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Article

Trichoderma spp. evaluation, disinfestation and soil chiseling in alfalfa

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Abstract

Crops of alfalfa *Medicago sativa* L. are limited by crown rot (*Rhizoctonia solani*), Texas root rot (*Phymatotrichopsis omnivora*) and soil salinity and compaction. To attenuate crop rots, the objectives were, at the laboratory level, to evaluate methods of inoculation of *Trichoderma* spp. in alfalfa seed and in the field, to evaluate agronomic parameters, crown rot and Texas root rot after inoculating the seed with *T. harzianum* (T), deep soil chiseling (C) and reductive soil disinfestation (D), the combinations C+T, C+D, D+C+T, and uninoculated seed and untreated soil as the control (Co). Dishes (PDA) of *T. harzianum* of 1, 2, 4, 8 and 10 d of growth were used to place previously germinated (GS) or non-germinated (NGS) alfalfa seeds; after 17 d, the size and survival of the NGS seedlings was greater than their corresponding GS (Tukey $p< 0.05$). In the field, the weight and size of D+C+T vs. Co plants were between (27-35) and (9-3)% higher, respectively, but without statistical significance. Only in the first sampling, a 30% higher yield of D+C+T was detected when compared to Co (Tukey $p= 0.05$). The quality, yield and plants per m^2 of alfalfa were similar to what had been previously reported.

Keywords:

diseases, endophytes, forages, fungi.

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Introduction

In 2019, in the region of La Laguna, located between the states of Coahuila and Durango, alfalfa crops occupied 32 000 ha and had a green yield of 90.3 t ha^{-1} (SIAP, 2020). The factors that limit this crop are the restricted availability of irrigation water, the high electrical conductivity of the soil, root diseases, and compaction (Chew-Madinaveitia and Santamaría-César, 2000; Lara-Macías and Jurado-Guerra, 2014).

Crown rot and Texas root rot are the most common and harmful diseases for alfalfa; the first is caused by the fungi *Fusarium* spp. and *Rhizoctonia solani* Kühn and the second, by *Phymatotrichopsis omnivora* Hennebert, 1973. From the establishment of alfalfa, these fungi infect its roots; as the second and third years go by, the population and size of the plants decrease, forcing the farmer to remove the crop (Chew-Madinaveitia and Santamaría-César, 2000).

To attenuate alfalfa root rots, its seeds can be inoculated with *Trichoderma* spp. (Chew-Madinaveitia and Samaniego-Gaxiola, 2007; Redman *et al*., 2021). Likewise, the biotic effect (pests, weeds, and diseases caused by fungi, bacteria, nematodes) and the abiotic effect (drought, extreme temperatures, pH, salinity) are attenuated by endophytic microorganisms, where *Trichoderma* spp. stands out (Lata *et al*., 2018); these fungi induce systemic defense responses and promote growth in plants, they do so through the interaction of gene expression initiators, which includes epigenetic marks (Morán-Diez *et al*., 2021).

Reductive soil disinfestation is a method for controlling harmful organisms from plants, where the anaerobic decomposition of organic matter rich in easily degradable carbohydrates is used (Samaniego-Gaxiola *et al*., 2019 b). Therefore, the objectives of this work were: i) in the laboratory, to evaluate methods of inoculation of alfalfa seed with *Trichoderma* spp. and its responses in seedlings; and ii) in the field, to evaluate the yield, health, and quality of alfalfa in response to treatments of seed inoculated with *T. harzianum* (T), application of deep chiseling (C), reductive soil disinfestation (D), and some of their combinations.

Materials and methods

Inoculation of alfalfa seed and seedling with *T. harzianum* in the laboratory

The seed used was from Royal Ten® commercial alfalfa from two packages of 20 kg each, which was mixed, washed under running water until its coating was removed, dried in an oven with air flow at 28 °C, and stored at 10 °C until used as needed. PDA dishes (prepared with juice of 100 g potato, 10 g dextrose and 7.5 g agar per 0.5 L distilled water) were inoculated separately with *T. harzianum* to obtain colonies of 1, 2, 4, 8, and 10 d of growth in the dark at 28 °C. Each of these dishes received 25 non-germinated seeds (T1-NGS, T2-NGS, T4-NGS, T8-NGS, and T10-NGS) or 25 three-day germinated seeds (T1-GS, T2-GS, T4-GS, T8-GS, and T10-GS). The treatments, each with eight replications, were the seed germinated or not and placed in the plates with *T. harzianum*.

