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Cultivation and growth dynamics of endophytic fungi in a solid culture medium based on sugar beet pulp

Running title: Endophyte growth in beet pulp

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Abstract

Background

The production of mycelium from endophytic fungi has interest for applications ranging from inoculants and biofertilizers for crop production to fermentations for enzyme and metabolite production. The purpose of this study was to test the capacity of a solid growth medium based on beet pulp for growing different strains of endophytes.

Results

The ergosterol content of inoculated medium was measured to estimate fungal growth. Several parameters related to the preparation of the growth medium, such as water content, calcium salts and incubation time, were evaluated. The greatest fungal biomass production was observed in a medium prepared with a 1:2 (beet pulp:water) ratio, containing calcium sulphate and carbonate. Strains belonging to different fungal species grew well in the growth medium finally selected, producing yields ranging from 50 to 500 g of mycelium per kg of dry culture, after 22-27 days. Cultures containing up to 400 g of beet pulp grew successfully, and could be scaled up.

Conclusion

A solid culture medium based on beet pulp supported the growth of diverse taxa of fungal endophytes. Both the water and calcium salt content of the growth medium affected the efficiency of mycelium production. Considering these factors, beet pulp medium was an excellent endophyte cultivation medium because of the high yield of fungal biomass observed, plus its ease of handling and scaling up production.

Keywords: Inoculum, endophyte, fungi, sugar beet pulp, mycelium

INTRODUCTION

Certain components of plant microbiomes have functions with adaptive value for their plant hosts. For example, some fungal endophytes can increase the tolerance of their plant hosts to abiotic and biotic stress factors such as heat, salinity, or herbivory.¹ Because of this, there is an increasing interest in using endophytes as a resource for the improvement of agronomic crop species.² Searching for fungal endophytes that can confer beneficial characteristics to agricultural plant species often

involves to survey and identify the microbiome of plant species adapted to stressful habitats, followed by a screening of individual microbes by means of artificial inoculation into the new host targeted.^{3,4} Such endophyte screening procedures require the preparation of fungal inoculum to infect the target plants. Spores are the inoculum of choice for many plant inoculation procedures.^{3,5} However, not all fungal strains sporulate in artificial growth media, and in such situations, inoculum must be based on fungal mycelium.^{4,6}

During the course of research with sterile endophytic fungi isolated from roots of wild grasses we sought methods to obtain adequate amounts of mycelium to inoculate agricultural grasses during germination. Beet pulp proved satisfactory as a basic substrate to produce mycelium for this purpose. Sugar beet is one of the main industrial crops with a world annual production of 301 million tonnes in 2017⁷, and beet pulp is a byproduct of sugar refining from sugar beet. After the sugar is extracted from beet slices by diffusion into hot water, the beet slices are pressed to extract the residual juice, and the solid residue is dried and sometimes pelleted. Sugar beet pulp is used mainly as a feed for livestock, because it is rich in carbohydrates, micro and macronutrients.^{8,9} This product has been shown to support fungal growth, for instance, Vogel⁸ tested the fermentation of beet pulp with *Fusarium oxysporum* to increase its protein content for use as feed, and Mohammadyani et al.¹⁰ used it to produce spores of entomopathogenic fungi. However, its use for mycelial biomass production, or how the preparation of the medium affects fungal growth have not been studied.

The main objective of this study was to test the effectiveness of beet pulp medium as a solid substrate for the growth of several endophytic fungi. The specific objectives were (1) to evaluate how some factors such as the water content, calcium

salts added to the medium, and incubation time affect fungal growth in beet pulp medium, and (2) to find out whether beet pulp medium was adequate for the growth of different fungal species.

