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Trichoderma – based inoculants an eco-friendly option to enhance Eruca sativa and Lactuca sativa productivity and sanity.

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RESUMEN

En este estudio, seis cepas nativas de *Trichoderma* fueron aisladas y testeadas como potenciales inoculantes para mejorar la productividad y la sanidad de plantas de rúcula (*Eruca sativa)* y lechuga (*Lactuca sativa*). Las cepas aisladas de la rizósfera de estas hortalizas fueron identificadas taxónomicamente como pertenecientes a las especies *Trichoderma harzianum* y *Trichoderma asperellum*. La inoculación con *Trichoderma* tuvo efectos positivos en el crecimiento de las hortalizas en condiciones de vivero y a campo. En rúcula, la inoculación con la cepa *T. harzianum* R2 fue el tratamiento más efectivo y produjo incrementos del 75% y del 63% en la biomasa aérea fresca en vivero y a campo, respectivamente. En lechuga, la inoculación con *T. harzianum* LMR3 incrementó 147% la biomasa aérea fresca a campo, mientras que en condiciones de vivero la inoculación no produjo un efecto positivo significativo. Con respecto al potencial biocontrolador de las cepas, se observó que las plantas de rúcula inoculadas con *T. asperellum* R1 presentaron una incidencia del 2,8% a causa de mildew comparadas a las plantas control que registraron un 98% de incidencia. En el ensayo de lechuga, las plantas inoculadas con *T. harzianum* R2 presentaron una incidencia del 8% por pudrición gris comparadas con un 100% de incidencia en el tratamiento control. Estos resultados demuestran que la inoculación de rúcula y lechuga con cepas nativas de *Trichoderma* genera incrementos en el rendimiento productivo y reduce la incidencia de enfermedades como mildew y pudrición gris. Este estudio preliminar sugiere el uso de inoculantes en base a cepas nativas de *Trichoderma* como una estrategia sustentable para mejorar la productividad y sanidad de estas hortalizas de hoja.

ABSTRACT

In this study, native *Trichoderma* sp. strains were tested as potential inoculants to improve *Eruca sativa* and *Lactuca sativa* productivity and sanity. Six strains assigned to *T. harzianum* and *T. asperellum* were isolated from the rhizosphere of this leafy vegetables. A significant positive effect of inoculation with all the native isolates was observed in rocket seedlings cultivated in nursery and in field conditions. The highest increase was observed in seedlings inoculated with *T. harzianum* R2 with an increment in the fresh shoot biomass of 75% in nursery and of 63% in field. In lettuce, an increase up to 147% was obtained in plants inoculated with *T. harzianum* LMR3, however in nursery any of the inoculation treatments did not enhance plant biomass. Moreover, rocket seedlings inoculated with *T. asperellum* R1 presented only 2,8% of mildew disease incidence caused by *H. parasitica* while lettuce seedlings inoculated with *T. harzianum* R2 presented only 8% of grey mold incidence compared to un-inoculated seedlings with 98% and 100% of disease incidence, respectively. In conclusion, bio-inoculation of rocket and lettuce with native *Trichoderma* strains increased yield of these crops and the decreased the incidence of mildew in rocket and gray mold in lettuce in nursery. Therefore, *Trichoderma*-based inoculants could represent a promising sustainable strategy to enhance productivity and sanity of leafy vegetables.

PALABRAS CLAVE

Eruca sativa, *Lactuca sativa*, *Trichoderma*; biofertilización, biocontrol, mildew, pudrición gris

KEY WORDS

Eruca sativa, Lactuca sativa, *Trichoderma*; biofertilization, biocontrol, mildew, grey mold

