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Isolation of *Trichoderma* Spp. from Desert Soil, Biocontrol Potential Evaluation and Liquid Culture Production of Conidia Using Agricultural Fertilizers

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Abstract

Three *Trichoderma* isolates were obtained from sandy soils collected at the "Gran Desierto de Altar" in the northwest of Mexico and characterized by morphologic and molecular analyses as *Trichoderma harzianum* 8.4, *Trichoderma asperellum* 12-2 and *Trichoderma asperellum* BP60. Isolate *T. asperellum* BP60 inhibited *Setophoma terrestris*, grew above 50°C, and produced chitinases and siderophores, therefore it was chosen to obtain enough biomass and conidia for field applications. Conidia production was intended in liquid culture fermentation using food grade ingredients and agricultural fertilizers. Assays were done using baffled Erlenmeyer flasks containing 75 mL of culture media, kept under constant agitation at 150 RPM, with initial pH adjusted to 6.5 (NaOH 1N) at 28 ± 2°C and evaluated at 3, 6, 9 and 12 days after inoculation (DAI). Among the carbon sources, sucrose and vinaze; the former induced higher yields of biomass and conidia. Regarding nitrogen sources, the fertilizer $(NH_4)NO_3$ induced higher conidia yield. V8 juice (V8) induced the highest effect on production of both biomass and conidia. Therefore, maximum yield was 1.06×10^9 conidia.mL^{-1}, with the formulation with 5 g of KH_2PO_4 (MKP, Greenhow®), 1.3 g of $MgSO_4 \cdot 7H_2O$ (Sul-Mag, Peñoles®), 20 mg of $FeCl_3 \cdot 6H_2O$ (Fermont®), 150 ml of V8, 10 ml of vinaze and 2.5 g.L^{-1} of $(NH_4)NO_3$. Results presented here prove the potential for using an alternative, low cost, liquid media to produce conidia of *T. asperellum*.

Keywords: Biocontrol; Liquid culture media; Conidia production

Introduction

Horticultural crops in the northwest of Mexico have increased in area and diversity. An example is the valley of San Luis Rio Colorado; where agriculture has diversified from traditional crops such as cotton and wheat, to vegetables including green onions, celery, broccoli, radish, asparagus, watermelon, and Brussels sprouts. The eastern section of the valley of San Luis Rio Colorado, is located within the limits of the "Gran Desierto de Altar" [1]. In this area, green onions have a high economic impact, since most of the production is exported to United States of America and the United Kingdom [2]. On average this crop is grown on 5,000 ha yearly, generating an approximated income of $12,800.00 USD.ha^{-1} [3]. Pink root rot in green onion is caused by *Setophoma terrestris* (H.N. Hansen) Gruyter, Aveskamp and Verkley, comb. nov. MycoBank MB514659. The disease is enhanced by high temperatures and it is especially important in soils with low organic matter, high plant densities and minimal crop rotations [4,5]. According to the growers, the disease in the valley of San Luis Rio Colorado has increased from 2008 to 2014 growing seasons, which accounts for losses of up to 15% of the crop yield, and therefore has great economic impact. Traditional chemical control of pink root rot is not an option for growers in this area, because to export vegetables to international markets there are standards on food quality and food safety to fulfill; and thus it encourages the use of alternative techniques with less environmental impact. The practices of integrated agricultural management, where chemicals are replaced by bioproducts, are the most suitable option [6]. Biological control agents (BCA) are bioproducts based on microorganisms that cause harmful alterations to plant pathogens by chemical or physical processes [7,8]. BCA differ from chemical agents in that to be effective, they need to grow and successfully colonize and therefore they need

to be applied in high and frequent quantities [9]. Fungi from the genus *Trichoderma* spp. have a long history of successful control of plant diseases [7,10,11]. Several mechanisms have been described as responsible for their biocontrol activity, including competition for space and nutrients, biofertilization and stimulation of the plant defense systems, rhizosphere modification, secretion of chitinolotic enzymes, mycoparasitism and production of inhibitory compounds [7,8,12]. Since all these mechanisms produce an effective control after the colonization of plant roots; the ability to suppress a disease is directly proportional to their population density. *Trichoderma* spp. produce three types of propagules: hyphae, chlamydospores and conidia [13,14]. All can be used in formulated bioproducts, however since hyphae cannot withstand some scale-up processes, chlamydospores and conidia are used as the active ingredients; normally the production focuses on conidia, because of their higher production [15,16]. For an agroindustry producing their own *Trichoderma*-based products, liquid fermentation has several advantages, among others, conidia