MATERIAL AND METHODS

Beet pulp

Beet pulp pellets were obtained from the ACOR sugar beet factory in Olmedo (Spain). The cylindrical pellets had a diameter of approximately 1 cm, and 1 to 5 cm in length. A chemical analysis of the fiber and mineral contents of four different samples of beet pulp pellets from the same lot used for the experiments is shown in Supplementary Table 1. Variation for these parameters is likely to occur among different lots of beet pulp, although the observed values of NDF, ADF and some elements are similar to those reported in other analyses of beet pulp composition.^{9,11}

Fiber contents (acid detergent fiber (ADF), neutral detergent fiber (NDF), cellulose, and hemicellulose) in beet pulp pellets and cultures were determined using the filter bag technique, with an Ankom Automated Fiber Analyzer A2000, based on the analytical method of Goering and Van Soest¹². The pH of the pellets dissolved in water was also determined. After combustion of dried and ground beet pulp samples at 450°C for 8 hours, ashes were dissolved in an acid mixture HCl:HNO₃:H₂O (1:1:8, v/v/v) and mineral content (P, K, Ca, Mg, Na, S, Fe, Mn, Al, Cu, Zn, B, Cr, Ni) analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES) with a Varian 720-ES. Nitrogen was determined with an automated dry combustion method (Dumas method) with a Leco TruMac CN-analyzer.

Fungal strains

The fungal strains used in this study were isolated from surface-sterilized asymptomatic roots of the grass *Stipa gigantea*¹³. Two strains (A33, T17) were used to check their capability to grow in beet pulp medium under different hydration conditions, and added supplements. Five strains were used to optimize the beet pulp formulation, and to estimate fungal growth dynamics in the medium: *Leptodonthidium* T17, *Fusarium* A33, *Trichoderma* A3, *Trametes* T40, and *Diaporthe* T6. In addition, another 15 fungal strains, each one belonging to a different genus, were tested to check for their capability to grow in this medium.

Effect of the amount of water used to hydrate beet pulp

To determine how the amount of water present in beet pulp medium affects fungal growth, 3.0 g of beet pulp pellet were hydrated with different amounts of deionized water (3.0 mL, 4.5 mL, 6.0 mL and 9.0 mL) in 50 mL plastic Falcon tubes, and autoclaved at 121°C for 30 min. The sterilized medium was then inoculated with a 5x5 mm block of mycelium from PDA cultures of fungal strains *Leptodonthidium* T17 and *Fusarium* A33. Three replicates of each fungal strain and water treatment were prepared, and the cultures were maintained at room temperature (20-22°C) for 27 days. After this period the cultures were freeze-dried and ground for ergosterol analysis.

To estimate fungal biomass we measured the amount of ergosterol present in the beet pulp cultures following the method of Gessner¹⁴. Ergosterol is a component of fungal cell membranes which is absent from plant cells.¹⁵ To extract it, freeze-dried and ground samples of beet pulp cultures (0.10 g) were placed in tubes with 10 mL of 0.14

M KOH in methanol. The mixture was incubated at 80 °C for 30 min in a block heater. After cooling, the extract was acidified with 5 mL of 0.75 M HCl and transferred to a SPE column reservoir (Waters Sep-Pack Vac RC, 500 mg, tC18) pre-conditioned with an acidified extract solution (0.14M KOH in methanol: 0.75M HCl; 6:1 v/v). After sample addition, the column was washed with 2.5 mL of 0.4M KOH in methanol:water 60:40(v/v) and dried under a N₂ stream. Ergosterol was eluted with 1.6 mL of isopropanol. The final separation of ergosterol from matrix lipids was performed in a HPLC system (Waters 2690) with a Sunfire C18 Waters column (5 µm; 5.6 × 250 mm), at 33 °C. The mobile phase was pure methanol with a flow rate of 1.4 mL min⁻¹. Ergosterol was detected at λ=282 nm with a Photodiode Array Detector (Waters 2996).

To estimate the amount of ergosterol present in pure fungal mycelium, several strains (*Leptodonthidium* T17, *Fusarium* A33, *Trichoderma* A3, *Trametes* T40, *Diaporthe* T6) were grown on top of cellophane membranes layered on PDA plates for 12 days. Pure mycelium was then harvested from top of the cellophane membrane, freeze-dried and weighed, and the ergosterol content of each strain was analyzed. Thus, for each fungal strain the mass of fungal mycelium in beet pulp cultures was estimated based on the ergosterol content analyzed.

