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Trichoderma harzianum T-22 and BOL-12QD inhibit lateral root development of *Chenopodium quinoa* in axenic co-culture

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Abstract: To investigate the symbiotic interaction of *Trichoderma harzianum* Rifai on *Chenopodium quinoa* Willd. in isolation, we studied axenic co-culture of the *T. harzianum* isolates T-22 and BOL-12QD and the *C. quinoa* cultivars Kurmi and Maniqueña real. Neither T-22 nor BOL-12QD affected seedling growth during two days of co-culture in the early growth phase of rapid primary root extension. However, after longer axenic co-culture, T-22 and BOL-12 were found to significantly inhibit the overall growth of *C. quinoa* cv. Kurmi and Real, affecting also vitality parameters as seen for chlorophyll and betalains. Lateral root development was strongly inhibited in all plant–fungal combinations, leaving stunted lateral roots. These results suggest that *T. harzianum* has a general capacity to inhibit the growth of *C. quinoa* plants with a main effect on the lateral root development.

Subjects: Environment & Agriculture; Pest Management; Plant Biology; Bioscience; Mycology

Keywords: *Chenopodium quinoa*; axenic co-culture; lateral root; root growth inhibition; *Trichoderma harzianum Rifai*

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PUBLIC INTEREST STATEMENT

Cultivation of quinoa (*Chenopodium quinoa*) is expanding because it has a high nutrient content grain. Quinoa has a high abiotic stress resistance but is sensitive to pathogens. The fungus *Trichoderma harzianum* is a biocontrol agent widely used against agricultural diseases, because it can enhance plant growth, counteract detrimental microbes and activate the plant immune system. However, the mechanism of plant–*Trichoderma* interactions is not completely understood, and some plant varieties can even experience negative effects from these fungi. We have developed a sterile co-culture system for two *Trichoderma* strains and two quinoa cultivars. *T. harzianum* did not affect seedling growth during two days of co-culture, but over longer time, they inhibited the overall growth of quinoa. Lateral root development was strongly inhibited in all plant–fungal combinations. Thus, *T. harzianum* has the capacity to inhibit the growth of *C. quinoa* plants in long co-culture under sterile conditions.

1. Introduction

Chenopodium quinoa Willd. (Amaranthaceae) is an emerging crop because of its high nutritional value combined with its ability to endure and grow under harsh cultivation conditions (Bazile et al. 2016; Jacobsen, Mujica, & Jensen, 2003; Ruiz et al., 2014; Vega-Gálvez et al., 2010). However, quinoa crops in highlands, e.g. in the Andean plateau, are heavily affected by the downy mildew disease caused by the oomycete *Peronospora variabilis* (Danielsen & Ames, 2000; Danielsen, Bonifacio, & Ames, 2003; Gandarillas, Saravia, Plata, Quispe, & Ortiz-Romero, 2015; Testen, Del Mar Jiménez-Gasco, Ochoa, & Backman, 2014), which can result in drastic yield reductions (Danielsen & Munk, 2004). In order to halt the development of the downy mildew disease, heavy loads of chemical pesticide are being applied, but this is not the solution for countries like Bolivia that have chosen an organic production. Therefore, novel organic solutions like biological control strategies need to be developed. In this respect, *Trichoderma*, a fungal genus known for improving crop production, could be an interesting option to alleviate the damages caused by the downy mildew disease as observed for other crops (Nagaraju, Sudisha, Murthy, & Ito, 2012; Nandini, Hariprasad, Niranjana, Shetty, & Geetha, 2013; Perazzolli et al., 2012).

Trichoderma is known by many beneficial effects on plants like promoting plant growth, inducing plant systemic resistance and also directly antagonizing phytopathogens (Harman, Howell, Viterbo, Chet, & Lorito, 2004; Kubicek et al., 2011). Therefore, strains like *Trichoderma harzianum* T-22 are in commercial use for several crops (Contreras-Cornejo, Macías-Rodríguez, del-Val, & Larsen, 2016; Harman, 2011). However, studies of maize and tomato growth promotion by T-22 have shown a highly variable response depending on the plant genotype and its combination with *Trichoderma* strains (Harman, 2006; Schuster & Schmoll, 2010; Tucci, Ruocco, De Masi, De Palma, & Lorito, 2011). Hence, the optimal *Trichoderma* effect will depend on the choice of plant cultivar and *Trichoderma* strains, e.g. *Trichoderma* strains from the same species from different geographic regions might cause different growth effects. Native strains of *Trichoderma* from quinoa soils (*T. harzianum* and *Trichoderma koningiopsis*) have been reported to improve agricultural quinoa yields (Ortuño et al., 2013; Ortuño, Castillo, Miranda, Claros, & Soto, 2016). These two strains as well as a recent *T. harzianum* BOL-12QD were also shown to have antimycotic properties (Espinal Churata, Huanca, Terrazas Siles, & Giménez Turba, 2010; García-Espejo, Mamani-Mamani, Chávez-Lizárraga, & Álvarez-Aliaga, 2016).

