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The effects of EDDHA and Ca on suppression of *Fusarium solani* and growth improvement of hopbush (*Dodonaea viscosa*)

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Abstract: The effect of various soil pH (6 and 8) and iron chelating agent (EDDHA) concentrations (0, 10 and 20 mmol/L) on disease severity of *Fusarium solani*-infested plants was determined. EDDHA significantly ($p<0.05$) reduced disease severity by *F. solani* and increased other evaluated characteristics of root at both pH levels. Also, *F. solani*-infested plants grown at pH= 6 and different EDDHA concentrations showed more disease severity and poorer growth characteristics compared with plants grown at pH=8. In other experiments, growth of non-infested plants at pH=6 and different levels of EDDHA was better than that at pH=8. In fact, a soil with pH=6 has a bio-availability of iron and other elements for absorption for both plant and *F.solani* and thus, disease severity will be higher. But, if the soil is not infested, growing conditions of plant will be better. The study on the effects of calcium and EDDHA in infested and non-infested soils at pH=6 showed that when both EDDHA and Ca were applied together, maximum decrease in disease severity (only 6.6%) as well as highest growth characteristics were obtained simultaneously. Greatest disease severity and lowest growth characteristics were observed in the absence of EDDHA and Ca compared to other treatments**.** The results of the effect of EDDHA concentrations and Ca at soil pH=8 showed that the growth characteristics increased and disease severity was reduced significantly.

Keywords: *Dodonaea viscosa*, pH, Fe, Disease severity, Growth characteristics.

Introduction

Dodonaea viscosa (L). Jacq., (Family: *Sapindaceae*) is an evergreen shrub abundantly growing in tropical and subtropical regions, it used as an ornamental hedge plant and has valuable medicinal properties. This plant was used by indigenous people as a traditional medicine for the treatment of different ailments. The infusion of leaves was used to treat rheumatism, skin infections, gout, hemorrhoids, fractures and snake bites. Experimental studies have reported that the plant possesses local anesthetic and smooth muscle relaxant, anti-diabetic, anti-ulcer, anti-inflammatory and anti-microbial activities [1, 2]. Its cultivation in Khuzestan province (Iran) is facing some difficulties including root and crown rot diseases caused by different soil borne pathogens. Studies have revealed that the major causal agent of disease in this province is *Fusarium solani* (Figure 1), although *Pythium aphanidermatum* and *Pythium ultimum* have also been reported [3].

Obviously, host, pathogen and environmental conditions (disease triangle) are the three factors needed for disease incidence. Therefore, a change in any of them may affect severity and incidence of the disease. In most cases, changes in environmental conditions such as pH or unavailability of elements required for germination of propagules which can create unfavorable conditions for pathogens [4- 8].

One of the limiting factors for plant growth and other organisms is iron deficiency. Although the quantity of iron in soils is high, its availability is generally low; therefore iron deficiency is a common problem. Most of iron on earth is in the form of Fe^{3+}, but the Fe^{2+} form is physiologically more important for organisms. The solubility of Fe^{2+} is relatively high, but it is easily oxidized to Fe^{3+}, which is finally precipitated. In alkaline and calcareous soils, Fe^{3+} is insoluble, making iron unavailable to organisms. Most organisms use various iron uptake mechanisms such as chelation, other organisms release compounds called siderophores with the ability to bind to iron and enhance its solubility. Furthermore, the release of protons (H^+) is another mechanism, which may reduce pH levels [8]. Therefore, unavailability of iron by using chemical iron-binding

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agents can cause induction of soil suppressiveness to pathogens due to iron deficiency [4-7].