The control treatment was non-germinated seed, disinfected with NaOCl and placed on a dish with *T. harzianum* of a day of growth (T1-NGS-NaOCl). The treatments were incubated at 28 °C in a regime of 16 h light and 8 h dark. After three days of incubating the treatments, 10 of the 25 seedlings were randomly selected from each treatment, which were sown in germinating trays of 200 cavities 7 cm deep filled with Peat moss premier®. The trays were incubated at 28 °C with 16 h of light and 8 hours of darkness. After incubating the trays for 3, 7, 10, 14 and 17 d, the variables evaluated were the height and survival of the seedlings.

Field treatments

The work was established with a producer from the region in the agricultural area Beta Santa Monica SPR of RL de CV, 25° 44’ 12.6” north latitude, 103° 11’ 10.3” west longitude, located in the municipality of San Pedro, Coahuila, Mexico. The sowing was carried out on February 14, 2020, with a Brillion seeder at a sowing density of 35 kg ha^{-1}, the management given to the crop was

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that of the producer, except for the treatments. Seven treatments were established in randomized blocks in an area of 0.5 ha, each with four replications, each replication was a plot 11.6 by 15 m.

Treatment 1 was the control (Co) and consisted of establishing the crop in a simple chiseled soil, using a six-pointed chisel plow at a depth of 60 cm. Treatment 2 (C) soil with double chiseling in a crosswise direction. Treatment 3 or disinfestation with molasses dissolved in water (7 cm layer), then the surface of the plot was covered with plastic for the next 14 d (Samaniego *et al*., 2019 a); on the third day after applying the molasses, the plastic was uncovered and 2 L of sulfuric acid was added to 3 000 L of water per plot; after 14 d, the soil was left to aerate for a week and then planted.

In treatment 4 (T), the seed was inoculated with a solution of 10^8 CFUs of *T. harzianum* at a rate of 3 ml per 20 g of seed. Treatments 5, 6 and 7 were the combinations of C+T, C+D and D+C +T, respectively. For combined treatments that included C or D, it was first chiseled, then D was applied and finally, it was sown.

The agronomic variables were the following: the density of plants per m^2 was counted by randomly counting 1 m^2 within each plot (replication). In each replication, the alfalfa was cut on a randomly selected area of 2 m^2. The fresh weight (t ha^{-1}) of each cut was recorded *in situ*, and a sample of 500 g was taken there to determine, in the laboratory, its dry weight (t ha^{-1}) and forage quality: neutral detergent fiber NDF, acid detergent fiber ADF, and protein in (%) (Rohweder *et al*., 1978).

Diseases in the field

For each treatment, two plant samplings were carried out in the field, the first at 113 and the second at 365 days after sowing (das). In each sampling and for each replication, 20 plants were randomly extracted. In the roots of each plant, the variables determined were the incidence and severity of crown rot and the incidence of Texas root rot, which was done by observing the cords on the root. The severity of crown rot was assessed with the modified scale of Chew-Madinaveitia and Santamaría-César (2000), where the value 1≤ 10, 2≥ 10< 25 and 3 > 25% of the crown rotten. The height of each sampled plant and the dry weight of its aerial part were also measured.

Statistical analysis

The results of the trial response of alfalfa seed and seedling to *T. harzianum* were analyzed with an Anova (SAS Institute Inc., 2000) in a completely randomized design. Field trial data were analyzed with an Anova in a randomized block design; the results of the two sampling dates were analyzed separately. When the value of F was significant ($p< 0.01$), the means were compared with Tukey $p< 0.05$.