To gain more detail on the interaction of *C. quinoa* and *T. harzianum* in isolation, we have here developed a system for axenic co-culture of *C. quinoa* and two biocontrol strains T-22 and BOL-12QD that allow both shoot and root analysis. The outcome of these interactions produced a general growth inhibition, where a lateral root growth inhibition appears to be the main cause.

2. Materials and methods

2.1. Biological material

Seeds of quinoa (*Chenopodium quinoa* Willd.) cultivars Maniqueña Real (*Real*) and Kurmi were kindly supplied by PROINPA (Quipaquipani, Bolivia). *Trichoderma harzianum*, Rifai, T-22, anamorph ATCC 20847 originated from a protoplast fusion of T12m, isolated from NY, USA (Hadar, Harman, & Taylor, 1984) and T95, which is a UV-induced mutant of T-Co isolated from Bogotá, Colombia (Ahmad & Ralph, 1987; Chet & Baker, 1981; Stasz, Harman, & Weeden, 1988). T-22 was purchased from the American Type Culture Collection (Manassas, VA, USA). *Trichoderma harzianum* BOL-12QD (BOL-12) was isolated and provided by the Instituto de Investigaciones Farmaco-bioquímicas (IIFB-UMSA, La Paz, Bolivia).

2.2. Fungal growth

T-22 and BOL-12QD were maintained on potato dextrose agar (BD-Difco, Detroit, USA) at 25°C. To isolate spore suspensions, 1 mL of sterile water was added to 2-week-old *Trichoderma* cultures on potato dextrose agar and collected conidia were filtered through a sterile piece of absorbent cotton. The spores were washed twice with sterile ddH_2O and pelleting at 3700g

for 5 min at 4°C in an Allegra X-12R centrifuge (Beckman, Brea, CA, USA). Spores were resuspended in sterile ddH_2O and kept at 4°C until experiments.

Germination of T-22 and BOL-12QD spores for *C. quinoa* treatment was performed as described by Yedidia, Benhamou, and Chet (1999) using 15 mL tubes shaken at 200 rpm for 18 h. The germinated spore suspension was washed twice by centrifugation as described above and finally resuspended in sterile ddH_2O. The final spore concentration was adjusted to be 1 germinated spore/µl and verified by colony forming unit (CFU) counts on potato dextrose agar Petri dishes.

For DNA extraction, growth tubes with 10 mL of Potato Dextrose Broth (BD-Difco, Detroit, USA) were inoculated with *Trichoderma harzianum* BOL-12QD and incubated for two days at 25°C in darkness. The biomass developed (<100 mg) was transferred to a microcentrifuge tube for later use.

2.3. Sterilization of *C. quinoa* seedlings and germination

Seeds of *C. quinoa* were surface-sterilized by soaking in commercial bleach (NaClO; 27 gkg^{-1}) for 20 min., followed by 6 rinses in sterile ddH_2O. Immediately thereafter, the seeds were placed on sterile water agar (8 gL^{-1}) in Petri dishes and incubated in darkness at 24°C for 14 h.

2.4. Co-culture of quinoa and *T. harzianum* in growth boxes

Three germinated axenic seedlings of each cultivar Kurmi and Real with similar root length were aligned on a straight line in 11.4 cm × 8.6 cm × 10.2 cm growth boxes (Phytatray II, Sigma). These contained 0.1× Murashige and Skoog Basal Salts Mixture (MS; Duchefa, Haarlem, The Netherlands), supplemented with 8 gL^{-1} agar in which the agar medium had solidified while the boxes were tilted to 45°. The seedlings were incubated at 24°C for 4 h under regular light (fluorescent tubes; 50 µmol $m^{-2}s^{-1}$) prior to T-22 and BOL-12QD treatment.

C. quinoa seedlings were treated by adding 10 µL [1 CFU $µL^{-1}$] of either T-22 or BOL-12QD germinated spore suspension on top of the neck of the primary root. Ten microliters of sterile ddH_2O were added to each seedling in the mock control group. After treatment, the seedlings were incubated for 14 days at 24°C under regular light with a 16 h light/8 h darkness photoperiod.