Many studies have been done on the effects of chelating agents on plant nutrition and other aspects of agriculture. The effect of foliar application of synthesized zinc-amino acid chelates and zinc sulfate on the yield and grain nutritional quality of wheat was evaluated by Ghasemi et al and the results showed that zinc-amino acid chelate significantly increased grain yield, grain Zn and Fe concentrations and grain protein content [9]. Scher and Baker reported that EDDHA (ethylene diamine-N, N'-bis (2-hydroxyphenylacetic acid)), one of the strongest iron chelating agent that had been used in different aspects, when added to the soil reduced germination of chlamydospores of seven special forms of *F. oxysporum* (*vasinfectum*, *dianthi*, *conglutinans*, *melonis*, *lini*, *cucumerinum* and *pisi*) from 70 to 36 % [7]. The study of the effect of iron availability on the induction of systemic resistance to Fusarium wilt of chickpea by the application of Pseudomonas spp. and EDDHA showed disease control and induced systemic resistance (ISR) [10]. Jones and Woltz studied the effect of soil pH and micronutrients on *Fusarium* wilt of tomato [11]. Their results indicated that raising pH to 7 or 7.5 decreased *Fusarium* wilt incidence and severity. Farokhi Nejad found that when pea plants were grown in soil infested with *Fusarium* at 610 CFU/g of soil, disease incidence of control plants was 47.96%, in contrast to plants treated with calcium chloride which was 16.41% [4].

Since the use of chemical pesticides to control plant diseases causes environmental pollution and other problems. We aimed to study the influence of synthesized chelating agents (EDDHA) and calcium on root and crown rot disease severity caused by *F. solani* (the major pathogen of this disease in the province) at acid and alkaline pH. Also, hopbush growth characteristics in *F. solani*-infested and non-infested soils were investigated.

Materials and methods

Sampling, isolation and purification of fungi

Infected plants that showed yellowing and declining symptoms were collected from some areas of the Khuzestan province during 2013. We isolated fungi associated with root and crown rot after surface sterilization. Four tissue sample pieces from root and crown (5mm length) were surface sterilized with 1% and 5% sodium hypochlorite for 1 min and 30 second respectively and washed two times in sterile distilled water. In order to isolate causal agent of disease, these pieces were transferred onto Potato Dextrose Agar (PDA) and WA (Water Agar). Also, *Fusarium* spp. were isolated from infected crown and root by Nash & Snyder medium. Isolates were then purified using single spore and hyphal tip methods.

In order to identify *Fusarium* species, fungi were transferred onto Carnation-Leaf Agar (CLA), incubated 12 h photoperiod at 20/25 C° night/day temperature cycle. The important characteristics used in the identification of *Fusarium* species were: shape of the macroconidium; presence or absence of microconidia; shape and mode of formation of microconidia; nature of the conidiogenous cell bearing microconidia; presence or absence of chlamydospores and colony morphology on PDA, using *Fusarium* key [12].

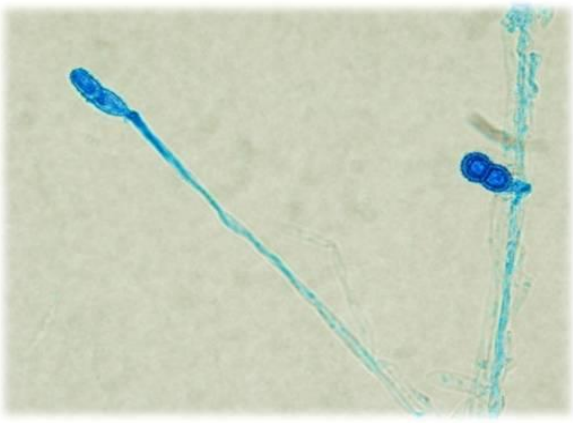

Figure 1. Phialide and chlamydospore of *F.solani*

Preparation of alkaline soil at pH=8

Alkaline soil used in this investigation was a sandy loam with the following characteristics: pH=8, Ec=6 ds/m, Ca=14 meq/L, Mg=10 meq/L. Alkaloid soil was taken from agricultural land of Chamran University campus (Ahwaz). For sterilization, soil samples were autoclaved twice at 121°C and 15 psi before usage.

Preparation of acidic soil at pH=6

For the preparation of acidic soil (pH=6) from alkaline soil (PH= 8), the soil was acidified by adding 0.1 M HCl. Due to the high calcium carbonate content of alkaine soil, the buffering capacity of the soil is high, so it is necessary to use acid to neutralize calcium carbonate and pH. In order to prevent pH fluctuations, plants were irrigated every day with water adjusted to pH=6.

Measurement of calcium

Calcium measurements were carried out using EDTA, based on complex formation with the metal ions [13].

