCl$_2$ or FeCl$_3$ were used as controls. Subsequently, a series of MgCl$_2$, CaCl$_2$ or MnCl$_2$ concentrations (0, 0.15, 0.3, 0.45, 0.6, 1.2 mM) was added to the media to saturate the 0.15 mM EDTANa$_2$ to different degrees, and the fungi were cultured for 52 h to measure the mycelial biomass at OD$_{290}$. The mycelia that were treated with 0.3 mM cations were collected at 7 d, frozen and dried for chitin content and trace element measurements [61]. Three independent experiments were performed, and the average was calculated.

4.10. Statistical Analysis

Statistical analysis was performed using Duncan’s multiple comparison test (for multiple comparisons) and Student’s $t$-test, all at a significance level of 0.05.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6651/13/1/17/s1, Table S1: Minimum inhibitory concentrations of EDTANa$_2$; Table S2: Strains used in this study; Table S3: Primers used in TRI genes qPCR; Figure S1: Internal transcribed spacer (ITS) sequences alignment of *Fusarium* isolates used in this study. ITS sequences of different strains were amplified using primers ITS-F/ITS-R (ITS-F: TCCGTAGGTGAACCTGCGG, ITS-R: TCCTCCGCTTATATGC). Sequences alignment was performed using NCBI Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with default parameters.

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References

1. Ma, L.J.; Geiser, D.M.; Proctor, R.H.; Rooney, A.P.; O’Donnell, K.; Trail, F.; Gardiner, D.M.; Manners, J.M.; Kazan, K. *Fusarium* pathogenomics. *Annu. Rev. Microbiol.* 2013, 67, 399–416. [CrossRef] [PubMed]
2. Summerell, B.A. Resolving *Fusarium*: Current status of the genus. *Annu. Rev. Phytopathol.* 2019, 25, 323–339. [CrossRef] [PubMed]
3. Nelson, P.E.; Dignani, M.C.; Anaissie, E.J. Taxonomy, biology, and clinical aspects of *Fusarium* species. *Clin. Microbiol. Rev.* 1994, 7, 479–504. [CrossRef] [PubMed]
4. Bai, G.H.; Shaner, G. Management and resistance in wheat and barley to *Fusarium* head blight. *Annu. Rev. Phytopathol.* 2004, 42, 135–161. [CrossRef] [PubMed]
5. Gordon, T.R. *Fusarium oxysporum* and the *Fusarium* Wilt Syndrome. *Annu. Rev. Phytopathol.* 2017, 55, 23–39. [CrossRef] [PubMed]
6. Kazan, K.; Gardiner, D.M.; Manners, J.M. On the trail of a cereal killer: Recent advances in *Fusarium graminearum* pathogenomics and host resistance. *Mol. Plant. Pathol.* 2012, 13, 399–413. [CrossRef]
7. Loffler, M.; Kessel, B.; Ouzunova, M.; Miedaner, T. Population parameters for resistance to *Fusarium graminearum* and *Fusarium verticillioides* ear rot among large sets of early, mid-late and late maturing European maize (*Zea mays* L.) inbred lines. *Theor. Appl. Genet.* 2010, 120, 1053–1062. [CrossRef]
8. Xu, X.; Nicholson, P. Community ecology of fungal pathogens causing wheat head blight. *Annu. Rev. Phytopathol.* 2009, 47, 83–103. [CrossRef]
Toxins 2021, 13, 17

9. Edwards, J.E., Jr.; Bodey, G.P.; Bowden, R.A.; Buchner, T.; de Pauw, B.E.; Filler, S.G.; Ghannoum, M.A.; Glauser, M.; Herbrecht, R.; Kaufman, C.A.; et al. International conference for the development of a consensus on the management and prevention of severe candidal infections. Clin. Infect. Dis. 1997, 25, 43–59. [CrossRef]

10. Treikale, O.; Priekule, I.; Javoisha, B.; Lazareva, L. Fusarium head blight: Distribution in wheat in Latvia. Commun. Agric. Appl. Biol. Sci. 2010, 75, 627–634.