CONTEXT & COLLABORATORS

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INTRODUCTION

The consumption of fresh leafy vegetables such as rocket (*Eruca sativa* Gersault) and lettuce (*Lactuca sativa* L.) increased five times in Argentina the last years ([1] Del Pino, 2012). Rocket yields achieve up to 10 tons/ha per year in field and up to 15 tons/ha in greenhouse ([2] Botto, 2003), while lettuce yields on average 96 tons/ha per year ([3] Pineda, 2018). These leafy vegetables are produced under intensive agricultural systems that rely on a high amount of chemical fertilizers and phytosanitary products ([4] Colla et al. 2010), that applied constantly on the same plots can contaminate surfaces and groundwater sources ([5] Tilman et al., 2002). Diseases reported to affect rocket productivity in Argentina are white rust (*A. candida*) ([6] Zapata, 2005), mildew (*H. parasítica*) ([7] Larran, 2006; [8] Sandoval, 2017), black rot (*Xanthomonas campestris* pv campestris) ([9] Romero, 2008) and leaf spot (*Alternaria alternata*) ([8] Sandoval, 2017); while rot (*Sclerotinia sclerotiorum*, *S. minor* and *Fusarium* spp.) ([3] Pineda, 2018), gray rot (*Botrytis cinerea*), and mildew (*Bremia lactucae*) ([10] Adlercreutz, 2016) are reported in lettuce.

Due to the increasing demand of more sustainable and eco-friendly agricultural practices, the application of biological products that enhance crop productivity and sanity are increasing in popularity. Among the microbes that are commercialized in agriculture, the species of the genus *Trichoderma* are well reported as effective biological control agents of many plant diseases as well as bioestimulants and biofertilizers of a wide range of crops ([11] Harman, 2004; [12] Woo, 2014; [13] Copolla, 2019). In horticulture, *Trichoderma* inoculation has shown an effective strategy to reduce diseases caused by *Sclerotium rolfsii, Rhizoctonia solani* y *Fusarium oxysporum* ([14] Torres, 2008; [15] Guédez, 2012; [16] Martínez, 2013). A recent, study has shown that *Trichoderma* inoculation on leafy vegetables enhances plant biomass particularly in low N fertility soils ([17] Fiorentino, 2018). The rhizosphere and roots of several crops are relevant micro niches for the isolation of native plant growth promoters adapted to endemic soils conditions from which effective bio-inoculants can be formulated ([18] Bergottini, 2015).

In this context, *Trichoderma* inoculation could represent an eco-friendly and economic option to increase rocket and lettuce productivity in nursery and in field conditions.

According to previous studies, we hypothesized that native *Trichoderma* strains associated to the rhizosphere of this crops will be more effective biocontrollers and biofertillizers due to their adaption to local environmental conditions.

OBJETIVE

The aim of this study was to isolate, characterize and select native *Trichoderma* strains associated to *E. sativa* and *L. sativa* to assess their potential as inoculants for this crops.

MATERIALS AND METHODS

Trichoderma isolation and identification

For *Trichoderma* isolation, ten gram of roots with the rizospheric attached soil of *E. sativa* and *L. sativa* plants were dissolved in 90 mL of sterile physiological solution (0.85% NaCl) and incubated under agitation at 30 °C for 30 minutes. This suspensions were diluted ten-fold serially, spread onto YGC agar plates and incubated at 25°C for 7 days. *Trichoderma*-like colonies were analyzed microscopically with lactofenol blue and then purified by streaking out on PDA. In order to confirm their taxonomical affiliation, the sequence of the ITS1–5.8S–ITS2 region was analyzed. DNA extraction, PCR amplification with the primer pair ITS1 TCTGTAGGTGAACCTGCGG and ITS4 TCCTCCGCTTATTGATATGC and sequencing was performed according to Macrogen (South Korea). The search for similarity amongst ITS sequences available in GenBank database was performed using BLASTn (NCBI, www.ncbi.nlm.nih.gov) and with Database Fungal barcoding (www.fungalbarcoding.org/). The sequences are available in GenBank under the following accession numbers MN552151-MN552156. Prior to test the isolates as bio-inoculants in plants, their ability to inhibit the growth of damping-off attack species *Fusarium oxysporum* (National University of Quilmes collection), *Rhizoctonia solani* (Universidad Argentina de la Empresa collection) and *Botrytis cinerea* Pers (National University of Lomas de Zamora collection) was analyzed in dual culture assay on PDA. The radial growth of all fungi was analyzed daily during 7 days in order to measure the percentage of inhibition of radial growth (PIRG) = (a-b)/a * 100, where a = the radius of each damping-off fungi colony without the biocontrol agent and b = radius of each damping-off fungi colony in the presence of *Trichoderma* strains ([19] Desai, 2012). An IR value higher than 50% suggests that strains are effective antagonists.