Study of the effect of EDDHA

Two concentrations of EDDHA (10 and 20 milli moles per liter) (Serva, USA) were added to both soils prepared in the previous steps (pH=8 and pH=6) after planting seedlings in pots. This chelating agent was added before and after inoculation with *F. solani* during the growth period of plants.

Study of the effect of calcium

The addition of acid for pH adjustment led to increased solubility of calcium carbonate and released calcium ions into soil. So after determining the amounts of calcium in both soils (14 meq/L and 26 meq/L (pH=8 and pH=6) respectively), we adjusted them by adding 12 meq/L calcium to the pH= 8 soil. In order to equate calcium level, 876 mg/l calcium chloride ($2H_2O$. $CaCl_2$) 1 molar was used. Finally, two soils with different pH and equal calcium contents were obtained.

Preparation of inoculum and inoculation

One flask was filled with 250 g of moistened wheat seeds and autoclaved twice. A plug of one week old *F. solani* in PDA culture (5mm in diameter of medium) was put in a flask and incubated for two weeks, shaken occasionally by hand. Six months old hopbush seedlings were planted in pots containing 985g of sterilized soil and were inoculated with 15g of *Fusarium* inoculum. Seventy days after inoculation, the characteristics parameters were studied.

Statistical analysis

This investigation was conducted in a greenhouse using factorial experiments (2×3) in a completely randomized design with three replications. Four variables were studied including: fresh and dry weights of shoots, fresh weight of roots and disease severity. In the experiments on the effect of pH and EDDHA concentrations in infested and non-infested soils, factor 1 consisted of two values of pH (6 and 8) and factor 2 consisted of three concentrations of EDDHA chelating agent (0, 10 and 20 mmol/L). Experiments on the effect of EDDHA and Ca at pH=6 were conducted with inoculated and non-inoculated pH=6 soil, factor 1 consisted of two concentrations of calcium (0, 12 meq/L) and factor 2 consisted of three concentrations of EDDHA chelating agent (0, 10 and 20 mmol/L).

The same experiments were conducted at pH=8 in inoculated and non inoculated soils and factors were same as for soil at pH=6. Disease severity was assessed using the modified method of Kim et al. as follows [13], 0% of severity = Healthy plant and no lesion evident, 20% = roots with surface brown lesion and no cankers, 40% ≤ 20% roots with typical cankers, 60% ≤ 20% roots with typical brown cankers, 80% ≤ 40% roots with typical brown cankers, 100% = death of plant.

Statistical analyses were performed using the SAS, Sigma-Plot (version 16) SPSS Inc., and the graphs were plotted by Excel 2013.

Results

Study of isolated fungi

Sixty-three fungal isolates including: *Fusarium solani* (55), *F. equiseti* (5) and *Lasiodiplodia hormozganensis* (3) were obtained. Based on their morphological characteristics, *Fusarium* isolates were identified as *F. solani* and *F. equiseti*. Current study revealed that the major pathogen of this disease is *Fusarium solani* in this province. *F. solani* and *F. equiseti* were isolated by other researchers in the Khuzestan province [3]. *L. hormozganensis* has not been reported on hopbush previously.

Morphological characteristics of *Fusarium solani*

Macroconidia were formed abundantly in cream sporodochia on CLA (Carnation Leaf Agar) medium. Macroconidia were wide, curved and 3-4 septate. The macroconidia were formed from monophialid on branched conidiophore. Also on this medium, 1-2 cells microconidia were formed abundantly in false-heads on very long monophialid. The chlamidydospores were formed abundantly on the CLA culture. This specie produced white to cream, floccose mycelium on PDA (Potato dextrose agar) medium. Characteristics of *F. solani* in our study were consistent with Burgess et al. Key [11].