are produced in a short time, their production does not require much space and labor, and any contamination can be controlled easily [15]. Studies on growth media for *Trichoderma* spp. showed that nitrogen sources are essential for the production and germination of conidia, but the effect of carbon sources on conidial and biomass production, differ among isolates easily [16]. The use of agricultural fertilizers as ingredients in culture medium for the growth of *Trichoderma spp.* is uncommon, but they have been used in microalgae scaling processes [17,18]. In *Trichoderma*, nitrogen fertilizers stimulate growth and conidia production and, in large amounts may have a synergistic effect on biocontrol effectiveness [19]. In general, the use of food grade ingredients for BCA is uncommon because of the potential risk of promoting growth of undesirable microorganisms [20], however since these ingredients are low-cost alternatives to media formulations, here we tested agricultural fertilizers and food grade ingredients to develop a media capable of inducing a high yield of conidia from a selected *Trichoderma* sp.

Materials and Methods

Area of study

Soil samples were collected on the agricultural farm "Los Pivotes del Desierto" located at the "Gran Desierto de Altar" (32°19'58.4" N, 114°52'08.4" W), near the city of San Luis Rio Colorado, Sonora, Mexico. Average temperatures range from a maximum of 32.0°C to a minimum of 13.5°C, with an annual average of 22.7°C. Although, in summer temperatures can reach up to 50°C [21]. Soil composition is sandy, belonging to the Entisol group in the Soil Taxonomy classification of USDA [22].

Isolation and identification of fungal strains

An isolate of *S. terrestris* was obtained from green onion showing pink root rot disease, and identified morphologically after cultivation on PDA media. For *Trichoderma* spp. isolation, soil samples were taken using a soil auger of 2.5 cm diameter from the 0-15 cm top layer, fifteen samples were mixed to make a composite sample of around 0.5 Kg. Fourteen composite samples were obtained, placed in plastic bags, kept cool and brought to the laboratory of phytopathology of the Center for Scientific Research and Higher Education of Ensenada (CICESE). From each composed sample, 10 g of soil was weighed and mixed 1:1 w/v with sterile distilled water and a two drops of Tween20, vortexed (3500 RPM), to suspend soil particles and let stand for five min. From the supernatant, serial dilutions were done up to 10^{-3}. From each dilution, 100 μL were plated onto PDA amended with rose Bengal (25 mg.L^{-1}) and chloramphenicol (10 mg.L^{-1}). Plates were maintained at 28 ± 1°C, for 10 days in darkness and checked daily. Well-defined colonies that had the characteristic features of the genus *Trichoderma* spp. were recovered on fresh PDA plates. To confirm *Trichoderma* identity, morphology was observed using a Zeiss inverted microscope (100X). Mycelium with conidia of putative *Trichoderma* strains were harvested by scraping, suspended in sterile distilled water and stored at 4°C for further use. For long- term preservation, strains were placed in 5 mL.L^{-1} of glycerol (20%) and kept at -80°C. To molecularly identify *Trichoderma*-like isolates, DNA was extracted using a commercial kit (Qiagen DNeasy kit®), DNA integrity was verified by gel electrophoresis and PCR the elongation factor-1α (*EF1a*) and their Internal transcribed spacer (ITS) were amplified. Primers used were EF1 (ATGGGTAAGGA(A/G) GACAAGAC) and EF2 (GGA(G/A)GTACCAGT(G/C)ATCATGTT) for *EF1a* [23], and ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) for ITS [24]. DNA amplification was done using 50 ng of DNA, 5 μl of 10 × Taq polymerase buffer,

0.5 U of Taq polymerase, 10 mM of dNTPs mix, 1 mM of reverse and forward primers and deionized water to complete a total volume of 50 μl. For the amplification of EF1a the BioRad Thermocycler was programmed with an initial denaturing at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min and the final extension at 72°C for 7 min. For ITS, PCR amplification was done as for EF1a, except that the annealing temperature was adjusted to 59°C. PCR products were analyzed by gel electrophoresis and amplified fragments purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Calif, USA), according to the manufacturer's indications. Cleaned PCR products were sent to Clemson University Genome Institute (CUGI) for sequencing. Resulting sequences were compared using the NCBI GenBank database.