11. Pestka, J. Deoxyxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. Arch. Toxicol. 2010, 84, 663–679. [PubMed]

12. Woloshuk, C.P.; Shim, W.B. Aflatoxins, fumonisins, and trichothecces: A convergence of knowledge. FEMS Microbiol. Rev. 2013, 37, 94–109. [PubMed]

13. Brauer, E.K.; Balcerzak, M.; Rocheleau, H.; Leung, W.; Scherthanher, J.; Subramaniam, R.; Ouellet, T. Genome editing of a deoxynivalenol-induced transcription factor confers resistance to Fusarium graminearum in wheat. Mol. Plant Microbe Interact. 2020, 33, 553–560. [CrossRef] [PubMed]

14. Dill-Macky, R.; Jones, R.K. The effect of previous crop residues and tillage on Fusarium head blight of wheat. Plant Dis. 2000, 84, 71–76. [CrossRef]

15. McMullen, M.; Bergstrom, G.; De Wolf, E.; Dill-Macky, R.; Hershman, D.; Shaner, G.; Van Sanford, D. A unified effort to fight an enemy of wheat and barley: Fusarium head blight. Plant Dis. 2012, 96, 1712–1728. [CrossRef]

16. D'Angelo, D.L.; Bradley, C.A.; Ames, K.A.; Willyerd, K.T.; Madden, L.V.; Paul, P.A. Efficacy of fungicide applications during and after anthesis against Fusarium head blight and deoxynivalenol in soft red winter wheat. Plant Dis. 2014, 98, 1387–1397. [CrossRef]

17. Zhang, Y.J.; Yu, J.J.; Zhang, Y.N.; Zhang, X.; Cheng, C.J.; Wang, J.X.; Hollomon, D.W.; Fan, P.S.; Zhou, M.G. Effect of carbendazim resistance on trichothecene production and aggressiveness of Fusarium graminearum. Mol. Plant Microbe Interact. 2009, 22, 1143–1150. [CrossRef]

18. Qiu, J.; Shi, J. Genetic relationships, carbendazim sensitivity and mycotoxin production of the Fusarium graminearum populations from maize, wheat and rice in eastern China. Toxins 2014, 6, 2291–2309. [CrossRef]

19. Chen, C.J.; Wang, J.X.; Luo, Q.Q.; Yuan, S.K.; Zhou, M.G. Characterization and fitness of carbendazim-resistant strains of Fusarium graminearum (wheat scab). Pest. Manag. Sci. 2007, 63, 1201–1207. [CrossRef]

20. Chen, Y.; Chen, C.; Wang, J.; Jin, L.; Zhou, M. Genetic study on JS399-19 resistance in hyphal fusion of Fusarium graminearum by using nitrate nonutilizing mutants as genetic markers. J. Genet. Genom. 2007, 34, 469–476. [CrossRef]

21. Mikaberidze, A.; Paveley, N.; Bonhoeffer, S.; van den Bosch, F. Emergence of resistance to fungicides: The role of fungicide dose. Phytopathology 2017, 107, 545–560. [CrossRef] [PubMed]

22. Al-Hatmi, A.M.; Meis, J.F.; de Hoog, G.S. Fusarium: Molecular diversity and intrinsic drug resistance. PLoS Pathog. 2016, 12, e1005464. [CrossRef] [PubMed]

23. Cordova-Alcantara, I.M.; Venegas-Cortes, D.L.; Martinez-Rivera, M.A.; Perez, N.O.; Rodriguez-Tovar, A.V. Biofilm characterization of Fusarium solani keratitis isolate: Increased resistance to antifungals and UV light. J. Microbiol. 2019, 57, 485–497. [CrossRef] [PubMed]

24. Muregi, F.W. Antimalarial drugs and their useful therapeutic lives: Rational drug design lessons from pleiotropic action of quinolines and artemisins. Curr. Drug Discov. Technol. 2010, 7, 280–316. [CrossRef] [PubMed]

25. Deising, H.B.; Reimann, S.; Pascholati, S.F. Mechanisms and significance of fungicide resistance. Braz. J. Microbiol. 2008, 39, 286–295. [CrossRef] [PubMed]

26. Al-Bakri, A.G.; Othman, G.; Bustanji, Y. The assessment of the antibacterial and antifungal activities of aspirin, EDTA and aspirin-EDTA combination and their effectiveness as antibiofilm agents. J. Appl. Microbiol. 2009, 107, 280–286. [CrossRef]

27. Taha, M.O.; Al-Bakri, A.G.; Zalloum, W.A. Discovery of potent inhibitors of pseudomonal quorum sensing via phosphochromophore modeling and in silico screening. ACS Med. Chem. Lett. 2006, 16, 5902–5906. [CrossRef]

28. Cikes, M.; Vranjesic, B.; Tomic, M.; Jamnickyi, O. A successful treatment of formation damage caused by high-density brine. Spec. Prod. Eng. 1990, 5, 175–179. [CrossRef]

29. Crabtree, M.; Eslinger, D.; Fletcher, P.; Miller, M.; King, G. Fighting scale-destruction and prevention. Oilfield Rev. 1999, 11, 30–45.

