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 vinaza; the former induced higher yields of biomass and conidia. Regarding nitrogen sources, the fertilizer (NH₄)NO₃ induced higher conidia yield. V8 juice (V8) induced the highest effect on production of both biomass and conidia. Therefore, maximum yield was 1.06 × 10⁷ conidia mL⁻¹, with the formulation with 5 g of KH₂PO₄ (MKP), Greenhow®, 1.3 g of MgSO₄•7H₂O (Sul-Mag, Peñoles®), 20 mg of FeCl₃•6H₂O (Fermento®), 150 mL of V8, 10 mL of vinaza and 2.5 g L⁻¹ of (NH₄)NO₃. 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 Río Colorado; where agriculture has diversified from traditional crops such as cotton and wheat, to vegetables including green onions, celery, radish, asparagus, watermelon, and Brussels sprouts. The eastern section of the valley of San Luis Río 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, low rainfall and soil stresses. The disease is controlled by, bacterial and chemical agents, among others. Chemical agents have been used for space and nutrients, biofertilization and stimulation of the plant defense systems, rhizosphere modification, secretion of chitinolytic 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 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 chitinolytic 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

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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 microalgal 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 Pavites 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 to 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 10⁻⁶. From each dilution, 100 μL were plated on PDA amended with rose Bengal (25 mg.L⁻¹), T6 (V8/vinaze/(NH₄)₂SO₄•2SO₃), T5 (V8/vinaze/(NH₄)₂SO₄•2SO₃•K₂SO₄), T3 (V8/sucrose/ (NH₄)₂SO₄), T1 (V8/(KNO₃/Sulka®), T2 (V8/sucrose/NH₄NO₃), T4 (V8/SUCAZINE/(NH₄)₂SO₄), T7 (NA/sucrose/(NH₄)SO₄•2SO₃), T8 (NA/sucrose/ (NH₄)NO₃), T9 (NA/sucrose/(NH₄)SO₄), T10 (NA/vinaze/KNO₃/K₉SO₄), T11 (NA/vinaze/(NH₄)NO₃) and T12 (NA/vinaze/(NH₄)₂SO₄). An amendment of 150 mL.L⁻¹ V8 juice (8 Verduras, Herdez®) was used. Assayed carbon sources were 8 g.L⁻¹ of sucrose (Sulka¹), and 10 mL.L⁻¹ of vinace, a residual product from *Agave tequilana* blue Weber from the tequila distillation process (Tecno Ferti-V, Vida Verde¹). Nitrogen sources used were, KNO₃•K₂SO₄(Nitro K Sul, Greenhow¹), (NH₄)₂NO₃ (Sulfonit, ISAOSA²) and (NH₄)₂SO₄ (granulated ammonium sulfate, ISAOSA²) at 10 g.L⁻¹.

**Assay 1**

To select from the suitable ingredients, an assay was established using all the possible combinations, thus treatments were as follows: T1 (V8/sucrose/KNO₃•K₂SO₄), T2 (V8/sucrose/(NH₄)NO₃), T3 (V8/sucrose/(NH₄)₂SO₄), T4 (V8/vinaze/KNO₃•K₂SO₄), T5 (V8/vinaze/(NH₄)NO₃), T6 (V8/vinaze/(NH₄)₂SO₄), T7 (NA/sucrose/KNO₃•K₂SO₄), T8 (NA/sucrose/(NH₄)NO₃), T9 (NA/sucrose/(NH₄)₂SO₄), T10 (NA/vinaze/KNO₃•K₂SO₄), T11 (NA/vinaze/(NH₄)NO₃) and T12 (NA/vinaze/(NH₄)₂SO₄). After adding all ingredients the pH was adjusted to 6.5 using NaOH 1N. Autoclaved media was distributed into Erlenmeyer flasks with three baffles (Avitrol², 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 quinases 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 × 10⁶ conidia.mL⁻¹ in T5 at 12 DAI (Figure 2C). Among treatments without V8, maximum conidia concentration obtained was 4.38 × 10⁵ 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.
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 \((\text{NH}_4\text{})\text{NO}_3\), 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 of its ingredients; V8 juice and vinaze were first assayed at 50, 100 or 150 mL/L and at 10, 15 or 20 mL/L, respectively. As before, all treatments were inoculated with \(1 \times 10^6\) conidia/mL 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 was obtained when using 100 mL of V8 and 15

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**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\(_3\)•K\(_2\)SO\(_4\) (T1, T4, T7 and T10), (\text{NH}_4\text{})\text{NO}_3\) (T2, T5, T8 and T11) and (\text{NH}_4\text{})•2SO\(_4\) (T3, T6, T9 and T12). All treatments were inoculated with \(1 \times 10^6\) conidia/mL 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.
ml of vinaze (T5.5), while maximum conidia yields of 1.63 × 10^8 conidia.mL^-1 were 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 × 10^6) than when using 7.5 g.L^-1 (1.06 × 10^7), 5.0 g.L^-1 (8.57 × 10^6) or 2.5 g.L^-1 (1.06 × 10^7). 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.PO_4 (MKP, Greenhow^®), 1.3 g of MgSO_4•7H_2O (Sul-Mag, Petolex^®), 20 mg of FeCl_3•6H_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 × 10^6 conidia.mL^-1, which are comparable to concentrations of 1 × 10^6 UFC.ml^-1, 2 × 10^6 conidia.ml^-1, 1 × 10^5 UFC.g^-1 and 1 × 10^6 UFC.g^-1 reported in labels of Trichoderma spp. liquid products. Other studies reported yields of 2.28 × 10^6 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 underestimate 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 conidal concentration. Formulations containing carbon sources with non-defined composition has shown good results on T. harzianum, reaching concentrations up to 1 × 10^6 spor.e.g 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•K_2SO_4, whereas the lowest values were obtained with (NH_4)NO_3 and (NO_3)•2SO_4. Monga et al. (2001) used NH_Cl 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_3)•2SO_4 and KNO_3•K_2SO_4, respectively (Figure 2B). Treatments with KNO_3•K_2SO_4 showed a tendency to have a neutral pH; (NO_3)•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 Papazivas 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 conidium 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 from 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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**References**


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**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.

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

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 ≤ 0.05).