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Research Article

Production of Coconut Aroma in Solid-State Cultivation: Screening and Identification of *Trichoderma* Strains for 6-Pentyl-Alpha-Pyrone and Conidia Production

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The polyketide, 6-pentyl-alpha-pyrone (6-PP), has a characteristic coconut aroma and is produced by many *Trichoderma* species. In this study, we screened formerly isolated *Trichoderma* strains for 6-PP production grown using solid-state fermentation (SSF). Sugarcane bagasse supplemented with a nutrient solution was used as the support material for the culture. We detected outstandingly strong 6-PP production in the case of two *Trichoderma* strains in this experiment. The strains were identified based on internal transcribed spacer (ITS) sequence analysis as *Trichoderma asperellum* (G7 and G18). We also investigated the production of 6-PP in the case of the best *Trichoderma* strain selected (*T. asperellum* G7) from two types of cultivation (SSF and liquid cultivation (LC)). The same liquid medium was used to impregnate sugarcane bagasse. Results led to a 6-PP production 80 times higher (85.11 $\mu g \cdot g \cdot DM^{-1}$) in SSF than that found in LC (10.00 $\mu g \cdot L^{-1}$) after eight days of cultivation. For this, the SSF is the best option to produce 6-PP compared to liquid cultures.

1. Introduction

Solid-state fermentation (SSF) is an ancestral process, initially used for human alimentation [1]. Numerous studies have shown advantages of SSF over its liquid homologues, advantages related to quantitative and qualitative aspects in the productions of enzymes and secondary metabolites by filamentous fungi [2, 3]. Moreover, one of the main driving forces in the gain of popularity of SSF over the last decades is the growing concern about sustainability in bioprocess development linked to the possibility of using agroindustrial wastes as cheap culture media to perform fermentations [4, 5]. Agroindustrial residues are generally considered the best substrates for solid-state fermentation processes, mainly due to their low cost [6, 7]. In SSF, the solid medium provides support and nutrients for the microorganism's development. A large number of microorganisms including bacteria, yeasts, and fungi have been used for cultivation on different solid substrates such as sugarcane bagasse, coffee husk, jatropha cake, sorghum pulp, olive pomace, and fruit seeds [8, 9]. However, filamentous fungi especially Hypocreacea are the best choice for secondary metabolites production [4]. The fungal genus *Trichoderma*, known since 1887 for its antagonistic properties, has been used as a biological control organism against several plant pathogens [10]. During their development, *Trichoderma* produce biomass, primary metabolites (enzymes and organic acids), conidia, and secondary metabolites like 6-pentyl-alpha-pyrone (6-PP), a fungicidal compound [11]. This lactone is also called "coconut lactone" because of its characteristic smell. *Trichoderma* has been well investigated because of its commercial importance used in food and pharmaceutical products [12]. On the contrary, 6-PP has attracted considerable attention as a control agent due to its powerful

antifungal activity. Hence, the ability of 6-PP to control the growth of *Botrytis cinerea* has been highlighted by Walter et al. [13]. Several workers have also reported that 6-PP production is apparently related to the antagonism response of some *Trichoderma* species to disadvantageous culture conditions and that the solid-state culture can give higher yields of 6-PP than submerged fermentation [14–16]. Furthermore, chemical synthesis of 6-PP requires high temperature (400°C) and seven reaction steps [17]. For this reason, production of this compound by a biotechnological way can be an interesting alternative to chemical synthesis.

A previous work has already been published for the production of 6-PP using SSF and *Trichoderma* strains [18].

This work aimed to screen *Trichoderma* strains derived from different natural sources on solid media for 6-PP production and to characterize the best strains able to produce a large quantity of 6-PP and conidia. Moreover, solid and submerged conditions were tested for the best *Trichoderma* strain selected to compare the production of 6-PP using the same composition of liquid culture medium.

2. Materials and Methods

2.1. Fungal Strains. Seventeen *Trichoderma* strains of the present study derived from various fungi collection were investigated: (i) Biotechnologie Environnementale et Chimiométrie, IRD/IMBE (Marseille, France); (ii) the Department of Agricultural Parasitology of UAAAN (Universidad Autónoma Agraria Antonio Narro) (Coahuila, Mexico); (iii) the Laboratory for Improvement and Protection of Olive Genetic Resources, Olive Tree Institute (Sfax, Tunisia); and (iv) the Tissue Culture Laboratory, Fitotechniki (Filothei-Arta, Greece).