2.5. Co-culture of quinoa and *T. harzianum* in Petri dishes

Co-culture of *T. harzianum* and *C. quinoa* on square Petri dishes were carried out as for the growth box system, with the following changes: After germination, 5 seedlings of each cultivar (Kurmi and Real) with similar length were aligned in a straight line on 12 × 12 cm square Petri dishes containing 0.1× MS, 8 gL^{-1} agar, in which the agar had been solidified with the Petri dishes in a horizontal position. The Petri dishes were then tilted 45° during growth with the agar/air interface facing upwards and seedlings having the roots pointing towards the bottom part of the Petri dish. The seedlings were incubated at 24° C for 4 h under regular lights and then treated with T-22 or BOL-12QD as described above. After treatment, the seedlings were incubated at 24°C in a 16 h light/8 h darkness photoperiod either at regular or at high light intensity.

2.6. Co-culture under different light systems in Petri dishes

Co-cultivation under regular light intensity was done with fluorescent lights (Polylux XLr 30W, GE, Budapest, Hungary) 50 µmol $m^{-2}s^{-1}$ for 2 and 7 days. Co-cultivation under higher light intensity was done with white LED growth lights (UFO LED Grow Light 90W, JDSweden AB, Råå, Sweden) 175 µmol $m^{-2}s^{-1}$ for 10 and 6 days. For six days of co-cultivation, growth incubation was carried out as described above but on larger square Petri dishes (24 × 24 cm).

2.7. Seedling growth analysis

Main root length, shoot length, hypocotyl length, lateral root number and lateral root length were analyzed through images taken with a Digital Camera Canon EOS Rebel T3. Measurements from the photographs were done with the segmented line tool of *ImageJ* 1.49 (Abramoff, Magalhães, &

Ram, 2004). For fresh weight analysis, shoots were separated from the roots with a scalpel and mass determined using an analytical scale (Sartorius ED 124S, Goettingen, Germany). Root weights were not determined because of lateral root damage upon lifting. All seedlings with an incomplete expansion (cotyledons remaining attached to the seed husk) were discarded from analysis.

2.8. Chlorophyll and betalain determination

Intact whole shoots were placed in a pre-cooled mortar and ground under liquid nitrogen. One milliliter of water was added and the whole sample recovered and centrifuged at 12,300g for 5 min. The supernatant was used for betalain quantification and the pellet for chlorophyll quantification.

Betalain concentration was determined according to Castellar, Obón, Alacid, and Fernández-López (2003) from the absorbance at 535 nm in a Multiskan GO plate reader (Thermo Fisher Scientific, Vantaa, Finland), using an extinction coefficient of $E^{1\%}_{1cm}$ = 1120 (Kujala, Loponen, & Pihlaja, 2001).

For chlorophyll determination, the pellet was resuspended with acetone to a final concentration of 80% (v/v), left in the dark for 1 min and centrifuged for 5 min. The absorbance was read at 645, 663 and 750 nm in a Multiskan GO plate reader (Thermo Fisher Scientific, Vantaa, Finland). The total chlorophyll in the seedling was calculated according to Ni, Kim, and Chen (2009): µg chl/mg tissue = $8.02 \times (A_{663} - A_{750}) + 20.2 \times (A_{645} - A_{750})] \times (V/1000) \times W$ based on Arnon, Allen, and Whatley (1954).

2.9. Fungal DNA extraction and PCR

For DNA extraction, fungal biomass was collected in microcentrifuge tubes, frozen with liquid nitrogen and ground with a disposable tissue grinder. Then, 400 µL of lysis solution buffer AP1 (Qiagen, Valencia, CA, USA) was added, vortexed and ground again. The remaining procedure was carried out according to the manufacturer's instructions using DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

PCR of the Internal Transcribed Spacer (ITS) region was done with primer pairs ITS1/ITS4 as described by White, Bruns, Lee, and Taylor et al. (1990) using DreamTaq polymerase (Thermo Scientific, Carlsbad, CA, USA) supplemented with 0.2 mM dNTP Mix (Thermo Scientific), 1.25 mM $MgCl_2$ and with 0.25 µM of each primer. The PCR program had the following conditions: 1 cycle of: 95°C, 5 min; 30 cycles of 95° C, 30 s; 53°C, 30s; 72°C, 45s; 1 final cycle of 72°C for 5 min.

2.10. ITS sequencing

PCR products (150 ng) from the ITS region of the biocontrol strain *T. harzianum* BOL-12QD were directly sequenced by the Sanger method (Eurofins, Ebersberg, Germany) and confirmed for the complementary strand. *T. harzianum* BOL-12QD ITS sequence was deposited in the NCBI GenBank under accession number KY644517.1.