Effect of additives in the beet pulp medium

CaCO₃ and CaSO₄ are additives used for the preparation of cereal grain substrates for the growth of edible mushroom mycelium.^{16,17} The effect in fungal growth of these salts added to the growth medium was tested. To a standard mixture of 3.0 g of beet pulp pellet hydrated with 6.0 mL of water in 50 mL Falcon tubes the following calcium salt treatments were added: (i) no additives ; (ii) CaCO₃ 0.3 g; (iii)

CaCO₃ 0.9 g; (iv) CaSO₄ 0.3 g; (v) CaSO₄ 0.9 g; (vi) CaCO₃ 0.3 g + CaSO₄ 0.15 g; (vii)

CaCO₃ 0.9 g + CaSO₄ 0.45 g. After autoclaving, the mixture was inoculated with a block of mycelium measuring about 5x5 mm from a PDA culture of *Leptodonthidium* T17 and *Fusarium* A33, with three replicates for each strain and treatment, and maintained at room temperature (20-22°C) during 27 days. Then, samples were freeze dried and ground for ergosterol analysis as above explained.

Effect of the incubation time on fungal growth

To measure the growth over time of several fungal strains in beet pulp medium, 3.0 g of beet pulp pellet mixed with 0.9 g CaCO₃ and 0.45 g CaSO₄ were hydrated with 6.0 mL of water and autoclaved in 50 mL plastic Falcon tubes. The medium was inoculated with a 5x5 mm block of mycelium from a PDA culture of strains *Trichoderma* A3, *Fusarium* A33, *Diaporthe* T6, *Leptodonthidium* T17 or *Trametes* T40, and incubated at room temperature (20-22°C) for 7, 12, 17, 22, 27, and 32 days. At each time period three replicates of each fungal strain, plus a control consisting of three uninoculated tubes with beet pulp medium were freeze-dried and ground. These samples were used to estimate the ergosterol and the neutral detergent fiber (NDF) content of the cultures. The first parameter was used to estimate the amount of fungal mycelium produced, and the second the change in cellulose, hemicellulose and lignin of the medium, giving clues to the nutritional sources used by the cultured fungi.

Growth of fungi of different taxa

To find out if the beet pulp medium was adequate for the growth of different fungal species, we tested strains belonging to 15 different genera, in addition to those

five tested before. Each strain was inoculated in a 50 mL Falcon tube containing beet pulp hydrated with two parts of water (w:w) and containing with $0.3 \text{ gL}^{-1} \text{ CaCO}_3$ and $0.15 \text{ gL}^{-1} \text{ CaSO}_4$. After four weeks the fungal colonization of the medium was assessed visually.

Larger scale cultures

Larger scale cultures were prepared on 1 L glass containers filled with 60g beet pulp pellets hydrated with 120 mL of H_2O containing 6.0 g CaCO_3 and 3.0 g CaSO_4 , and in autoclavable polypropylene bags with filters to allow gas exchange (Microsac, SacO₂, Belgium) containing 150 to 400 g of beet pulp prepared as above. These containers were autoclaved, inoculated with blocks of mycelium from PDA cultures, and observed over a period of four weeks. Some mature cultures were extracted from the containers to observe the extent of fungal colonization within the medium.

Statistical analyses

The effect of each factor (amount of water, additives, period of incubation) on the growth of mycelium was analyzed by means of one-way ANOVA for each fungal strain separately. When significance of an effect was detected by the ANOVA, Duncan's test was used to compare the means among different treatments. These analyses were made using IBM SPSS Statistics 23.

RESULTS AND DISCUSSION

Ergosterol content in fungal mycelium

In order to estimate the amount of mycelium present in beet pulp cultures we first estimated the amount of ergosterol present in pure mycelium obtained from the top of cellophane disks layered on top of PDA. For several fungal strains the ergosterol content of their mycelium was the following: *Trichoderma* A3= 2.16 g kg⁻¹ dry mycelium, *Fusarium* A33= 2.72 g kg⁻¹, *Diaporthe* T6= 2.40 g kg⁻¹, *Leptodonthidium* T17= 4.15 g kg⁻¹, *Trametes* T40= 3.95 g kg⁻¹. These values were later used to estimate the mass of fungal mycelium in beet pulp cultures of each of these strains, based on their ergosterol content. Gessner and Chauvet¹⁸ reported similar values for ergosterol content in strains belonging to 12 different species of aquatic fungi (2.3 – 11.5 g kg⁻¹; average: 5.5 g kg⁻¹), also observing variation among species.