The fungal conidia were stored at 4°C in a 5 ml bottle on potato dextrose agar (PDA) culture medium.

2.2. Inoculum Preparation. The preculture medium had the following composition: glucose: 30.0 g·L^{-1}; KH_2PO_4: 7.0 g·L^{-1}; Na_2HPO_4: 2.0 g·L^{-1}; $NaNO_3$: 2.0 g·L^{-1}; $MgSO_4 \times 7H_2O$: 1.5 g·L^{-1}; $CaCl_2 \times 6H_2O$: 0.1 g·L^{-1}; $FeCl_3 \times 6H_2O$: 8.0 mg·L^{-1}; $ZnSO_4 \times 7H_2O$: 1.0 mg·L^{-1}. The pH was adjusted to 5.6 before sterilization. Bottles (100 ml) containing 50 ml of this medium were inoculated with 5-day-old strains (1.0 cm^2 from PDA medium) and incubated under agitation (150 rpm, 5 cm diameter stroke) at 25°C for 4 days. The mycelium was homogenized in an Ultra-Turrax homogenizer, and this mycelial homogenate was used as the inoculum.

2.3. Culture Conditions for LC. Cultures of *Trichoderma* strains were grown in the following culture medium: glucose: 30.0 g·L^{-1}; $NaNO_3$: 0.6 g·L^{-1}; KH_2PO_4: 1.0 g·L^{-1}; KCl: 0.5 g·L^{-1}; $MgSO_4 \times 7H_2O$: 0.5 g·L^{-1}; extract yeast: 1.0 g·L^{-1}; and 2 ml·L^{-1} of trace element solution containing (in mg·L^{-1}in distilled water) $CaCl_2 \times 6H_2O$ (8.0), $ZnSO_4 \times 7H_2O$ (1.0), and $FeSO_4 \times 7H_2O$ (0.1). The pH was adjusted to 6.0 before sterilization. Bottles (250 ml) containing 100 ml of this medium were inoculated with 3.0 ml of the above mycelial homogenate and incubated under agitation (150 rpm) at 27°C. Each experiment has been performed in triplicate.

2.4. Culture Media and Conditions for SSC. The solid substrate comprising 4.0 g sugarcane bagasse was placed in 250 ml bottles supplemented with 10 ml of the same nutriment solution used for liquid culture. The humidity was set to 66% before sterilization. Sugarcane bagasse and nutriment solutions were individually autoclaved at 120°C during 1 hour. Each bottle was then inoculated with 3.0 mL of mycelial cell suspension. The incubation temperature was 27°C. Culture conditions such as incubation temperature, moisture level, inoculum concentration, and pH are fixed according to previous studies [18].

2.5. Production Kinetics. Kinetic profiles of 6-PP, conidia (for SSF), biomass, pH, and glucose were studied at the second, fourth, sixth, eighth, and tenth day of culture.

2.6. Conidia and 6-PP Analysis. All the results being expressed in gram of dry matter, the water content of each sample was measured as follows: 1.0 g of fermented material was introduced into a lab oven at 105°C to analyze the relative humidity of the sample.

Conidia suspension was prepared by mixing 1.0 g of the fermented material and 100 mL of distilled water containing tween 80 (0.01% v : v). The conidia were counted using a traditional hemocytometer as described by Roussos et al. [19].

Sugar concentration was determined after each enzyme reaction according to the amount of resulting reducing sugar using 3,5-dinitro salicylic acid (DNS) following the assay method described by Miller [20].

The extraction of aroma produced by fungi in SSF was performed by the Soxhlet system from 10 g of wet solid fermented material using pure heptane (99.7%, Sigma-Aldrich, USA). For the extraction of 6-PP produced in the liquid medium, a 10 ml aliquot of the filtered fermented broth was extracted with 100 ml of heptane.