2.11. Statistics

Data of each set of experiments was analyzed separately for cultivars and was carried out by one-way analysis of variance. Statistical differences between treatments were tested with Tukey's HSD post-hoc test. Student's *t* test was used to compare controls of Kurmi and Real. Statistical analysis was carried out using R packages plyr (Wickham, 2011) and stats (R Core Team, 2016). Images were produced using ggplot2 (Wickham 2016).

3. Results

3.1. Molecular identification of *T. harzianum* BOL-12QD by ITS sequencing

In order to verify the native *Trichoderma* BOL-12QD isolate with molecular tools and provide a barcode, we sequenced the ITS region. The BOL-12QD strain, previously annotated as *Trichoderma*

inhamatum based on a morphological description (Espinal Churata et al., 2010), had an ITS sequence that was 524 bp long and had a match identity of 100% to 13 *T. harzianum* accessions registered at NCBI. Eleven nucleotides varied as compared to the T-22 ITS region. Therefore, the fungal BOL-12QD isolate is classified as *T. harzianum* BOL-12QD.

3.2. Growth of *C. quinoa* in axenic systems for treatment with*T. harzianum*

The effects of T-22 or BOL-12QD on *C. quinoa* in isolation were studied through axenic growth systems, where *C. quinoa* seedlings were grown on 0.1× MS and 0.8% agar in square Petri dishes or in growth boxes. To allow analyses of root growth on an agar surface, we made initial test with square Petri dishes standing vertically. However, seedling primary root tips were prone to lift from the agar surface and grow into the air, with consequentially restricted primary root growth. In contrast, in square Petri dishes tilted 45°, and in growth boxes with a similarly slanted agar surface, the roots followed the agar surface with a minimum of root lifting into the air phase or root growth into the agar medium. In this system, the seedlings grew fast and homogenously, extending to 8.0 ± 0.3 cm and 8.6 ± 0.3 cm (mean ± SE; n = 15) in Kurmi and Real, respectively, over 2 days of cultivation (data not shown).

3.3. Short-term treatment of *C. quinoa* with T-22 and BOL-12QD

C. quinoa seedlings of 18 h old and having similar sizes were treated with a T-22 or BOL-12QD germinated spore suspension or mock control and incubated for two days under regular light in square Petri dishes (Figure 1). The primary root length of each cultivar 2 days postinoculation (dpi) with T-22 or BOL-12QD was similar to the control (Figure 1(a,b)). However, the primary root length of the mock control real was significantly larger than control Kurmi ($p < 0.05$). Shoot length of both cultivars was unaffected after 2 days of co-culture with T-22 or BOL-12QD (Figure 1(c)). Comparing the mock controls, the shoot length of Kurmi was larger than real ($p < 0.05$). After this time of co-cultivation, there was not a measurable effect on the growth of the plant (Figure 1(a,c)), and quinoa seedlings were as large as the side of the square Petri dish, and thus close to reaching the Petri dish walls.

3.4. Long-term treatment of *C. quinoa* with T-22 and BOL-12QD in growth boxes

For investigating long-term effects, we co-cultivated 18-h-old quinoa seedlings with T-22 or BOL-12QD in growth boxes with slanted agar medium for 14 days. Shoot fresh weight was significantly reduced, similarly in cultivars Real and Kurmi (Figure 2(a)). In controls, the shoot fresh weight of real was similar to Kurmi. Root fresh weight was not quantified because lateral roots were stuck on the agar, thus detaching unevenly from the primary root when lifted.

Functional differences between cultivars and treatments were investigated through pigment shoot contents. Chlorophyll content was measured as an indicator of general vitality and Caryophyllales-specific betalain pigments as a marker for general defense response activation level (Polturak & Aharoni, 2018). The chlorophyll concentrations of mock-treated control seedlings were similar in real and Kurmi (Figure 2(b)). However, chlorophyll concentration was significantly decreased in both cultivars upon interaction with either strain of *Trichoderma*. The betalain concentration of the mock-treated control seedlings was significantly larger ($p < 0.05$) in Kurmi than in real (Figure 2(c)). Upon interaction with T-22 and BOL-12, the betalain level increased in both cultivars, being significantly different in Kurmi seedlings treated with T-22 and in real seedlings treated with BOL-12. Under these growth conditions, quinoa seedlings had very long hypocotyls, indicating a possible restriction by light.