Results and discussion

Inoculation of alfalfa seed and seedling with *T. harzianum* in the laboratory

Dishes with *T. harzianum* allowed an alfalfa seed germination of 96 to 100%, except for the T1-NGS-NaOCl treatment with 93%. The survival of seedlings that were established in Peat moss is indicated in Table 1. During the 17 days of evaluation, only seedlings of the T1-NGS and T1-NGS-NaOCl treatments gradually decreased their survival.

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Table 1. Survival (%) of alfalfa seedlings from 3 to 17 d. Where seeds or seedlings were exposed to treatments and germination or not of the seed and its contact with *T. harzianum* were combined.

Treatments	Days after being planted				
	3	7	10	14	17
T1-GS	98.8 a	92.5 a	92.5 a	91.3 a	83.8 b
T2-GS	98.8 a	92.5 a	92.5 a	91.3 a	82.5 b
T4-GS	96.3 a	92.5 a	92.5 a	92.5 a	87.5 b
T8-GS	98.8 a	98.8 a	98.8 a	98.8 a	98.8 a
T10-GS	98.8 a	98.8 a	97.5 a	96.3 a	93.3 a
T1-NGS	100 a	86.3 b	86.3 b	86.3 b	66.3 c
T2-NGS	98.8 a	96.3 a	95 a	95 a	95 a
T4-NGS	98.8 a	96.3 a	96.3 a	96.3 a	93.3 a
T8-NGS	100 a	100 a	100 a	100 a	100 a
T10-NGS	98.8 a	96.3 a	92.5 a	91.3 a	91.3 a
T1-NGS-NaOCl	92.5 a	63 c	63 c	63 c	47.1 d

T1, T2, T4, T8 and T10= growth of the *T. harzianum* colony for 1, 2, 4, 8 and 10 days respectively, before placing the seed; GS and NGS, germinated or non-germinated seed, respectively, before being placed on the fungus colony. T1-NGS-NaOCl= non-germinated seeds that remained 10 min in NaOCl before being placed on the *T. harzianum* colony. Numbers followed by the same letter within the column denote that there is no significant difference according to the means separation test (Tukey $p< 0.05$).

The height of seedlings at 17 d was significantly higher (Tukey $p< 0.05$) in the treatments of non-germinated seed (NGS) placed in dishes of 2, 4 and 8 days of growth of *T. harzianum*; the seedling heights ranged from 73 to 77.4 mm, which contrasted with the height of the seedlings with the T1-GS treatment, which was 28.1 mm (Table 2). The 100% seed survival coincided with the largest seedling size (77.4 mm at 17 d), which occurred in the T8-NGS treatment; this agreement in germination and seedling size also occurred in the T2-NGS and T4-NGS treatments (Tables 1 and 2).

Table 2. Alfalfa seedling heights (mm) from 3 to 17 d. Where seeds or seedlings were exposed to treatments and germination or not of the seed and its contact with *T. harzianum* were combined.

Treatments	Days after being planted				
	3	7	10	14	17
T1-GS	6.3 c	12.4 c	18.4 c	24.6 d	28.1 d
T2-GS	7.3 c	14.4 c	21.6 c	28.6 c	34 c
T4-GS	8.8 c	17.3 ab	25.9 bc	34.4 bc	42.3 b
T8-GS	6.8 c	13.5 c	20.1 c	26.8 c	33.9 b
T10-GS	9.3 c	18.3 ab	27.4 bc	36.4 bc	48.6 b
T1-NGS	12.5 b	11.4 c	23.1 c	30.4 c	41.5 b
T2-NGS	20.7 a	23 a	46.2 a	61.4 a	73 a
T4-NGS	22.6 a	21.8 a	43.8 a	58.4 a	74.6 a
T8-NGS	27.5 a	16.9 ab	33.8 b	45 b	77.4 a
T10-NGS	10 c	18.1 ab	35.9 a	47.5 b	45.3 b
T1-NGS-NaOCl	7.5 c	11.5 c	22.8 c	30.5 c	41 b

T1, T2, T4, T8 and T10= growth of the *T. harzianum* colony for 1, 2, 4, 8 and 10 days, respectively, before placing the seed; GS and NGS, germinated or non-germinated seed, respectively, before being placed on the fungus colony. T1-NGS-NaOC= non-germinated seeds that remained 20 min in NaOCl before being placed on the *T. harzianum* colony. Numbers followed by the same letter within the column denote that there is no significant difference according to the means separation test (Tukey $p< 0.05$).