Samples were then stirred for 20 minutes and concentrated under a rotatory evaporator at 40°C. 1 ml of the obtained heptanoic extract was then filtered with a 0.2 μm Millipore filter into vials and then analyzed with a gas chromatograph 7890A (GC) (Agilent Technology, USA) equipped with a split/splitless injector (T = 200°C) and a flame ionization detector (T = 260°C). 6-PP was separated using a Supelcowax capillary column (internal diameter: 0.25 mm; length: 60 m; film thickness: 0.25 μm). The carrier gas was dihydrogen (column flow 1 mL·min^{-1}), and the split ratio was 2 : 1. The oven temperature was set as follows: 30 min at 180°C, from 180 to 230°C at 10°C·min^{-1}, and 10 min at 230°C. Quantitative analysis of 6-PP was carried out using the internal calibration method, using γ -undecanolactone (99%, Sigma-Aldrich) as an internal standard.

2.7. Molecular Identification. Total DNA was extracted from 50–200 mg of mycelium (fresh weight) using the

NucleoSpin® Plant II Genomic DNA Purification Kit (Macherey-Nagel) according to the manufacturer's procedure and stored at −20°C. Amplification of the ITS rDNA was performed by using the universal primers ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′), respectively. The PCR amplification was performed in 20 μL reaction mixtures containing 4 μl reaction buffer (5X), 2.6 μl $MgCl_2$ (25 mM), 3.5 μl dNTPs (1.25 mM), 0.65 μl of each primer (10 μM), 0.2 units of GoTaq® DNA polymerase (Promega), and 2 μl (20 ng) of template DNA. The PCR program consisted of an initial step at 94°C for 3 min, then 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 50°C, and elongation for 30 s at 72°C, followed by a final elongation step at 72°C for 5 min. The ITS-PCR amplification patterns were migrated on standard 1% agarose gels in 0.5X Tris-acetate-EDTA (TAE). The amplified ITS fragments were sequenced (GATC Biotech). Phylogenetic analyses were performed using MEGA7 [21].

3. Results

3.1. Screening and Identification of 6-PP Producing Fungi. The 6-PP production by a total of 17 strains of *Trichoderma* strains isolated from different natural sources was examined. These experiments were performed on solid media with sugarcane bagasse impregnated with a nutritive solution. All strains were cultivated at 27°C and harvested after 8 days of total incubation. Only seven produced 6-PP (Table 1).

Characterization by ITS sequences analyses (Table 1) and morphological observations (Table 2) of the selected strains were conducted on the strains that produced the highest amount of 6-PP. Although they did not produce 6-PP, the strains G13 and G17 were also identified in order to compare the relationship between species and 6-PP and conidia production. Morphological characterization was performed on pure cultures grown for 3–6 days on PDA and was based on the ten characters (Table 2). Most discriminating ones were conidial shape, mycelial form, colony color, and culture smell.

The molecular identification of our fungal strains was performed by sequencing the ITS 1 regions of rDNA and compared NCBI GenBank nucleotide database. The BLAST analyses showed that the ITS sequences of all sequenced strains were at least 99% similar to the corresponding GenBank sequences. All belonged to *Trichoderma*, with only two species identified: five strains were affiliated to the species *Trichoderma asperellum* (G7, G18, G3, G11, and G9) while three proved to be *Trichoderma longibrachiatum* (G13, G14, and G17). One of the *T. asperellum* strains (G7) showed the best 6-PP production with an average of 86.69 μg·g·DM^{-1} of 6-PP (Table 1). Two other strains, *T. asperellum* G18 and *T. asperellum* G3, produced 80.79 μg·g·DM^{-1} and 39.83 μg·g·DM^{-1}, respectively. The highest value of conidia production was shown by *T. asperellum* G9 (5.78×10^8 conidia·g·DM^{-1}) followed by *T. asperellum* G18 and *T. longibrachiatum* G17 with 3.95 and 2.81×10^8 conidia·g·DM^{-1}, respectively. Comparing the production of conidia and 6-PP, the maximum conidia production was showed by *T. asperellum* G9; however, this strain also showed the most limited amount of 6-PP (16.30 μg·g·DM^{-1}). Currently, *Trichoderma* strains are able to produce a large quantity of conidia and a green-colored fermented product characteristic due to conidia color. In this experiment, *T. asperellum* G7, presenting a limited conidia production and white to transparent color, is defined the best strain to produce 6-PP under the conditions tested. Based on these results, in order to evaluate the effect of conidia on 6-PP production and due to the interesting 6-PP properties, we selected *T. asperellum* G7 strain for further 6-PP production survey.