Biocontrol potential evaluation

Biological control capabilities of the isolated strains against *S. terrestris* were evaluated by performing volatile organic compounds, non-volatile organic compounds, and competition assays on agar plates according to the methods reported by Dennis and Webster, Dennis and Webster and Royse and Ries, respectively [25-27]. Also, Siderophore Siderophore [28] and chitinase [29] production were evaluated. Finally, to test for thermic tolerance, isolates were placed on PDA, and grown at 30 ± 1°C, 40 ± 1°C, 50 ± 1°C and 60 ± 1°C, for 5 days in full darkness. Obtained results were analyzed to select an isolate for further experiments.

Inoculum recovery for conidia production assay

From the selected isolate, conidia were harvested by scraping the surface of a 7 day-old culture grown on a PDA plate. The concentration was adjusted to $1 × 10^6$ conidia mL^{-1} in 10 mL centrifuge tubes using sterile deionized water. In all following assays, 1% (v/v) of this initial concentration was used as starting inoculum.

Selection of culture media

All tested media contained a basic formulation per liter consisting in: KH$_2$PO$_4$ (MKP, Greenhow®), 5 g; MgSO$_4$•7H$_2$O (Sul-Mag, Peñoles®), 1.3 g; and FeCl$_3$•6H$_2$O (Fermont®), 20 mg. An amendment of 150 mL.L^{-1} V8 juice (8 Verduras, Herdez®) was used. Assayed carbon sources were 8 g.L^{-1} of sucrose (Sulka®), and 10 mL.L^{-1} of vinaze, a residual product from *Agave tequilana* blue Weber from the tequila distillation process (Tecno Ferti-V, Vida Verde®). Nitrogen sources used were, KNO$_3$•K$_2$SO$_4$ (Nitro K Sul, Greenhow®), (NH$_4$)NO$_3$ (Sulfonit, ISAOSA®) and (NH$_4$)•2SO$_4$ (granulated ammonium sulfate, ISAOSA®) at 10 g.L^{-1}.

Assay 1

To select from the suitable ingredients, an assay was stablished using all the possible combinations, thus treatments were as follows: T1 (V8/sucrose/KNO$_3$•K$_2$SO$_4$), T2 (V8/sucrose/(NH$_4$)NO$_3$), T3 (V8/sucrose/(NH$_4$)•2SO$_4$), T4 (V8/vinaze/KNO$_3$•K$_2$SO$_4$), T5 (V8/vinaze/(NH$_4$)NO$_3$), T6 (V8/vinaze/(NH$_4$)•2SO$_4$), T7 (NA/sucrose/KNO$_3$•K$_2$SO$_4$), T8 (NA/sucrose/(NH$_4$)NO$_3$), T9 (NA/sucrose/(NH$_4$)•2SO$_4$), T10 (NA/vinaze/KNO$_3$•K$_2$SO$_4$), T11 (NA/vinaze/(NH$_4$)NO$_3$) and T12 (NA/vinaze/(NH$_4$)•2SO$_4$). After adding all ingredients the pH was adjusted to 6.5 using NaOH 1N. Autoclaved media was distributed into Erlenmeyer flasks with three baffles (AvitroLab®, 250 mL) placing 75 mL in each. Flasks were maintained under continuous agitation at 150 RPM and 28 ± 2°C and placed in a complete randomized array. Sampling to estimate biomass and conidia production was done at 3, 6, 9 and 12 days after inoculation (DAI) as described below, and pH was

measured directly for each experimental unit. All experiments were done in triplicate.

Assay 2

Once results were evaluated, and in order to increase conidia production, a second assay was done by selecting the culture media that showed the higher yield in the first assay. All growing conditions, basic culture media, evaluation of conidia and biomass production were performed exactly as in the first experiment.

Evaluation of conidia and biomass production

To evaluate the produced conidia in each experimental unit, a 1 mL aliquot was taken into a microcentrifuge tube and counting was performed directly using an improved Neubauer Chamber (Housser Scientific®) as described before [15,25-31]. Concentration values are expressed in conidia.mL^{-1}. To quantify the produced biomass, the rest of the culture was passed through a Whatman® grade 4 qualitative filter paper, using a vacuum pump at 24 ± 1 in Hg vac (Gast™ V4BG608X, Fisher Scientific Inc.). Filters were dried at 95°C overnight to obtain a constant weight (± 0.005 g) and cooled at room temperature in a desiccator. Dry weight values were obtained (Explorer® OHAUS®), and reported as g.L^{-1} [13,15,32].