Growth inhibition in axenic co-culture of *Arabidopsis thaliana* with *Trichoderma* sp. has been reported to be due to acidification of the growth media (Pelagio-Flores, Esparza-Reynoso, Garnica-Vergara, López-Bucio, & Herrera-Estrella, 2017). However, the roots of 2-day-old quinoa seedlings acidified the medium to a pH below 3.5 in the absence of *Trichoderma* (Supplementary Figure 1). *Trichoderma* was found to acidify the medium during the first days of mycelial growth (2 dpi), whereas as conidiation started, the pH increased to above 6 (5 dpi;

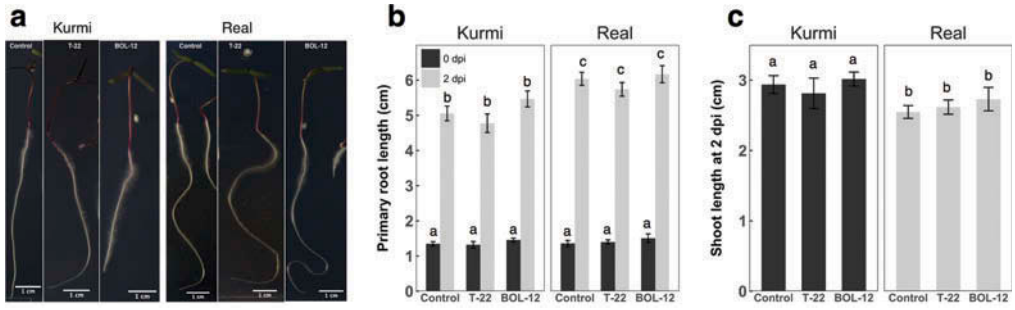

Figure 1. Short-term treatment of *C. quinoa* seedlings with T-22 and BOL-12 on square Petri dishes. (A) Representative images of at least 10 quinoa seedlings treated with T-22 or BOL-12 at 2 dpi. (B) Effect of T-22 and BOL-12 on primary root growth in *C. quinoa* seedlings at inoculation time (0 dpi; black bars; *n* = 10) and 2 dpi (gray bars; *n* = 24). (C) Effect of T-22 and BOL-12 on shoot growth in *C. quinoa* seedlings at 2 dpi (*n* = 15). Data shows means ± SE per treatment. Shoots were not measurable at time of inoculation (0 dpi) because they were still surrounding the seed coat.

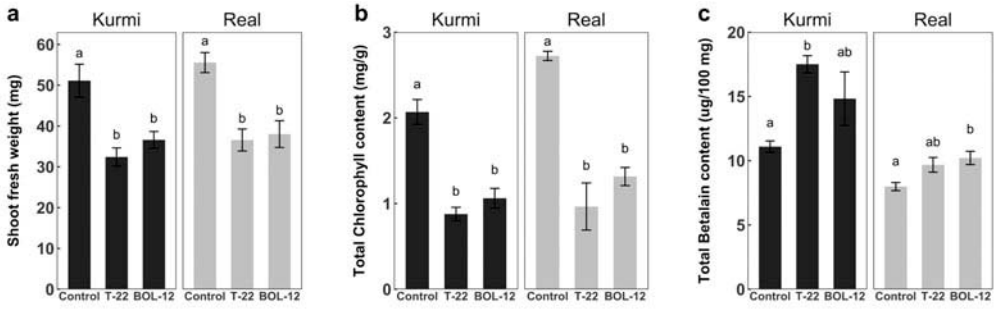

Figure 2. Long-term treatment of *C. quinoa* seedlings with T-22 and BOL-12 in growth boxes. Effect of T-22 and BOL-12 in *C. quinoa* seedlings at 14 dpi on (A) shoot growth (*n* = 17) (B) chlorophyll content (*n* = 3) and (C) betalain content (*n* = 3). Data shows means ± SE per treatment. Statistically significant differences ($p < 0.05$) are denoted with different letters.

Supplementary Figure 1). This indicates that the *Trichoderma* inhibition of the quinoa growth was not due to a lowered pH.

3.5. Trichoderma interactions at a higher light intensity

We then investigated co-cultivation under higher light intensity (175 µmol m^{-2} s^{-1}) in 45° angled square Petri dishes, which allows documentation of finer root and shoot structures without lifting seedlings from the agar surface. Quinoa seedlings grown under higher light intensity were almost as large as the sides of square Petri dishes (24 × 24 cm) after 6 days of incubation (Figure 3(a)) and had shorter hypocotyls than under regular light intensity (Figure 3(b)). Quinoa seedlings co-cultivated for 7 days under regular light intensity had already significantly longer hypocotyls than seedlings co-cultivated for 10 days under higher light intensity (Figure 3(b)). Real hypocotyls were significantly shorter when treated with BOL-12, and this was only observed under higher light intensity (Figure 3(b)).