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In general, *Trichoderma* spp. stimulates both the germination and growth of cultivated plants (Stewart and Hill, 2014). Nevertheless, germinated seed (GS) and T1-NGS-NaOCl treatments had the lowest seedling sizes at 17 d compared to non-germinated seed (NGS), suggesting that seedling development is impaired by seed germination and subsequent contact with *T. harzianum* as well as by disinfection with NaOCl.

Nonetheless, seedling size increased in treatments of NGS with *T. harzianum*, it did not happen in T1-NGS and T10-NGS, where alfalfa seed could be negatively affected by compounds generated by this fungus, early (1 d) or late (10 d) (Table 2); this idea is supported by the results by Schweiger *et al*. (2021), who determined that corn seeds inoculated with endophytic *Trichoderma virens* generated seedlings and roots of smaller size than the non-inoculated ones, which was attributed to competition for nutrients and production of toxic metabolites of the fungus towards the seedling.

In short, the responses of *T. harzianum*-seedlings are given by the age of the inoculum in the dish, the germination or not of the seed, and the disinfection of the seed with NaOCl. Non-disinfected pepper and tomato seeds inoculated with *Trichoderma* spp. endophytes were found to have increased their root and seedling size compared to non-inoculated seeds (Moin *et al*., 2021; Rokni *et al*., 2021); in contrast, seeds of corn and soybean (*Glycine max*) disinfected with NaOCl decreased their root and seedling size compared to non-disinfected seeds (Bilal *et al*., 2020; Schweiger *et al*., 2021).

For Figueiredo dos Santos *et al*. (2021), the NaOCl used to disinfect corn seed reduces the population of bacteria needed to germinate and increase root growth. According to Schweiger *et al*. (2021), smaller corn seedlings are due to metabolic changes caused by endophytic *T. virens*, which will result in smaller plants. The results of this research also showed that the dynamics of small seedlings at 3 d remain up to 17 d (Table 2).

Field evaluation

For crown rot, there was only a difference between treatments in the incidence and severity of the second sampling (Tukey $p< 0.05$); the control treatment had the lowest incidence with 61.3%, which was similar to what was previously reported (62%) in one-year-old alfalfa fields (Chew-Madinaveitia and Santamaría-César, 2000); however, the rest of the treatments exceeded 80% incidence (Table 3). For its part, the lowest severity value of crown rot was in the C+D treatment with 1.4, which contrasted with the control, C, and T treatments, the values of which were ≥ 2 (Table 3).

Table 3. Incidence, severity, weight, and height of alfalfa plants after applying treatments against crown rot and Texas root rot.

Treatments	Crown rot Incidence (%)		Severity[€]	Sampling[††]	Texas root rot Incidence (%)		Weight (g)		Height (cm)	
	1	2	1	2	1	2	1	2	1	2
Co	81.3 ns	61.3 a	1.1 ns	2.1 a	0 ns	12.5 ns	14.5 ns	34.2 ns	38.5 ns	61.9 ns
C	78.8 ns	80 ab	1.1 ns	2.2 a	0 b	8.8 ns	12.5 ns	33.9 ns	34.7 ns	58.3 ns
D	77.5 ns	83.8 ab	1 ns	1.6 a	3.7ab	12.5 ns	15.7 ns	47.3 ns	47.3 ns	64.5 ns
T	75 ns	88.8 b	1.1 ns	2 a	0 b	11.3 ns	12.7 ns	44.9 ns	39 ns	64.2 ns
C+T	80 ns	87.5 b	1.1 ns	1.9 a	0 b	5 ns	12.9 ns	38.9 ns	36.9 ns	63.5 ns
C+D	80 ns	91.3 b	1.1 ns	1.4 b	0 b	0 ns	13.2 ns	49.5 ns	37 ns	63.3 ns
D+C+T	82.5 ns	88.8 b	1.1 ns	1.6 b	7.5 a	12.5 ns	19.9 ns	52.9 ns	42.1 ns	63.9 ns
Average	79.3	83	1.1	1.5	1.6	8.9	14.5	43.1	39.3	62.8