Statistical analysis

Data was analyzed by a factorial analysis of variance (ANOVA) and Tukey HSD test was applied when ANOVA revealed significant differences (P ≤ 0.05). Software used was Statistica 12©, StatSoft, Inc. 1984-2013.

Results and Discussion

Isolation, identification and evaluation of biocontrol potential

Three strains were isolated from desert sandy soils and identified by morphological and molecular analysis as *T. harzianum* 8.4, *T. asperellum* 12-2, and *T. asperellum* BP60 (Figure 1A-1C), respectively. While *Trichoderma* has been reported as the most abundant fungi in soil [8,11,25,26], the dry environment and lack of organic matter in the site might be reducing the occurrence and diversity of this species. Similarly,

low *Trichoderma* recovery was obtained before in sandy soils of Egypt [33] and in beaches of Brazil [34]. Isolates grew well at 30°C, 40°C and 50°C without noticeable changes, *T. asperellum* BP60 also grew at 60 ± 1°C although it showed reduced growth. Inhibition percent against *S. terrestris* were on average 33, 35 and 30%, respectively. In the evaluation of the production of volatile compounds, all *Trichoderma* isolates reduced the size of *S. terrestris* when compared with the control, also the color changed from purple to white, especially in the presence of *T. asperellum* T12-2. When evaluating the production of volatile compounds, only *T. asperellum* BP60 and *T. harzianum* T8.4 reduced the size of the colony of *S. terrestris,* but it was not significantly different from the control. All three isolates were capable of produce chitinases, but only *T. asperellum* BP60 produced siderophores. Among the isolates, *T. asperellum* BP60 showed the most promising characteristics as a biological control agent. It presented adverse effects on *S. terrestris* (Figure 1D), produces quitinases and siderophores (Figure 1E and 1F), grows at higher temperature, as well as producing volatile compounds and to inhibit *S. terrestris* growth. Therefore isolate BP60 was chosen to perform the next experiments. Previous reports indicate *that T. asperellum* had biological control activity over *Phytophthora capsici, P. megakarya* and *Rhizoctonia solani* [35-37]. *T. asperellum* BP60 was also capable of growing in a broad range of temperatures, since temperatures in the zone can reach up to 50°C, the isolate is well adapted to the predominant conditions and could therefore tolerate and prevail in the region.

Selection of culture media for conidia production of *T. asperellum* BP60

Assay 1: Higher biomass and conidia production of *T. asperellum* BP60 were obtained in all treatments containing V8 juice. Regarding biomass, T4 was the more efficient media containing V8, where *T. asperellum* BP60 reached 4.18 g.L^{-1} at 6 DAI (Figure 2A); this represents an increase of 79.6% when compared to T8, which showed the lowest value among the treatments without V8 (0.85 g.L^{-1} at 9 DAI) (Figure 2B). No statistical differences were detected between T1 and T2; and between T3 and T5 (Figure 2A). Meanwhile, maximum conidia production was 2.88 × 10^{8} conidia.mL^{-1} in T5 at 12 DAI (Figure 2C). Among treatments without V8, maximum conidia concentration obtained was 4.38 × 10^{6} conidia.mL^{-1} in T11 at 9 DAI (Figure 2D).
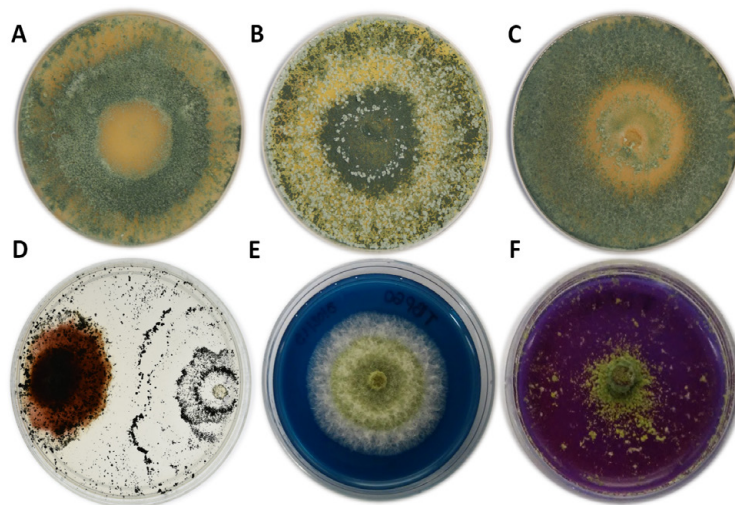