Plant-inoculation assays

Trichoderma isolates were tested as bio-inoculants in rocket and lettuce plants in pots assays in nursery. Then, the most promising isolates were tested as bio-inoculants in field conditions. The assays were conducted in two different nurseries, biofertilization was tested at Faculty of Agronomy of Buenos Aires (Tecnicatura de Producción Vegetal Orgánica- FAUBA) from September to October 2018, and the biocontrol assay was conducted at Faculty of Agricultural Sciences of Lomas de Zamora (UNLZ) from September to October 2019. For both assays the inoculum preparation consisted in growing *Trichoderma* isolates in PDA at 25°C with a photoperiod of 16 h day/8 h night until sporulation (7 days), then flooding the agar plates with sterile physiological solution (0.85% NaCl) and spreading with a Drigalsky spatula until recovering the total biomass. This biomass was then inoculated on sterile rice husk at 25°C with a photoperiod of 16 h day/8 h night until sporulation (15 days). The conidial suspensions obtained were adjusted to a final concentration of 1× 10^8 conidia ml^{-1} using a Neubauer chamber. Organic seeds of rocket and lettuce (var. four seasons) were coating with a 1:1 suspension of each *Trichoderma* isolate (1× 10^8 conidia ml^{-1}) with 0.5% (w/v) starch solution and incubated overnight at room temperature to air-dry. Control seeds were treated only with the starch solution. Pelleted seeds were placed in pots (80 cm^3) with approximately 80 g of soil. In both assays, two inoculation reinforcement were performed, firstly on ten-day seedlings and secondly on twenty-day seedlings by watering the soil pot with 1 ml of a fresh conidial suspension adjusted to 1× 10^8 conidia ml^{-1}.

The biofertilization assay consisted in six *Trichoderma* inoculated treatments and a non-inoculated control with 50 rocket/lettuce plants per treatment. After five weeks of growth, plants were harvested to determine fresh and dry biomass. Biocontrol assays consisted in testing the effect of *Trichoderma* inoculants in rocket infected with *Hyaloperonospora parasítica* and in lettuce plants infected with *Botrytis cinerea* Pers. *Trichoderma* inoculants were applied as a seed-coating treatment, hand-seeded to soil pots (70 cm^3) and every two weeks a reinforcement of inoculation was applied. Pathogen inoculation was performed in fifteen-days-old seedlings by watering plants with suspensions of conidia adjusted to 2 x 10^6 UFC ml^{-1}. After pathogen inoculation, the infected leaf area development was monitored weekly to estimate the incidence of the disease. Fifty-day old plants were harvested to determine fresh biomass. The effect of bio-inoculation in plant biomass was analyzed by a one-way ANOVA and pairwise differences were tested using Tukey's post-hoc test ($P \leq 0.001$).

RESULTS

Taxonomic identification and pathogen inhibition assays

Six *Trichoderma* strains were from the rhizosphere of rocket and lettuces plants cultivated in an organic nursery. Colonies of white aerial mycelium that with sporulation developed a yellowish-green periphery and with concentric ring-like zones were selected. Microscop-ically, the observation of branched conidiophore with flask-shaped phialides and green hyaline conidia clustered at the tip determined that isolates belong to *Trichoderma*. In order to confirm their taxonomic affiliation, the ITS 1-5.8S –ITS2 region sequences of the six isolates allowed to identify four isolates as *Trichoderma harzianum* and two *as Tricho-*

derma asperellum (Table 1). Based on the dual culture antagonist assays, all isolates presented a higher %RI than 50% suggesting their potential as effective bio controllers. A marked antagonistic effect was observed against *Botrytis cinérea,* showing the highest percentage of growth inhibition *T. asperellum* LMR2 (84,2%), *T. asperellum* R1 (82,7%) and *T. harzianum* LMR3 (81,9%) Strains LMR2 and LMR3 also showed the highest %RI against *F. oxysporum* (72,2 a 75,7%) *and R. solani* (53 a 56%).