The ergosterol content of a fungal species can change depending on the growth medium, and to a lower extent, on the age of the culture.¹⁸ Therefore, the fact that conversion factors from ergosterol to fungal biomass obtained from pure mycelium grown on top of cellophane disks in PDA plates, were later used to estimate mycelial biomass on sugar beet, a different medium, should be acknowledged.

Effect of the amount of water on fungal growth

The amount of water used to hydrate the beet pulp pellets had a significant effect on the biomass produced by the *Leptodonthidium* T17 strain ($F= 6.843$; $P= 0.003$) (Figure 1). This fungus barely grew when less than two parts of water were added to the beet pulp. In contrast, differences among water treatments were not statistically significant for the growth of *Fusarium* strain A33 ($F= 1.163$; $P= 0.386$). A water content lower than two parts per part of dry beet pulp might hinder the growth of some fungi, as exemplified by the T17 strain. This might be due to reduced solubility and

availability of nutrients. On the other hand, a water content greater than four parts per part of beet pulp, resulted in free water present in the growth medium containers, what may be undesirable in terms of manipulation and avoidance of contaminations in cultures. Considering the above results, the 1:2 pellet to water ratio was used for subsequent experiments. Nevertheless, given the large taxonomic and functional diversity existent among endophytes, some fungal strains or taxa might have an optimal growth condition with a different water content in the medium.

Effect of additives on fungal growth

The addition of both CaCO_3 and CaSO_4 to the substrate was more favorable for fungal growth than each compound alone. For *Leptodonthidium* T17, the addition of CaSO_4 alone was detrimental, and its greatest growth occurred with both salts at the highest dose ($F= 9.492$; $P<0.01$; Figure 1d). For *Fusarium* A33 no statistically significant differences among treatments were observed ($F= 1.895$; $P= 0.152$; Figure 1c). CaCO_3 and CaSO_4 are additives used for the preparation of cereal grain substrates for the growth of edible mushroom mycelium.^{16,17} The modification of the pH of the medium might be related to the improved growth observed in the presence of both additives. For the growth of many fungal species, pH values in the 6-8 range are better than more acidic or basic conditions (Yamanaka).¹⁹ The pH of beet pulp without additives in water was 4.5, addition of 0.3 g CaCO_3 and 0.05 g CaSO_4 per g of beet pulp increased the pH of the medium to 6.8. This modification could increase the availability of soluble sugars due to pectin depolymerization. It has been reported that the pectin fraction of polysaccharides in beet pulp is partially hydrolyzed at pH= 6 – 9, while cellulose, hemicellulose and lignin remain stable in the pH range 4 – 9.⁹ In contrast,

addition of 0.1 g or 0.3 g $\text{CaSO}_4 \text{ g}^{-1}$ beet pulp changed the original pH value of the beet pulp to 4.8.

Effect of the incubation time on fungal growth

To observe fungal growth dynamics in beet pulp five strains were assayed. Fast growing strains like *Diaporthe* T6 and *Fusarium* A33 reached a stationary growth phase after 17 days, while slower strains like *Leptodonothidium* T17 and *Trametes* T40 kept growing for longer time periods (Figure 3a). Ergosterol content in non-inoculated samples was zero. These results indicated that incubation periods of 22 to 27 days could be adequate for most strains. The growth of strains like *Diaporthe* T6, *Fusarium* A33 or *Trichoderma* A3 was remarkable; after three weeks more than 250 g kg^{-1} of the dry mass of the beet pulp cultures was fungal.

In fast growing strains such as *Diaporthe* T6 and *Fusarium* A33, the amount of neutral detergent fiber increased over time much faster than in other strains growing slower (Figure 2b). NDF is a measure of the hemicellulose, cellulose and lignin content of the beet pulp, and the increment of the relative amount of NDF implies that these strains might be making preferential use of other beet pulp components, such as pectin and other soluble sugars. In fact, in 27 day cultures there was a significant positive relationship between the amount of mycelium and NDF content of the beet pulp cultures, (Pearson's $r = 0.89$; $p = 0.044$). The cellular content fraction, which is the other component of the fiber quality analysis, is constituted mainly by undegraded protein and pectin²⁰, and this fraction seems to be the main nutritional source of the *Diaporthe* and *Fusarium* strains.