Effect of pH and EDDHA

Figures 2 and 3 show that, at both pH, addition of 10 and 20 mmol/L of iron chelating agent EDDHA significantly ($p<0.05$) reduced the severity of *Fusarium* crown and root rot and increased fresh and dry weight of shoots and fresh weight of roots compared to control. Also, no significant difference ($p<0.05$) was detected in disease severity and other characteristics, between 10 and 20 mmol/L EDDHA at both pH. Significant differences ($p<0.05$) in disease severity and other characteristics were detected between pH=6 and pH=8 for any EDDHA concentrations, such that all concentrations of EDDHA at pH=8 greatly reduced disease severity and increased fresh and dry weights of shoot and fresh weight of root when compared to the same characteristics at pH=6. Also, control plants (EDDHA=0) grown at pH= 8 had significantly ($p<0.05$) less severe disease symptoms (66.7%) and more fresh and dry weight of shoot and fresh root than those at pH=6 (disease severity at pH=6 is 93.3%). Although 10 and 20 mmol/L EDDHA at both pH, showed no significant difference yet lowest disease severity and highest fresh and dry weights of shoots and fresh weight of roots were obtained in 20 mmol/L EDDHA at pH= 8 (6.7%).

Figure 2. Inoculated and non-inoculated plants treated with different concentrations of EDDHA. (-) = No inoculation, (+) = Inoculation, E= EDDHA, P: pH

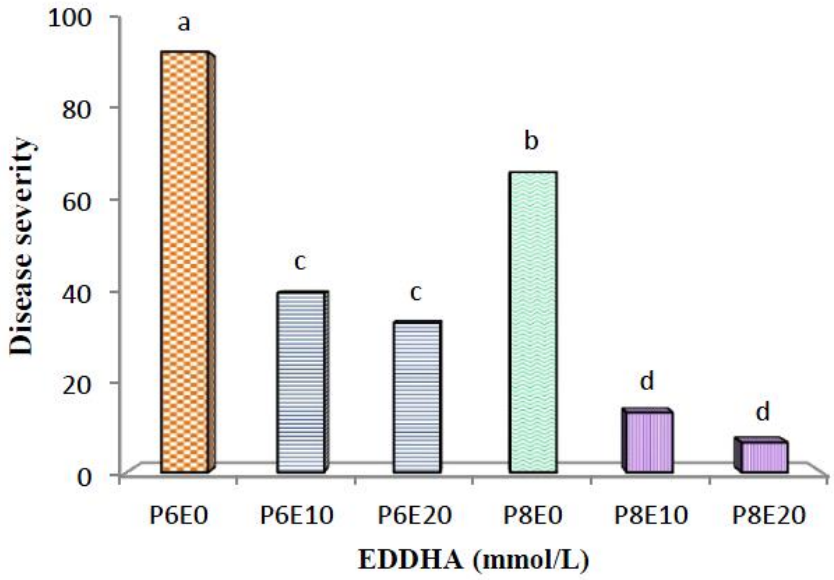

Figure 3. Comparison of mean disease severity in different EDDHA concentration at both pH (Columns followed by different letter are significantly different $P < 0.05$). E: EDDHA, P: pH

Non inoculated plants

No damping-off or rot symptoms were observed on any of non inoculated plants at both pH. Non inoculated plants grown with 0, 10 and 20 mmol/L EDDHA at pH=6 had greater fresh and dry weights of shoots and fresh weight of roots than plants grown at equal concentrations of EDDHA at pH=8. Moreover, no visual difference was detected in growth characteristics between 10 and 20 mmol/L EDDHA at both pH. The highest fresh and dry weights of shoot and fresh weight of root was obtained at pH=6. There was significant difference between no EDDHA and10 or 20 mmol/L EDDHA.

Effects of calcium and EDDHA at pH= 6

Inoculated plants

The results revealed that treatments of Ca + EDDHA (10, 20 mmol/L) had the lowest disease severity (6.6%) and highest fresh and dry weights of shoot and fresh weight of root compared with other

treatments (Fig. 4). Also, results revealed no significant difference among the effects of 10 and 20 mmol/L EDDHA with Ca (12 meq/L). Moreover, we found that there were significant differences among the effects of different levels of EDDHA (10 and 20 mmol/L) and Ca (12 meq/L) compared with non-application of chelating agent and calcium.