Table 1: Taxonomic assignment and antagonistic effect *in vitro* of *Trichoderma* isolates

Plant	Strain	Genbank accession	Phylogenetic assignment	*Botrytis cinerea*	*Fusarium oxysporum*	*Rhizoctonia solani*
				%RI on day 5	%RI on day 6	%RI on day 10
Eruca sativa	R1	MN552156	*Trichoderma asperellum*	82,7± 6,5 a	72,7±2,5 a	56,7±1,5 a
Eruca sativa	R2	MN552154	*Trichoderma harzianum*	78,2±1,3 a	56,7±2,0 c	37±2bc
Eruca sativa	R3	MN552153	*Trichoderma harzianum*	78,9±3,4 a	61±2,6bc	40±1bc
Latuca sativa	LMR1	MN552151	*Trichoderma harzianum*	78,9±1,3 a	56±1.7 c	36.3±3 c
Latuca sativa	LMR2	MN552155	*Trichoderma asperellum*	84,2±2,3 a	75,7±2,0 a	53±2 a
Latuca sativa	LMR3	MN552152	*Trichoderma harzianum*	81,9±3,9 a	65,7±2,0 b	42±1,5 b

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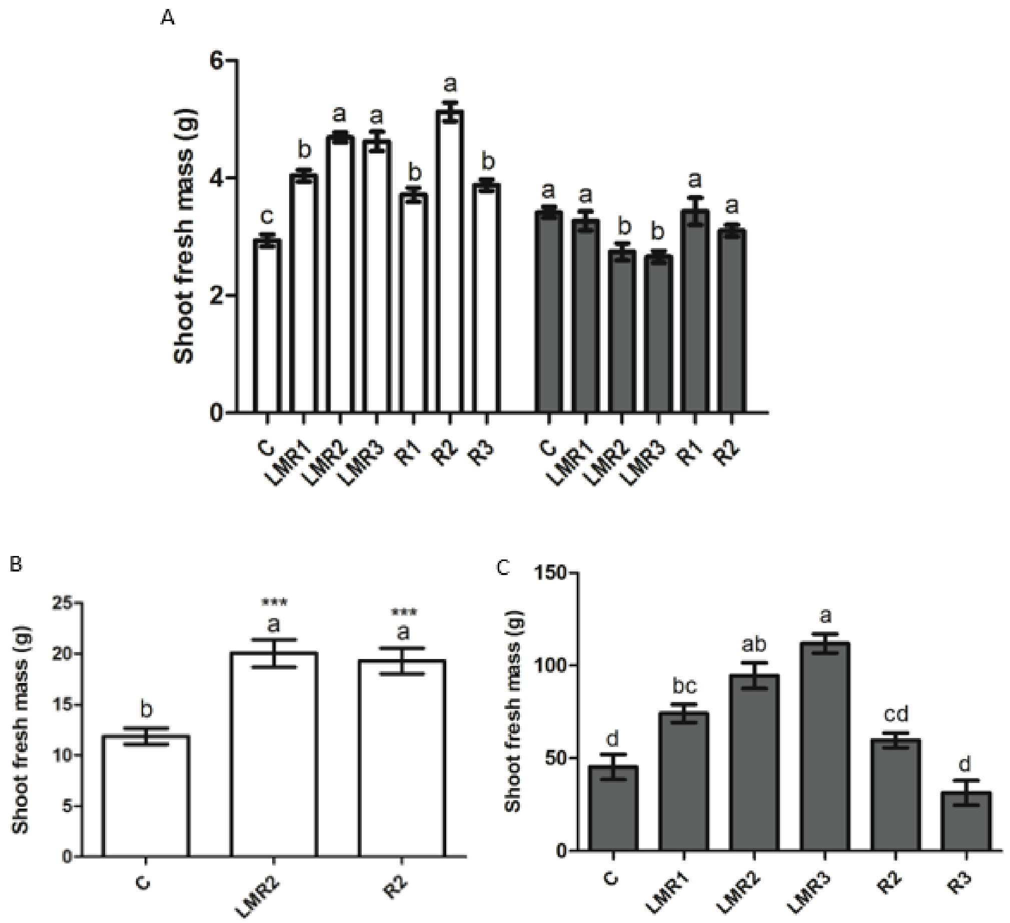
Biofertilization assay