Figure 1: Native *Trichoderma* isolated from agricultural sandy soils of "Gran Desierto de Altar" in Sonora, Mexico (32°19'58.4"N 114°52'08.4"W) growth after 7 days on PDA at 28°C: (A)*Trichoderma harzianum* 8.4, (B) *T. asperellum* 12-2, and (C) *T. asperellum* BP60. (D) Competition assay of *T. asperellum* BP60 with *Setophoma terrestris* after 10 days of growth, (E) production of siderophores and (F) chitinase production.
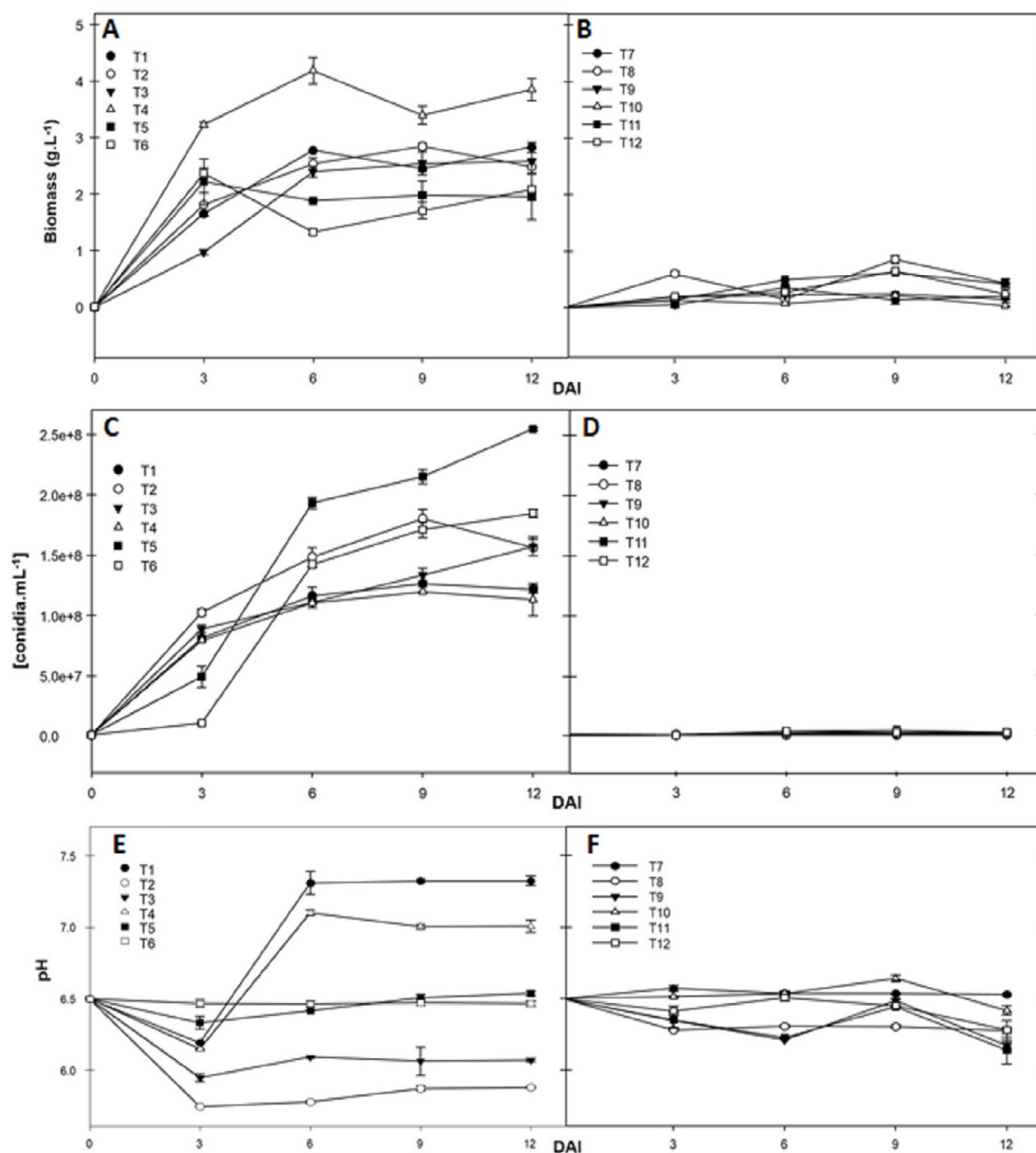
Figure 2: Biomass and conidia production and pH changes of *T. asperellum* in a fertilizer-based liquid media culture containing V8 juice (A, C and E) and without V8 juice (B, D and F). As carbon source treatments T1(●), T2(○), T3(▼), T7(●), T8(○) and T9(▼) contained sucrose; T4(△), T5(■), T6(□), T10(△), T11(■) and T12(□) contained vinaze. Used nitrogen sources were $KNO_3 \cdot K_2SO_4$ (T1, T4, T7 and T10), $(NH_4)NO_3$ (T2, T5, T8 and T11) and $(NH_4) \cdot 2SO_4$ (T3, T6, T9 and T12). All treatments were inoculated with 1×10^6 conidia.mL^{-1} at 1% (v/v), growth conditions were maintained during 12 days after inoculation (DAI) at 28 ± 2°C, 150 RPM and initial pH adjusted to 6.5 (NaOH 1N). Each data point indicates the mean (± S.E) of three replicates.