30. Banin, E.; Brady, K.M.; Greenberg, E.P. Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a biofilm. Appl. Environ. Microbiol. 2006, 72, 2064–2069. [CrossRef]

31. Gil, M.L.; Casanova, M.; Martinez, J.P. Changes in the cell wall glycoprotein composition of Candida albicans associated to the inhibition of germ tube formation by EDTA. Arch. Microbiol. 1994, 161, 489–494. [CrossRef] [PubMed]

32. Gray, G.W.; Wilkinson, S.G. The effect of ethylenediaminetetra-acetic acid on the cell walls of some gram-negative bacteria. J. Gen. Microbiol. 1965, 39, 385–399. [CrossRef] [PubMed]

33. Ramage, G.; Wickes, B.L.; Lopez-Ribot, J.L. Inhibition of Candida albicans biofilm formation using divalent cation chelators (EDTA). Mycopathologia 2007, 164, 301–306. [CrossRef] [PubMed]

34. Saadat, M.; Roudbarmohammadi, S.; Yadegari, M.; Khavarinejad, R. Evaluation of antibacterial effects of catechin and EDTA on planktonic and biofilm cells of Pseudomonas aeruginosa. Malays. J. Microbiol. 2013, 9, 184–188. [CrossRef]

35. Yakandawala, N.; Gawande, P.V.; LoVetri, K.; Madhyastha, S. Effect of ovo transferrin, proamine sulfate and EDTA combination on biofilm formation by catheter-associated bacteria. J. Appl. Microbiol. 2010, 102, 722–727. [CrossRef]
36. Chauhan, A.A. Full and broad-spectrum in vivo eradication of catheter-associated biofilms using gentamicin-EDTA antibiotic lock therapy. *Antimicrob. Agents Chemother.* 2012, 56, 6310–6318. [CrossRef]
37. Shaikh, A.I.; Musaddig, M. Antibiotic-EDTA combination induced dispersal of *Pseudomonas aeruginosa* biofilm. *J. Pure Appl. Microbiol.* 2012, 6, 363–367.
38. Bartrnicki-Garcia, S. Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annu. Rev. Microbiol.* 1968, 22, 87–108. [CrossRef]
39. Brent, K.J. *Fungicide Resistance in Crop Pathogens: How Can It Be Managed?* 2nd ed.; Fungicide Resistance Action Committee: Basel, Switzerland, 1995; Available online: http://www.frac.info (accessed on 27 December 2020).
40. Dekker, J. Prospects for the use of systemic fungicides in view of the resistance problem. *Labi. Phytopathol.* 1976, 3, 60–66.
41. Grimmer, M.K.; van den Bosch, F.; Powers, S.J.; Paveley, N.D. Evaluation of a matrix to calculate fungicide resistance risk. *Pest Manag. Sci.* 2014, 70, 1008–1016. [CrossRef] [PubMed]
42. van den Bosch, F.; Gilligan, C.A. Models of fungicide resistance dynamics. *Annu. Rev. Phytopathol.* 2008, 46, 123–147. [CrossRef] [PubMed]
43. Ates, M.; Akdeniz, B.G.; Sen, B.H. The effect of calcium chelating or binding agents on *Candida albicans*. *Oral Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod.* 2005, 100, 626–630. [CrossRef] [PubMed]
44. Ding, Q.; Alborzi, S.; Bastarrachea, L.J.; Tikekar, R.V. Novel sanitization approach based on synergistic action of UV-A light and benzoic acid: Inactivation mechanism and a potential application in washing fresh produce. *Food Microbiol.* 2018, 72, 39–54. [CrossRef] [PubMed]
45. Lai, Y.-W.; Campbell, L.T.; Wilkins, M.R.; Pang, C.N.I.; Chen, S.; Carter, D.A. Synergy and antagonism between iron chelators and antifungal drugs in Cryptococcus. *Int. J. Antimicrob. Agents* 2016, 48, 388–394. [CrossRef] [PubMed]
46. Mesterhazy, A.; Toth, B.; Varga, M.; Bartok, T.; Szabo-Hever, A.; Farady, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins* 2011, 3, 1453–1483. [CrossRef]