Trichoderma inoculants had a significant growth promoting effect of rocket seedlings in nursery and field conditions (Figure 1). The six native strains produced a significant increase on the shoot fresh biomass in comparison to un-inoculated controls in nursery. *T. harzianum* R2 was the most promising inoculant with an increase of 75% of the fresh weight followed by *T. asperellum* LMR2 and with 60% and *T. harzianum* LMR3 with 57%. The two most effective inoculants *T. harzianum*R2 and *T. asperellum* LMR2 were assayed under field conditions. For *T. asperellum* LMR2 the increase in fresh shoot biomass corresponded to 69% and for *T. harzianum* R2 to 63%. The performance of the inoculants was also tested in lettuce plants under nursery and field conditions. No significant increase was observed in lettuce seedlings inoculated with *T. harzianum* LMR1, R1 and R2 compared to non-inoculated controls in pots. Lettuce seedlings inoculated with *T. harzianum* LMR1 and *T. harzianum* LMR3 showed the lowest fresh shoot biomass. In contrast, under field conditions bio-inoculation with *T. harzianum* LMR1, *T. harzianum* LMR3, *T. harzianum* R2 and *T. asperellum* LMR2 increased the shoot fresh biomass of lettuce compared to the non-inoculated controls. Bio-inoculation with *T. harzianum* LMR3 enhanced by 147% and *T. asperellum* LMR2 by 109% the weight of lettuce shoots under field conditions.

Figure 1: Plant growth promoting effect of native Trichoderma strains on the growth of Eruca sativa and Latuca sativa in nursery and field conditions.



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Seedlings were inoculated with isolates of T. asperellum (LMR2, R1) and T. harzianum (R2, R3, LMR1 and LMR3). White and gray columns represent means obtained in rocket and in lettuce, respectively. Means of shoot fresh mass of rocket and lettuce were calculated from 40 plants pretreatment after 5weeks of cultivation in pots in nursery (A), and from 35 plants after 6 weeks of cultivation in soil under field conditions (B and C). One-way ANOVA and Tukey post hoc-test compared performances between isolates and non-inoculated controls. Statistical significant differences in strain's performances in comparison with non-inoculated controls are indicated by different letters ($P \leq 0.05$). Statistical differences between bio-inoculants and the control conditions in soil are shown as: *** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$.

Biocontrol assay

All *Trichoderma* inoculants highly decreased mildew incidence on *E. sativa* cultivated under nursery conditions (from 2.8 to 16% disease incidence). Seedlings inoculated with *T. asperellum* R1 and *T. harzianum* LMR1 were less susceptible to be infected by *H. parasitica* than seedlings inoculated with the other *Trichoderma* strains (Table 2). Non-inoculated seedlings infected with *H. parasitica* presented 98% of mildew incidence with consequences in yields. Yields values also suggested that *Trichoderma* inoculated seedlings infected with *B. cinerea* achieved higher shoot biomass than non-inoculated healthier control seedlings (104,07 g for R1 versus 83 g control). Similarly, in *L. sativa* all *Trichoderma* inoculants were effective to decrease the *B. cinerea* infection responsible of grey mold (from 8 to 87% of disease incidence). Non-inoculated seedlings infected with *B. cinerea* presented 100% grey mold incidence, which caused plant death after 9-10 days. *T. harzianum* R2 and *T. asperellum* R1 inoculated seedlings presented only 8% and 18% grey mold incidence and the highest yields.

Table 2: Plant growth and disease development in *E. sativa* and *L. sativa* inoculated with *Trichoderma.*

Treatments	Disease incidence %	Yield g/pot
E. sativa	Downy Mildew	30 plants
T. asperellum R1 + *H. parasitica*	2,8	104,07
T. harzianum R2 + *H. parasitica*	10,8	57,98

T. harzianum R3 + *H. parasitica*	10	61,72
T. harzianum LMR1 + *H. parasíti-*	7,1	88,44
T. asperellum LMR2 + *H. parasi-*	16	101,57
T. harzianum LMR3 + *H. parasitica*	10	66,35
Infected with *H. parasitica*	98	55,5
Non-infected non-inoculated	0	83
L. sativa	Grey mold	50 plants
T. asperellum R1 + B. cinerea	18	158,85
T. harzianum R2 + *B. cinerea*	8	89,92
T. harzianum R3 + *B. cinerea*	24	46,52
T. harzianum LMR1 + *B. cinerea*	78	25,73
T. asperellum LMR2 + *B. cinerea*	20	71,64
T. harzianum LMR3 + *B. cinerea*	24	84,130
Infected with *B. cinerea*	100	0
Non-infected non-inoculated	0	150,47

Own sourse.