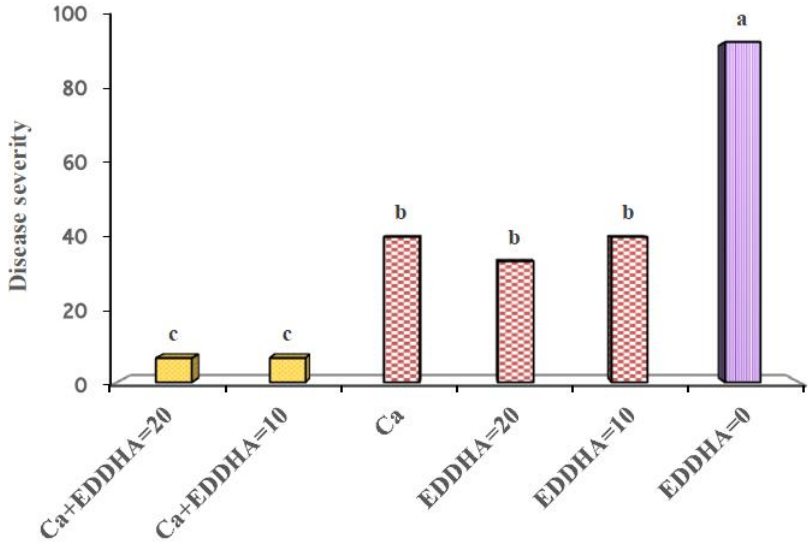

Figure 4. Comparison of mean disease severity in different EDDHA concentrations and Ca at pH=6 (Columns followed by different letter are significantly different $P < 0.05$).

Non inoculated plants

Experiment on the effect of Ca and different levels of EDDHA at pH=6 indicated that the highest fresh and dry weights of shoots and fresh weight of roots belonged to Ca+20 mmol/L and Ca+10 mmol/L EDDHA, although there were no significant differences between Ca+20 mmol/Land Ca+10 mmol/L EDDHA treatments. There was significant difference between both Ca+20 mmol/L and Ca+10 mmol/L EDDHA and the other treatments.

Effect of calcium and EDDHA at pH= 8

Inoculated plants

Study on the effect of Ca and EDDHA on disease severity and other characteristics of infected-plants at pH=8 has shown that Ca, 10 and 20 mmol/L EDDHA and EDDHA+Ca significantly increased all of the growth characteristics (Fig. 5), but there were no differences among Ca , 10 and 20 mmol/L EDDHA. On the other hand, inoculated-plants grown with EDDHA+Ca had the highest increase in growth characteristics comparing with other treatments. Unlike pH=6, according to the above assessment method, 6.6% rate of symptoms was observed on plants at any EDDHA concentration with Ca. (Fig. 5). So, addition of Ca and two levels of EDDHA (10 and 20 mmol/L) alone and together (Ca+10 mmol/L and Ca+20 mmol/L EDDHA) significantly decreased the disease severity.

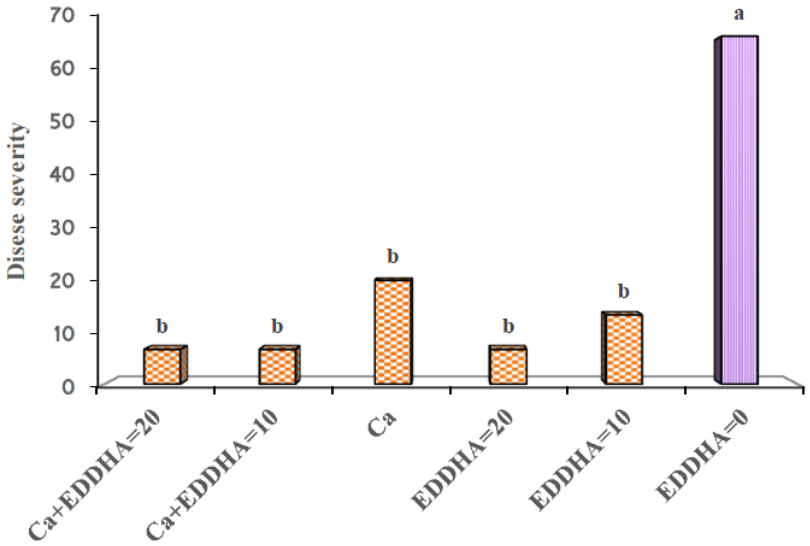

Figure 5. Comparison of mean disease severity in different EDDHA concentrations and Ca at pH= 8 (Columns followed by the different letter are significantly different at $P < 0.05$).