Glucose concentration, biomass, and 6-PP production in culture medium (Figure 1) were determined for 12 days in a stirred liquid culture of *T. asperellum* G7. As can be seen from Figure 1, the biomass increased sharply to a level of 5.84 g·L^{-1} at the end of 4 days, and thereafter, the increase was gradual, reaching a final value of 14.57 g·L^{-1}.

The residual sugar decreased sharply to a level of 5.66 g·L^{-1} at the end of fermentation, indicating rapid utilization of sugar. 6-PP production was started after 6 days of incubation, the maximum value of 6-PP production (10.00 μg·L^{-1}) was observed after the exponential phase, which is a common characteristic of secondary metabolites, and then decreased rapidly to attain values of 1 μg·L^{-1} after 10 days. There was a wide pH variation in the liquid culture, and the pH decreased from 6.0 to 4.7, after 8 days.

3.2. Conidia Production by T. asperellum G7 Cultivated in SSF. In order to compare the production of 6-PP by *T. asperellum* G7 in liquid and in SSF, the same liquid medium tested in both cultures was used to impregnate sugarcane bagasse. 6-PP and conidia production was determined during 10 days of culture (Figure 2). Under SSF conditions, conidia production was initiated after 1 day of incubation. The conidia production increased sharply to a level of 3.7×10^7 conidia·g·DM^{-1} at the end of the second day, and thereafter, the increase was gradual, reaching a final value of 5.1×10^7 conidia·g·DM^{-1}. After 2 days, 6-PP production started. The maximum value of 6-PP production was observed after 8 days (85.11 μg·g·DM^{-1}). After that, the 6-PP production decreased progressively to attain the value of 58.14 μg g·DM^{-1} at 10 days. As seen in Figure 2, conidiation and 6-PP production follow a similar trend at the beginning of cultivation, and the production of 6-PP was started after one day of conidiation.

4. Discussion

The production of secondary metabolites is an actual topic that is investigated following several ways, including bioreactors design, medium composition, and fungal strains [22, 23]. Concerning medium, substrates for SSF are usually homogeneous agroindustrial by-products which offer an economic advantage in this fermentation process [24]. Even so, to be fully both economically competitive and ecofriendly, the SSF must, to a larger extent, be carried out on local by-products.

It is well known that sugarcane bagasse is particularly suitable for SSF because of its porosity allowing good water

Table 1: Comparison of 17 strains of *Trichoderma* for their ability to produce 6-PP and conidia after 8 days of cultivation and molecular identification by ITS1 rDNA regions sequencing.

Strains	6-pentyl-α-pyrone ($\mu g \cdot g \cdot DM^{-1}$)	Conidia ($\times 10^8$ conidia$\cdot g \cdot DM^{-1}$)	Best BLAST hit: species	Strain	GenBank accession number	Maximum similarity (%)
G7	86.69	0.49	*T. asperellum*	TV104	KP263611	100
G18	80.79	3.95	*T. asperellum*	TF1	KU341007	99
G3	39.83	1.12	*T. asperellum*	QT22046	KY225608	99
G11	34.45	2.31	*T. asperellum*	DWG3	KM268676	99
G19	32.68	2.21	Not identified	—	—	—
G9	16.30	5.78	*T. asperellum*	E-117	KU059966	99
G14	5.30	0.92	*T. longibrachiatum*	QT22111	KY225659	99
G2	Nondetected	2.32	Not identified	—	—	—
G4	Nondetected	2.50	Not identified	—	—	—
G5	Nondetected	1.11	Not identified	—	—	—
G13	Nondetected	2.70	*T. longibrachiatum*	T22	JN108920	99
G15	Nondetected	0.31	Not identified	—	—	—
G16	Nondetected	0.93	Not identified	—	—	—
G17	Nondetected	2.81	*T. longibrachiatum*	QT22040	KY209918	99
G21	Nondetected	2.07	Not identified	—	—	—
G22	Nondetected	1.14	Not identified	—	—	—
G23	Nondetected	1.22	Not identified	—	—	—

Table 2: Morphological characters of *Trichoderma* selected strains grown on PDA Petri dish and incubated at 27°C for 4-5 days.