Quinoa seedlings grown at higher light showed also growth inhibition after treatment with T-22 or BOL-12 (Figure 3), like observed under regular light intensity (Figure 2). In cultivar real, shoot fresh weight was significantly decreased (by approximately 36%) when treated with T-22 or BOL-12, as compared to the mock control. Shoot fresh weight in Kurmi was less affected by treatment with T-22 and BOL-12, displaying a decrease of 12% and 23%, respectively, and only the latter being significant (Figure 3(c)). We observed changes in the quinoa root architecture after treatment with *T. harzianum*. The number of lateral roots per length unit of primary root was similar in

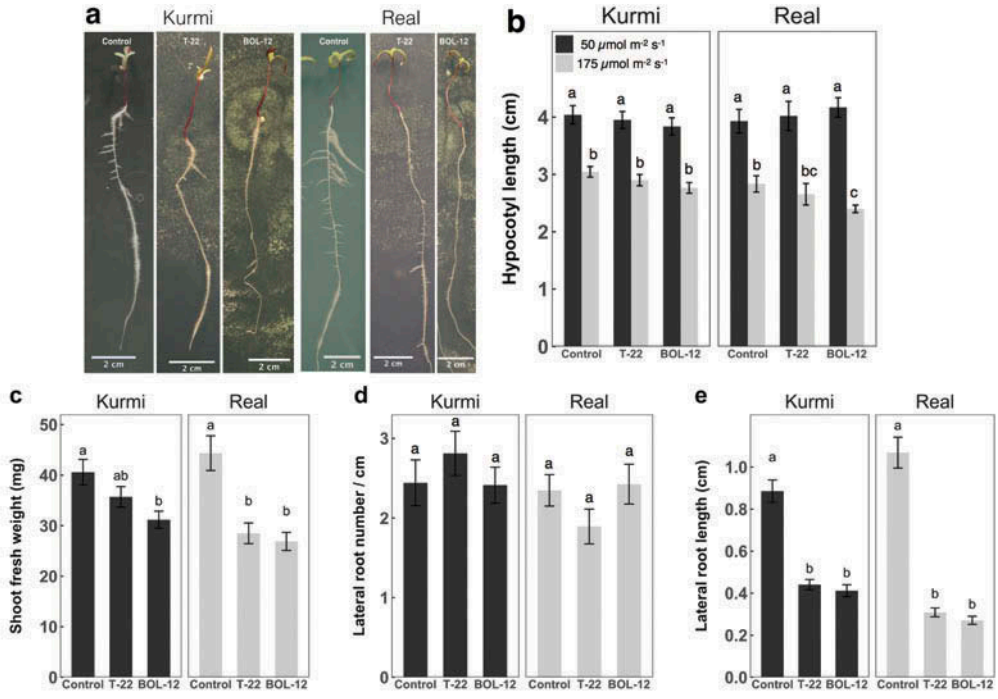

Figure 3. Co-cultivation of T-22 and BOL-12 with *C. quinoa* seedlings under higher light intensity in square Petri dishes. (A) Representative images of at least 3 quinoa seedlings treated with T-22 or BOL-12 at 6 dpi. (B) Effect of higher light intensity (black bars) on hypocotyl growth in *C. quinoa* seedlings treated with T-22 or BOL-12 compared with regular light intensity (gray bars; *n* = 17). Effect of T-22 and BOL-12 in *C. quinoa* seedlings at 10 dpi on (C) shoot growth (*n* = 19), (D) lateral root number (*n* = 12) and (E) lateral root growth (*n* = 19). Data shows means ± SE per condition. Statistically significant differences ($p < 0.05$) are denoted with different letters.

plantlets treated with T-22 or BOL-12 and in the mock control. Also, for both mock-treated cultivars, the number of lateral roots was similar (Figure 3(d)). In contrast, extension of lateral roots (Figure 3(e)) was strongly and significantly inhibited by treatment with either strain of *T. harzianum*. The reduction by the two *T. harzianum* strains was stronger in real (71–75%) than in Kurmi (50–53%) (Figure 3(a,e)). Lateral roots in both Kurmi and real seedlings treated with T-22 or BOL-12 had a stunted appearance (Figure 4), and this phenomenon was accompanied by browning of the lateral roots (Figure 4) and a decrease in lateral root hairs (not shown). Further, the primary root red color, especially in Kurmi, was decreased upon treatment with either strain of *T. harzianum*, indicating a loss of root betalain (Figure 4).