Co= control; C= chiseling; D= anaerobic soil disinfestation; T= *T. harzianum* in seed; C+T, C+D and C+T +D= combinations of the previous three treatments; [††]= samples 1 and 2, 113 and 365 das, respectively; [€]= scale 1 and 2 ≤10% and >10 <25 % of root rotten, respectively; ns= statistically not significant. Numbers with different letters indicate statistically different means (Tukey $p< 0.05$).

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The treatments (C, T, and D) favor the degradation of crop residues after each alfalfa cut, in addition to the initial fertilization of 185 kg ha^{-1} of nitrogen that the farmer applied; both crop residues and applied nitrogen are factors that favor the incidence of diseases caused by *R. solani* (Papavizas *et al*., 1975; Mayer, 2002; Bonanomi *et al*., 2007). Nonetheless, some organic residues can inhibit *R. solani* and the diseases it causes (Kasuya *et al*., 2006).

The incidence of Texas root rot was significant (Tukey $p< 0.05$) only during the first sampling, where D and D+C+T were the only treatments where plants with this disease were detected. In the second sampling, the incidence of Texas root rot was minor, although the C+D treatment did not manifest plants with symptoms. For both samplings, the plant weights between treatments were not statistically different, the weights of the first and second sampling ranged from 12.5 to 19.9 and from 33.9 to 52.9 g, respectively.

The height of the plants was not significant in both samplings either, its fluctuation for the first and second samplings was from 34.7 to 47.3 and from 58.3 to 64.5 cm, respectively (Table 3). Although there was no statistical significance of weight and height between treatments, the following trends were observed: i) the D+C+T treatment had the highest plant weight, 27 and 35% more than the control for the first and second sampling, respectively and ii) the same occurred for plant height, but only 9 and 3% more for the first and second sampling, respectively.

In the field, Chew-Madinaveitia and Samaniego-Gaxiola (2007) results revealed a higher density of plants per m^2 of alfalfa seed inoculated with *Trichoderma* spp. than in non-inoculated seed, but this effect was lost six months after establishing the crop; in this work, no difference was detected in plants per m^2 at four months (Table 3).

In the first sampling, the yield of alfalfa in all treatments exceeded the control (2.34 t h^{-1}), but the D+C +T treatment (3.36 t ha^{-1}) was significant (Tukey $p< 0.05$); this difference represented an increase in yield of ~30%. Redman *et al*. (2021) detected a 5% increase in alfalfa yield, evaluated over four years in 75 trials, with seed inoculated with *T. harzianum* and crop established under moderate stress conditions, but an average increase of 50% in yield in wheat and corn established under severe stress conditions.

The irrigation water used for alfalfa in this work ranged from 7.2 to 4.3 dS m^{-1} and the EC of the soil was 7 dS m^{-1}, which, together with nine cuts in the first year, can be considered stress factors for the crop, which would partly explain up to the 30% increase in yield mentioned in the first sampling. However, for the second sampling, ~9% more yield was detected between the T vs. Co treatments, although the difference was not statistically significant (Table 4).

Table 4. Characteristics of the crop and quality of alfalfa after applying treatments for the control of crown rot and Texas root rot.