Non inoculated plants

In the experiment on the effects of Ca and different concentrations of EDDHA on the growth characteristics at pH=8, we had the same results obtained at pH=6. So, the plants grown in calcium+EDDHA, were distinguishable from the other plants as evaluated by all of the growth characteristics, and no different was detected between 10, 20 mmol/L EDDHA and Ca (alone or together with EDDHA) in any of the characteristics**.**

Discussion

Chelating agents are complex compounds containing functional groups associated with free electron pairs which are able to create multiple bonds with metal ions (particularly transition metal elements) and stabilize metal ions and protect them from oxidation and precipitation. Synthesized chelate holds ions in different strengths at different pH levels (4-9) and therefore provides the ions for the plants when needed. Chelating agents, cause increased solubility of elements in the rhizosphere solution, therefore when competition for ions in the soil solution is high (e.g. in relatively saline soils and calcareous soils where sodium and calcium levels are high) the plant is not able to absorb zinc or iron. In these circumstances, chelating iron or zinc fertilizers can provide the required amounts of iron and zinc for plants. On the other hand, in alkaline soils, reduction of pH levels in order to increase the solubility of ions is difficult, using more stable iron chelates, such as EDDHA is advised [15].

In this study, we found that in *Fusarium*-infested soils there were significant differences between the two pH levels for all characteristics since plants grown at pH=8 showed significantly ($p<0.05$) less disease severity (66.7%) than plants grown at pH=6 with (99.3%). These results were consistent with the results of Jones et al. [16] and Woltz et al. [17]. Michael [5] also revealed that *Fusarium* crown and root rot severity of tomato at pH=8 was significantly less than at pH=6.

Many researchers suggested, competition for available Fe is implicated in the mechanism(s) associated with *Fusarium*-suppressiveness which finally reduces the *Fusarium* host colonization efficiency [18]. Farokhi Nejad also identified that induction of soil suppressiveness to *Fusarium* wilt disease of pea caused by chelating agent application, resulted from ferric ions competition for ferric ions [4]. In order to uptake Fe, at Fe- efficiency, pathogenic Fusaria produce siderophores. According to Scher and Baker's study if chelating agents were added to soil, only those microorganisms with higher stability constant than the ligands applied can use Fe [6]. The ligand EDDHA apparently has a higher stability constant than the siderophore produced by the *Fusarium* pathogen. The estimated stability constant for the iron complex ($\log_{10}$ K=29**)** of the Fusarinine siderophores (these siderophores are of Hydroxamate class) produced by *Fusarium* species is lower than EDDHA chaletor ($\log_{10}$ K=33.9). Therefore it may be easy to understand why Fe^{3+} is a limiting factor for the *Fusarium* [6]. Reduced pathogenicity of *Fusarium* in this investigation due to the application of chelate may be related to lower iron-binding ability of *Fusarium*. We hypothesize that Fe competition phenomenon is the mechanism contributing to suppressiveness of *Fusarium* root and crown rot by *Dodonea viscosa* containing-soil. This result has been presented by many researchers [4, 6].