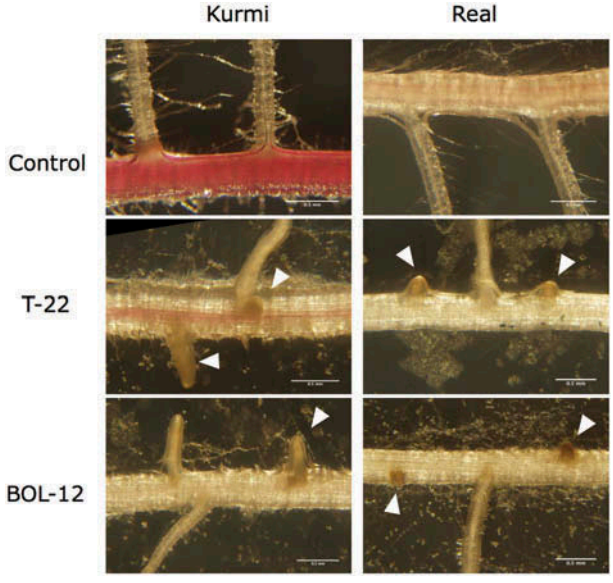

Figure 4. Effects of T-22 and BOL-12 on quinoa lateral roots. The figure shows representative images among three *C. quinoa* seedling replicate sets, examined for lateral roots emergences after treatment with T-22 or BOL-12 for 10 days. The images were taken at 3 cm from the root neck. Arrowheads denote stunted lateral roots. The scale bars represent 500 µm.

4. Discussion

Trichoderma species is the agriculturally most used microbial organism group for plant biocontrol and growth stimulation (Mukherjee, 2013). Growth stimulation by *T. harzianum* has been observed in many crop plants grown on soil (Harman, Taylor, & Stasz, 1989; Maag et al., 2013; Tucci et al., 2011; Yedidia, Srivastva, Kapulnik, & Chet, 2001), including also quinoa (Ortuño et al., 2013, 2016). However, the effect of *Trichoderma* on the growth outcome of plants has been found highly variable depending on fungal and plant genotypes as well as the growth conditions established (Harman, 2006; Tucci et al., 2011). For example, the commercially used biocontrol agent T-22 was found to inhibit the growth of certain cultivars of tomato (Tucci et al., 2011) and maize (Harman, 2006) in soil experiments. The large variation of outcomes by the plant-*Trichoderma* interactions is, however, difficult to investigate in soil systems because of the potential effects of other organisms, which cannot be controlled.

In axenic co-cultivation, we observed negative effects of two strains of *T. harzianum* on two cultivars of quinoa, affecting shoot growth as well as chlorophyll levels. The similar acidification of the medium by both quinoa and *T. harzianum* indicates that the inhibition of quinoa growth was not caused by *Trichoderma* acidification of the medium, which has been observed for *A. thaliana* (Pelagio-Flores et al., 2017). This is also consistent with the low pH observed for the germination of some quinoa cultivars (germination above 90% at pH 4.5) (González, Languasco, & Prado, 2014) and growth in a wide range of soils, ranging from acid soils with a pH of 4.5 to alkaline soils with a pH of 8.5 (Bhargava & Srivastava, 2013). In order to set a precise time for the beginning of the interaction in the axenic co-cultures, we inoculated pregerminated *T. harzianum* spores on top of the seedling root necks. In previous reports on sterile co-culture of plants and *Trichoderma*, interactions have generally been observed to promote plant growth. Examples include *A. thaliana*, tomato and tobacco seedlings in axenic co-cultures with *Trichoderma virens* Gv29.8, *Trichoderma atroviride* IMI 206040 and *T. harzianum* CECT 2413, respectively (Chacón, Rofríguez-Galán, & Al, 2007; Contreras-Cornejo, Macías-Rodríguez, Alfaro-Cuevas, & López-Bucio, 2014; Contreras-Cornejo, Macías-Rodríguez, Cortés-Penagos, & López-Bucio, 2009; Samolski, Rincón, Pinzón, Viterbo, & Monte, 2012). In these investigations, *Trichoderma* inoculum has been positioned a few centimeters away from the plant on the agar surface. Such physical separation yet indirect contact between *Trichoderma* and the plant allows for a period of time before physical interaction. During this period, the organisms can interchange molecular compounds and signals that may affect the outcome of the following physical interaction. For example, *Trichoderma* volatile organic compounds alone can strongly promote growth of *A. thaliana*, most likely by stimulating lateral root growth (Contreras-Cornejo, Macías-Rodríguez, Herrera-Estrella, & López-Bucio, 2014; Hung, Lee, & Bennett, 2013; Kottb, Gigolashvili, Großkinsky, & Piechulla, 2015). In addition, symbiotic mycorrhizal fungi start colonization only after chemical signaling, where plants secrete the appropriate signal into the soil and the fungi recognize and respond to it (Jones & Dangl, 2006; Oldroyd, 2013). Whether a similar preparatory signaling occurs between plant roots and *Trichoderma* is not known.