Larger scale cultures

In these cultures fungal growth was assessed visually. Fungal mycelium grew as well in cultures made in 1 L glass containers and in polypropylene bags with air filters as in the 50 ml Falcon tube cultures used for ergosterol-based determinations. As it can be observed in Figure 3, the beet pulp medium was profusely colonized by fungal mycelium. These solid cultures are easily disgregated to mix with a planting substrate in order to place on it the seeds or seedlings to be inoculated. For this purpose we mixed one volume of beet pulp culture with seven volumes of a 1:1 peat moss: perlite (v:v) substrate.

Fungal growth in an agar-based medium like PDA is limited to the flat surface of plates. In contrast, in beet pulp medium fungal growth is more three dimensional, occurring throughout the medium in flask type containers. Because of that, fungal production in beet pulp medium is much greater than in PDA or other agar-based solid media.

In addition to the fungal strains above reported, other strains belonging to different genera such as *Mycochaetophora*, *Periconia*, *Alternaria*, *Chaetomium*, *Lyconectria*, *Drechslera*, *Penicillium*, *Dactylonectria*, *Absidia* and *Embellisia* grew well in the beet pulp medium, as deduced from visual inspection. With these strains colonization of the medium was profuse after 22 to 27 days. The exceptions were fungal strains belonging to taxa like *Epichloë*, *Tolypocladium*, *Slopeiomyces*, *Darksidea*, and *Microdochium*, which did not grow in the beet pulp medium. Although these strains grew well in PDA, they may not have the metabolic capability to assimilate beet pulp nutrients.

CONCLUSION

Beet pulp was suitable for the growth of different fungal species. A culture medium prepared with a proportion of 2.0 g H₂O, 0.1 g CaCO₃, and 0.05 g CaSO₄ per g of beet pulp, gave the best results. High yield of mycelium was obtained, ranging from about 100 to 500 g kg⁻¹ of dry beet pulp after four weeks. Beet pulp is relatively unexpensive, the medium is easy to handle, and cultivation could be scaled up in containers of other types. Because of the above characteristics, beet pulp cultures could also be used for other applications, like soil amendments, or production of enzymes and metabolites.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure Captions

Figure 1. Biomass (mean $\pm$ SE) of fungal strains *Fusarium* A33 (a) and *Leptodonothidium* T17 (b) after 27 days in 3g of beet pulp hydrated with different amounts of water, in 50 mL plastic Falcon tubes. Biomass of *Fusarium* A33 (c) and *Leptodonothidium* T17 (d) after 27 days in 3 g dry beet pulp hydrated with 6 ml of water containing the following amounts of CaCO_3 and CaSO_4 additives per g of dry beet pulp: 1: no additives; 2: 100mg CaCO_3 ; 3: 300mg CaCO_3 ; 4: 100mg CaSO_4 ; 5: 300mg CaSO_4 ; 6: 100mg CaCO_3 +50mg CaSO_4 ; 7: 300mg CaCO_3 +150mg CaSO_4 . In each panel, different letters indicate significantly different means.

Figure 2. (a) Temporal evolution of the growth of several fungal strains in 50 mL tubes containing 3 g dry beet pulp medium hydrated in a 1:2 (w:w) proportion with water, and containing 100 and 50 mg CaCO_3 and CaSO_4 , respectively, per g of dry beet pulp (means $\pm$ SE). (b) Change in neutral detergent fiber (NDF) content of beet pulp cultures of fungal strains in the same period as above. NDF refers to the hemicellulose, cellulose and lignin content of the inoculated beet pulp medium.

Figure 3. (a) Fungal cultures in 1 L glass containers (60 g dry beet pulp), the container in the left has uninoculated beet pulp medium. (b) Fungal cultures in SacO_2 polypropylene bags with air filters (300 and 150 g dry beet pulp). (c) A block of a beet pulp culture showing profuse colonization by fungal mycelium.