Comparing treatments with the lowest concentrations of conidia (T6 and T11) with or without V8, conidial concentration was increased by 98.2%. Treatments that contained $(NH_4)NO_3$ as the nitrogen source have higher conidia concentrations (T5 and T2). pH was nearly constant amongst the group without V8 (Figure 2E) with a minimal value of 6.12 for T11 at 12 DAI and a maximum of 6.64 for T10 at 9 DAI. In contrast, the treatments with V8 showed a pH ranging from 5.74 for T6 at 3 DAI, up to 7.32 for T3 at 12 DAI (Figure 2F). Among all the ingredients used to formulate the culture media, V8 juice had a major effect over biomass and conidia production, becoming the most valuable ingredient in the formulation. All treatments without V8, showed a minimal growth in conidial concentration.

Assay 2: To improve the treatment T5, which yielded the highest conidia concentrations in assay 1, and in an attempt to decrease the use its ingredients; V8 juice and vinaze were first assayed at 50, 100 or 150 mL.L^{-1} and at 10, 15 or 20 mL.L^{-1}, respectively. As before, all treatments were inoculated with 1×10^6 conidia.mL^{-1} at 1% (v/v), maintained during 12 days at 28 ± 2°C, 150 RPM and initial pH adjusted to 6.5 (NaOH 1N). In all treatments, pH varied from 6.54 to 6.63. The highest biomass of 1.62 g.L^{-1} was obtained when using 100 ml of V8 and 15

Treatment	V8 (mL.L^{-1})	Vinaze (mL.L^{-1})	Biomass (g.L^{-1})	Conidia.mL^{-1}	pH
T5.1	50	10	0.54a	4.65×10^7a	6.42bc
T5.2	50	15	0.78b	4.84×10^7a	6.34a
T5.3	50	20	0.35a	5.36×10^7a	6.34a
T5.4	100	10	1.50de	1.05×10^8b	6.63f
T5.5	100	15	1.07c	9.63×10^7b	6.52e
T5.6	100	20	1.62e	1.01×10^8b	6.39b
T5.7	150	10	0.83b	1.63×10^8d	6.46cd
T5.8	150	15	0.74b	1.46×10^8c	6.46cd
T5.9	150	20	1.32d	9.20×10^7b	6.42bcd

All treatments were inoculated with 1×10^6 conidia.mL^{-1} at 1% (v/v), growth conditions were maintained during 12 days after inoculation at 28 ± 2°C, 150 RPM and initial pH adjusted to 6.5 (NaOH 1N). Values indicate the means of four samples with three replicates each. Letters indicate significant differences by Tukey HSD ($P \leq 0.05$).