Overall, the two *Trichoderma* strains did not qualitatively differ regarding the effect on the growth of either of the two quinoa cultivars or vice versa. As described above, a small number of *Trichoderma* strains and plant genotypes that are non-compatible for growth promotion have been reported (Harman, 2006; Tucci et al., 2011). Though it cannot be excluded that the quinoa and *T. harzianum* genotypes tested were untypical incompatible variants, the qualitatively similar effects on the two plant cultivars indicate that the *T. harzianum*-induced growth inhibition may be typical for quinoa in the described axenic co-cultures. The largest differences observed between Kurmi and real on the response to the strains of *Trichoderma* were a somewhat stronger inhibitory effect on growth in Real (Figure 3). This may be a consequence of real growing significantly faster than Kurmi (Figures 2 and 3). Trade-off between growth rate and stress resistance has been described for several plant species (Weih, 2003).

Consistently, a main differences between previously analyzed *Trichoderma*-plant interactions in axenic cultures and this *T. harzianum*-quinoa study is the low age of the quinoa seedlings upon treatment; 4–25 days in previous studies (Contreras-Cornejo, et al. 2014; Contreras-Cornejo et al., 2009; Saenz-Mata & Jimenez-Bremont, 2012; Sáenz-Mata, Salazar-Badillo, & Jiménez-Bremont, 2014; Samolski et al., 2012) versus 18 h in quinoa. The young age of treated quinoa was a necessity, due to the extremely high growth rate (approximately 4 cm/day) in axenic culture, also necessitating large growth vessels to be used. The young age and high rate of growth may have been contributing factors to the negative effect on quinoa by *T. harzianum*. In general, biotic stress resistance depends of plant age, the younger the plant the more susceptible it is. This correlation has been shown for several plant species against different pathogens, e.g. wheat against *Puccinia striiformis* (Farber & Mundt, 2017), broccoli against *Peronospora parasitica* (Dickson & Petzoldt, 1993) and *Arabidopsis* against *Pseudomonas syringae* (Carella, Wilson, & Cameron, 2015; Kus, Zaton, Sarkar, & Cameron, 2002; Panter & Jones, 2002).

In the axenic cultures of quinoa, lateral root growth was the parameter most strongly affected by *Trichoderma* treatment. Stunting of lateral root growth (Figure 3(a,e)) as well as browning of lateral root primordia and entire lateral roots was observed (Figure 4). This indicates that quinoa is directly damaged by *Trichoderma*. Similar lateral root damage has been observed in *Medicago truncatula* roots infected by the phytopathogenic fungi *Fusarium solani* f. sp. *phaseoli* (Salzer et al., 2000). Consistently, the general changes in betalain content in quinoa upon *T. harzianum* treatment (Figure 2(c)) may be a sign of plant defense as previously seen in red beets infiltrated with *P. syringae* (Sepúlveda-Jiménez, Rueda-Benítez, Porta, & Rocha-Sosa, 2004) and betalain-producing transgenic tobacco against *Botrytis cinerea* (Polturak et al., 2017). Interestingly, Kurmi had a larger content of betalain than Real (Figures 2(c) and 4) and was less negatively affected by *T. harzianum*. Betalains are analogous to anthocyanins, which likewise are induced by biotic and abiotic stress (Khan & Giridhar, 2015). A representative anthocyanin, camalexin, is usually induced in response to phytopathogenic attacks (Lemarié et al., 2015; Mert-Türk, Bennett, Mansfield, & Holub, 2003). However, an increase in camalexin concentration has also been observed when *A. thaliana* growth was treated with *Trichoderma* spp., inducing growth enhancement (Contreras-Cornejo, Macías-Rodríguez, Beltrán-Peña, Herrera-Estrella, & López-Bucio, 2011; Salas-Marina et al., 2011). Changes in betalain in quinoa may have an analogous function.

In summary, the plant growth inhibition and lateral root stunting observed when co-culturing *C. quinoa* with biocontrol strains of *T. harzianum* under axenic conditions indicate that *T. harzianum* in particular growth regimes can cause harm to plants. Elucidating the mechanisms behind this damage may explain the exceptions from growth enhancement that have been observed for *Trichoderma* treatment of crop plants growing on soil.

Supplementary material
Supplemental material for this article can be accessed here.

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