47. Latge, J.P. The cell wall: A carbohydrate armour for the fungal cell. *Mol. Microbiol.* 1997, 25, 219–228. [CrossRef] [PubMed]
48. Holmes, A.R.; Cannon, R.D.; Shepherd, M.G. Effect of calcium ion uptake on *Candida albicans* morphology. *FEMS Microbiol. Lett.* 2010, 77, 187–194. [CrossRef]
49. Robertson, E.J.; Wolf, J.M.; Casadevall, A. EDTA inhibits biofilm formation, extracellular vesicular secretion, and shedding of the capsular polysaccharide glucuronoxylomannan by Cryptococcus neoformans. *Appl. Environ. Microbiol.* 2012, 78, 7977–7984. [CrossRef]
50. Choi, W.J.; Cabib, E. The use of divalent cations and pH for the determination of specific yeast chitin synthetases. *Anal. Biochem.* 1994, 219, 368. [CrossRef]
51. Choi, W.J.; S Burlati, A.; Cabib, E. Chitin synthase 3 from yeast has zymogenic properties that depend on both the CAL1 and the CAL3 genes. *Proc. Natl. Acad. Sci. USA* 1994, 91, 4727–4730. [CrossRef] [PubMed]
52. Cheng, W.; Song, X.S.; Li, H.P.; Cao, L.H.; Sun, K.; Qiu, X.L.; Xu, Y.B.; Yang, P.; Huang, T.; Zhang, J.B.; et al. Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to Fusarium head blight and seedling blight in wheat. *Plant Biotechnol. J.* 2015, 13, 1335–1345. [CrossRef] [PubMed]
53. Orlean, P. Two chitin synthases in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 1987, 262, 5732–5739. [PubMed]
54. Kim, S.W.; Park, J.K.; Lee, C.H.; Hahn, B.S.; Koo, J.C. Comparison of the antimicrobial properties of chitosan oligosaccharides (COS) and EDTA against *Fusarium fujikuroi* causing rice banae disease. *Curr. Microbiol.* 2016, 72, 496–502. [CrossRef]
55. Chen, Y.; Zhou, M.G. Characterization of Fusarium graminearum isolates resistant to both carbendazim and a new fungicide J399-19. *Phytopathology* 2009, 99, 441–446. [CrossRef]
56. Duvick, J.P.; Rood, T.; Rao, A.G.; Marshak, D.R. Purification and characterization of a novel antimicrobial peptide from maize (*Zea mays*) kernels. *J. Biol. Chem.* 1992, 267, 18814–18820. [PubMed]
57. Song, X.S.; Li, H.P.; Zhang, J.B.; Song, B.; Huang, T.; Du, X.M.; Gong, A.D.; Liu, Y.K.; Feng, Y.N.; Agboola, R.S.; et al. Trehalose 6-phosphate phosphatase is required for development, virulence and mycotoxin biosynthesis apart from trehalose biosynthesis in *Fusarium graminearum*. *Fungal. Genet. Biol.* 2014, 63, 24–41. [CrossRef]
58. Audenaert, K.; Callewaert, E.; Hofte, M.; De Saeger, S.; Haesaert, G. Hydrogen peroxide induced by the fungicide prothiocanazole triggers deoxynivalenol (DON) production by *Fusarium graminearum*. *BMC Microbiol.* 2010, 10, 112. [CrossRef]
59. Zhou, Z.; Duan, Y.; Zhou, M. Carbendazim-resistance associated β2-tubulin substitutions increase deoxynivalenol biosynthesis by reducing the interaction between β2-tubulin and IDH3 in *Fusarium graminearum*. *Environ. Microbiol.* 2020, 22, 598–614. [CrossRef]
60. Xu, C.; Hou, Y.; Wang, J.; Yang, G.; Liang, X.; Zhou, M. Activity of a novel strobilurin fungicide benzothiostrobin against *Sclerotinia sclerotiorum*. *Pestic Biochem. Physiol.* 2014, 115, 32–38. [CrossRef]
61. Valentin, J.L.; Watling, R.J. Provenance establishment of coffee using solution ICP-MS and ICP-AES. *Food Chem.* 2013, 141, 98–104. [CrossRef] [PubMed]