Table 1: *T. asperellum* BP60 production of biomass, conidia concentration and pH response in liquid culture media containing different concentrations of V8 juice and vinaze.

ml of vinaze (T5.5), while maximum conidia yields of 1.63×10^8 conidia.mL^{-1} where obtained using 150 ml of V8 and 10 ml of vinaze (Table 1), since conidia are preferred over biomass, treatment T5.7 was considered the best. Consequently, to evaluate for the suitable amount of $(NH_4)NO_3$ in T5.7, concentrations of 10, 7.5, 5.0 or 2.5 g.L^{-1} were used. With 10 g.L^{-1}, conidia concentrations were statistically lower (1.6×10^8) than when using 7.5 g.L^{-1} (1.06×10^9), 5.0 g.L^{-1} (8.57×10^8) or 2.5 g.L^{-1} (1.06×10^9). No statistical differences were found among the last three treatments, This indicates that only 2.5 g.L^{-1} of $(NH_4)NO_3$ are needed in the media. Final improved liquid medium is prepared with 5 g of KH_2PO_4 (MKP, Greenhow®), 1.3 g of $MgSO_4\cdot7H_2O$ (Sul-Mag, Peñoles®), 20 mg of $FeCl_3\cdot6H_2O$ (Fermont®), 150 ml of V8, 10 ml of vinaze and 2.5 g.L^{-1} of $(NH_4)NO_3$. Studies showed that *T. asperellum* grows using different solid substrates [37-40]. In this work, media containing fertilizers and food grade ingredients provided good yields of conidia. In the lack of information on *T. asperellum* grown in liquid media, *T. harzianum* reports were used for comparison purposes. Biomass values obtained here were lower than the reported before for *T. harzianum* [13,15], but conidia concentrations for *T. asperellum* BP60 in liquid medium reached 1.06×10^9 conidia.mL^{-1}; which are comparable to concentrations of 1×10^8 UFC.ml^{-1}, 2×10^7 conidia.ml^{-1}, 1×10^9 UFC.g^{-1} and 1×10^7 UFC.g^{-1} reported in labels of leading *Trichoderma* spp. liquid products. Other studies reported yields of 2.28×10^7 conidia.mL^{-1} for *T. viride* using vegetable waste media [41]. It should be noted that direct counts, accounts only for supernatant conidia, and therefore might subestimate the total conidia concentration obtained in our assays. Between the carbon sources evaluated, vinaze induced more biomass than sucrose and a higher concentration of conidia. Evaluation of different concentrations of vinaze in the culture media indicates that adding 10 ml.L^{-1} increased conidial concentration. Formulations containing carbon sources with non-defined composition has shown good results on *T. harzianum*, reaching concentrations up to 1×10^9 spore.g^{-1} with molasses adjusted to 37% of total sugars on media [42]; also, media using molasses from sugarcane are used to induce *T. asperellum* to produce extracellular lignocellulosic enzymes [43]. Regarding the nitrogen source, the highest biomass was induced with $KNO_3\cdot K_2SO_4$, whereas the lowest values were obtained with $(NH_4)NO_3$, and $(NO_4)\cdot 2SO_4$. Monga et al. (2001) used NH_4Cl and KNO_3 as N-sources in minimal concentrations of 2.0 g.L^{-1}, and reported poor sporulation for *T. viride,* in contrast, *T. harzianum* had excellent response [44]. $(NH_4)NO_3$ induced the highest production of conidia, intermediate and lower concentrations were obtained using $(NO_4)\cdot 2SO_4$ and $KNO_3\cdot K_2SO_4$, respectively (Figure 2B). Treatments with $KNO_3\cdot K_2SO_4$ showed a tendency to have a neutral pH; $(NO_4)\cdot 2SO_4$ pH remained constant throughout the growing time, whereas $(NH_4)NO_3$ pH showed a minimal increase over time. Among all the experiments presented in this study, pH was little affected over time and probably did not influence conidia or biomass production, as stated from Lewis and Papavizas for *T. harzianum*, where conidial production was not influenced by the initial pH of the media or their continuous maintenance at pH 4 or 7 [45]. Moreover, other reports indicate that there are considerable differences in conidiation and growth in response to pH, including values as low as 2.8, concluding that there is a *Trichoderma* species-specific pH effect [46,47]. The highest conidia production obtained here using 2.5 g.L^{-1} of $(NH_4)NO_3$ is recommended for scaling experiments. Broadly, it appears that lowering the concentrations of $(NH_4)NO_3$ enhances conidia production.

Conclusions

In conclusion, three strains of *Trichoderma* with biocontrol capabilities were isolated form desert sandy soils. *T. asperellum* BP60 turned out to have the most effective control against *S. terrestris* on *in vitro* experiments. In addition, this study demonstrates that abundant conidia of *T. asperellum* can be produced in liquid media with the combination of V8, vinaze and $(NH_4)NO_3$.

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