DISCUSSION

In this study, six native *Trichoderma* strains were isolated from *E. sativa* and *L. sativa* rhizosphere. Many species of the genus *Trichoderma* are well reported to control plant diseases and to promote plant growth ([20] Karuppiah, 2019; [21] Contreras-Cornejo, 2016). We report strains assigned to *T. asperellum* and *T. harzianum* as potential inoculants for this crops. Previously, the high potential application of this species as inoculants in horticulture was demonstrated ([22] Bayiee, 2019; [17] Fiorentino, 2018). In this study, a marked antagonistic effect of the six *Trichoderma* isolates against *B. cinerea* in dual culture assays (from 78 to 84% IR) was confirmed in a biocontrol assay with lettuce seedlings under nursery conditions. Among all isolates both *T. asperellum* strains, R1 and LMR2, were the most effective antagonists against all the plant pathogens. In a recent study, the mechanisms of *T. asperellum* as a biological control agent and plant growth promoter was associated with the induction of genes involved in secondary metabolism, mycoparasitism and plant growth compounds ([20] Karuppiah, 2019). Several *Trichoderma* species have been reported as direct (antibiosis, mycoparasitism, competition of space and resources) and indirect (induction of the systemic plant immune response) biocontrol agents of *B. cinerea* in plant assays ([22] Bayiee, 2019, [23] Vos, 2014). Further experiments on the most performing isolates (R1 and LMR2) should be conducted to identify their mechanisms of action in lettuce. Regarding the biofertilization assay, inoculation with almost all *Trichoderma* isolates increased significantly the fresh biomass of lettuce under field conditions, although no significant increase was observed on seedlings cultivated in nursery. In a previous study, the most pronounced effect of *Trichoderma* on lettuce was observed when plants were cultivated under low and optimal N availability ([17] Fiorentino, 2018). We hypothesize that the use of a rich substrate like compost in our nursery assay could not favored a strong plant-microbe interaction which under field conditions was beneficial probably due to the low fertility of the plot soil (under intensive cultivation). In the other hand, in rocket, inoculation with all *Trichoderma* inoculants showed a significant increase of shoot fresh biomass, showing up to 75% increase in nursery and up to 69% in field conditions. Both assays, rocket and lettuce, were conducted in an unfertilized soil plot which may favored the role of *Trichoderma* as biofertilizer and bioestimulant. Several studies have reported different mechanisms of actions of *Trichoderma* to enhance plant growth, including the synthesis of hormone-like compounds that stimulate root development increasing the area of nutrient and water absorption and the modulation of soil microbial community that consequently impact on plant productivity and sanity ([24] Pelagio-Flores, 2017; [25] Vinale, 2008; [26] Hermosa, 2012). It can be hypothesized that a synergic combination of these mechanisms could promote rocket and lettuce productivity in our study. Regarding the biocontrol effect, *T. asperellum* R1 inoculation on rocket seedlings decreased mildew incidence caused by *H. parasitica* up to 2,8% in comparison to non-inoculated controls (98% of mildew incidence). Similar results were reported in *Arabidopsis* plants that presented a higher systemic resistance thanks to the inoculation with *T. asperellum* T34 ([27] Segarra, 2013).

CONCLUSIONS

Bio-inoculation of rocket and lettuce with native Trichoderma strains increased yield of these crops and the decreased the incidence of mildew in rocket and gray mold in lettuce. Albeit further experiments need to be performed in different geographical locations, the potential of this Trichoderma isolates to enhance rocket and lettuce productivity/sanity was demonstrated in nursery and field's assays. These results suggest to continuing with the isolation and selection of promising native Trichoderma inoculants since its represent an eco-friendly alternative to improve yields of this widely consumed leafy vegetables.

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