Treatments	Plants m^{-2} in samplings 1 2		Yield (t ha^{-1}) samplings 1 2		£ Protein, € ADF # NDF sampling 1			Protein, ADF, NDF sampling 2		
Co	391 ns	Nd	2.3 b	4.3 ns	15.8 ns	23.7 ns	34.6 ab	14 c	21.9 ns	31.4 ab
C	263 ns	Nd	2.6 ab	4.1 ns	16.2 ns	21.5 ns	33.8 ab	18.4 ab	22.4 ns	33.5 ab
D	366 ns	Nd	2.3 b	4.6 ns	14.42ns	21.4 ns	31.7 b	13.5 c	26.2 ns	35.4 a
T	320 ns	Nd	2.8 ab	4.6 ns	15.9 ns	21.4 ns	35.5 ab	21.1 a	21.1 ns	28.5 b
C+T	285 ns	Nd	2.8 ab	4.1 ns	14.4 ns	23.6 ns	36.2 ab	18.1 ab	21.9 ns	30.5 ab
C+D	353 ns	Nd	2.4 b	4.3 ns	16.4 ns	23.7 ns	34.2 ab	16 bc	24.7 ns	30.6 ab
D+C+T	363 ns	Nd	3.4 a	3.8 ns	13.9 ns	26.6 ns	36.9 a	15.3 bc	21.7 ns	34.1 a
Average	334	Nd	2.7	4.3	15.3	23.1	34.7	16.6	22.8	32

Co= control; C= chiseling; D= anaerobic soil disinfestation; T= *T. harzianum* in seed; C+T, C+D and C+T +D= combinations of the previous three treatments; samplings 1 and 2, 113 and 365 das, respectively; scale 1 and 2 ≤10% and >10 <25% of root rotten, respectively; ns= statistically not significant; Nd= not determined; £= Crude protein (%); €= detergent acid fiber (%); £= neutral detergent fiber (%). Numbers with different letters indicate statistically different means (Tukey $p< 0.05$).

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The quality of alfalfa between treatments was only significant (Tukey $p< 0.05$) for NDF in the first sampling and protein in the second sampling. In general, the quality of alfalfa was excellent for ADF and NDF and acceptable for protein according to Nuñez (2000).

Rush and Lyda (1984) results indicate that after deep chiseling, the incidence of Texas root rot was ~50% lower and the yield of cotton was ~90% higher compared to non-chiseling.

Alfalfa is considered an excellent crop for grooving the soil (biochiseling), Zhang and Peng (2021), which, together with optimal root-rhizosphere management, could optimize the management of this crop, including treatments for crown rot and Texas root rot. Optimizing the management of the root-rhizosphere has led to a reduction of almost two-thirds of the nitrogen applied to produce the same amount of corn, increasing the size and volume of roots, compared to the traditional method (Shen *et al*., 2013).

In addition, since alfalfa yield is the sum of factors such as rot, compaction, fertilization, and water and soil quality, when improving the last three, a more favorable response would be expected from the treatments evaluated in our work.

The strain evaluated in this study was shown to be endophytic to melon crops (Samaniego-Gaxiola *et al*., 2021), but this was not evaluated in this study. Future work could evaluate strains or isolates of endophytes of *Trichoderma* spp. or other fungi.

Harman *et al*. (2021) indicate that *Trichoderma* spp. endophytes, with specificity to the plant and region, confer tolerance to abiotic and biotic stress in agricultural crops. The reductive soil disinfestation (D) implemented in this work was carried out during the winter, where the soil temperature at 20 cm was around 20 °C; it is likely that with higher soil temperature, there will be better control of alfalfa rots. D has been implemented for a wide range of crops, using different substrates and at soil temperatures between 17 and >30 °C (Samaniego-Gaxiola *et al*., 2019b).

Conclusions

The age of the colony of *T. Harzianum*, before coming into contact with germinated or non-germinated seed, positively or negatively affected seedling survival and size at 17 d (Tukey $p< 0.05$).

In the field, only during the first sampling were the yields of the treatments higher than the control; D+C+T stood out, which was ~30% higher than the control. The one-year alfalfa control treatment had a lower incidence of crown rot (61%) (Tukey $p< 0.05$) than the rest of the treatments (~80%). The height and weight of the alfalfa plants showed no statistical difference.

The number of plants per m^2 and the quality of the alfalfa (protein, ADF, and NDF) were within the parameters considered for quality alfalfa. The conditions of the commercial management of the alfalfa field where the work was carried out, such as frequent cuts in the first year, the soil EC of 7 dS m^{-1}, and the initial fertilization of 185 nitrogen units, can be considered favorable for crown rot and Texas root rot.

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