The principal factor mediating Fe availability is soil pH, thus the more alkaline is the soil, the less Fe^{3+} would be available. Therefore, the pH can have significant influence in determining whether Fe^{3+} is limiting factor for the pathogen which ultimately, affects the disease severity [8]. Addition of EDDHA to *Fusarium* infested soils caused reduction in disease severity and increased the fresh and dry weights of shoots and fresh weight of roots (Figs.2, 3, 4 and 5). The results of this study are consistent with those of Farokhi Nejad that showed that the addition of Fe-EDDHA decreased significantly the severity of pea wilt at pH=6.5 and pH=8 [4]. Scher and Baker also added EDDHA and Fe-EDDHA to *F.oxysporum* f.sp. *lini*–infested soil (both with higher stability constants than *Fusarium* siderophores) and achieved same results [6]. Michael also showed that application of EDDHA at pH=6, reduced the severity of *Fusarium* root and crown rot of tomatoes [5].
According to the results of this study, there was no significant difference between 10 and 20 mmol/L EDDHA at both pH (Fig. 3). Scher and Baker also added Fe-EDDHA to *F. oxysporum* f.sp. *lini* and *F. oxysporum* f.sp. *conglutinans*-infested soils at 50µg/g and 100 µg/g and observed that diseases reduction with 50 µg/g was not significantly different from that of 100µg/g in either system [6]. On the other hand, study of the influence of Fe-EDDHA on crown and root rot disease of tomato had cleared that there were no significant differences among 0, 20, 50, 100 and 200 µL of EDDHA at pH=6.5 and pH=8 [5]. Active ions such as H^{+}, Al^{+3} and Fe^{+3} in acidic soils, preclude the presence of calcium ions in the soil (Washing of alkali cations in acid soils). But, in the alkaline soils of arid and semi-arid regions, if sufficient water for solubility of calcium is present, enough calcium will be available in the soils. The amount of free calcium ions is low for two reasons: competition with many of Na ions and formation of carbonate compounds such as calcium carbonate, dolomite, and calcium phosphates. Thus in such soils using plenty of water or reducing the pH soil the solubility of free calcium ions can increased [19].

Some plant pathologists have tried to explain calcium-mediated disease suppression with the hypothesis that calcification of pectate leads to concomitant reduction of pathogen's ability to produce enzymes involved in cell wall degradation.

According to this hypothesis, presence of high amount of calcium in the plants, inhibits the polygalacturonase activity and finally negatively affects pathogenicity [4].

deficiency occurred before infection and was followed by normal level of calcium, disease development was retarded. He determined that presence of low amounts of calcium in the vascular sap enhanced pathogen growth in the host. The author stated that calcium inhibited polygalacturonase activity that is necessary for *Fusarium* pathogenicity. Keane and Sackston studied the effect of calcium nutrition on *Fusarium* wilt of flax and reported similar results to those of Corden on the effect of calcium on disease development [20,21].

Furthermore, they found that with low calcium, disease was more severe, especially when calcium deficiency occurred after inoculation. The results of this study agree with Corden and Keane and Sackston and confirm their results [20,21]. So, in the current study, when soil was amended with calcium chloride (alone or in combination with iron chelate), results revealed significant improvement in growth and reduction in disease severity. This study also demonstrated that best plant growth characteristics and least disease severity were obtained when calcium and chelate were applied together. Indeed, we hypothesize that this was attributable to competition for available Fe in soil (by application of EDDHA, iron is unavailable to *Fusarium* Hydroxamate siderophores) and concomitantly calcification of pectate and increases calcium bonding of the pectic substances in the plant cell walls (that causes decrease in cell wall plasticity and inhibition of polygalacturonase enzymes activity). Our results were consistent with Farokhi Nejad [4] findings, who showed that application of Calcium chloride had significant effect on *Fusarium* wilt pathogen of pea. Everett and Blazquez also had found that the use of calcium carbonate is effective in reducing the severity of *Fusarium* wilt of watermelon, though the researchers concluded that the increasing of pH due to calcium carbonate was major factor in the disease control [22].

Conclusions

Fungal isolates obtained in this study included *F.solani*, *F.equiseti*, *Lasiodiplodia hormozganensis*. However, the major pathogen of this disease is *Fusarium solani* in this province. The effect of various levels of soil pH (6, 8) and EDDHA (0, 10 and 20 mmol/L) on disease severity of *F.solani*-infested plants was determined. EDDHA significantly ($p<0.05$) reduced disease severity of *F. solani* and increased other evaluated characteristics at both pH levels, also *F.solani*-infested plants grown at pH=6 and different EDDHA concentrations showed more disease severity and poorer growth characteristics compared with plants grown at pH=8.

Corden reported that disease severity of *Fusarium* wilt pathogen of tomato was increased by calcium deficiency, when it occurred after inoculation (regardless of calcium status before inoculation) [20]. On the other hand, if a calcium The current study also indicated that the application of EDDHA, significantly increased the plant characteristics at non-infested soils. Also, the effects of calcium and EDDHA in infested and non-infested soils at pH= 6 showed that when both EDDHA and Ca were applied, minimum disease severity was observed (6.6%) as well as highest growth characteristics were obtained.

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