Isolate/characters	Appearance				Mycelium			Conidia		
	Colony growth rate (cm)	Colony color	Colony edge	Reverse colony color	Culture smell	Mycelial form	Mycelial color	Conidial color	Conidial wall	Conidial shape
T. asperellum G7	7-8 in 5 days	White to transparent	Smooth	Colorless	Cocount	Arachnoid	Watery white	White	Smooth	3-4 μm
T. asperellum G18	8-9 in 5 days	Green to dark green	Wavy	Creamish	Cocount	Floccose to arachnoid	White	Dark green	Rough	3–5 μm
T. asperellum G3	8-9 in 5 days	Olive green to dark green	Wavy	Creamish	Cocount	Arachnoid	White	Green	Smooth	3.6–4.5 μm
T. asperellum G11	8-9 in 3 days	Green	Wavy	Creamish	Cocount	Floccose	White	Green	Rough	3–5 μm
T. asperellum G9	8-9 in 3 days	Dark green	Wavy	Creamish	Cocount	Floccose	White	Dark green	Rough	3–5 μm
T. longibrachiatum G14	7-8 in 3 days	Yellow to orange	Smooth	Light yellow	Malt	Floccose	White	Transparent	Smooth	2.5–3.5 μm
T. longibrachiatum G13	8-9 in 3 days	Yellow to green	Smooth	Light yellow	Cocount	Floccose	White	Green	Rough	2.5–3.5 μm
T. longibrachiatum G17	7-8 in 3 days	Yellow to green	Smooth	Deep yellow	Malt	Floccose	White	Green to yellow	Smooth	2.5–3.5 μm

absorption, indispensable to carry out the microbial metabolism [25]. Moreover, in terms of volume, it is the major solid agroindustrial by-product generated [26].

In this study, the use of sugarcane bagasse as support for fungal growth for conidia and secondary metabolites production using the solid-state culture was evaluated. Our main objective was to screen and identify *Trichoderma* strains producing an considerable amount of 6-PP. Among the 17 strains tested, 7 *Trichoderma* strains demonstrated the ability to produce 6-PP. The characterization of these strains by morphological and ITS gene analysis indicated that they were related to *T. asperellum* and *T. longibranchiatum*. Interestingly, while 6-PP is detected in traces in all the cultures of the tested *T. longibrachiatum* strains, the species *asperellum* is much capable of producing 6-PP under these conditions.

Among the strains that produce 6-PP, our results indicated that *T. asperellum* G7 was the best strain with an average 6-PP production of 85.11 $\mu g \cdot g \cdot DM^{-1}$. With a maximum value of 86.69 $\mu g \cdot g \cdot DM^{-1}$, our value was significantly higher than those reported in literature studies for other species (*T. harzianum* IM206040, *T. atroviride* IM206040, and *T. harzianum* 4040) cultivated under the same conditions; [12, 15, 27–29].

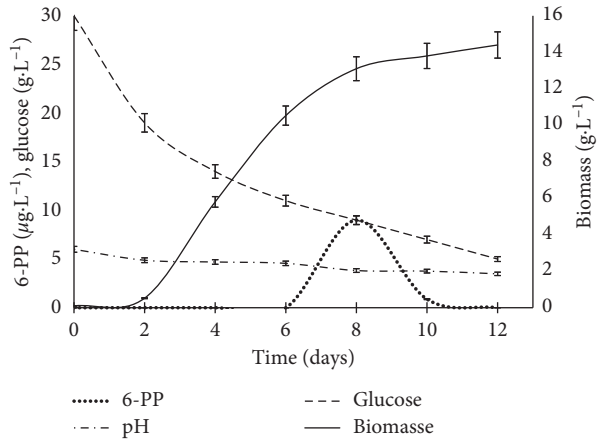

Figure 1: Evolution of 6-PP, glucose, and biomass in the liquid medium with *Trichoderma asperellum* G7.

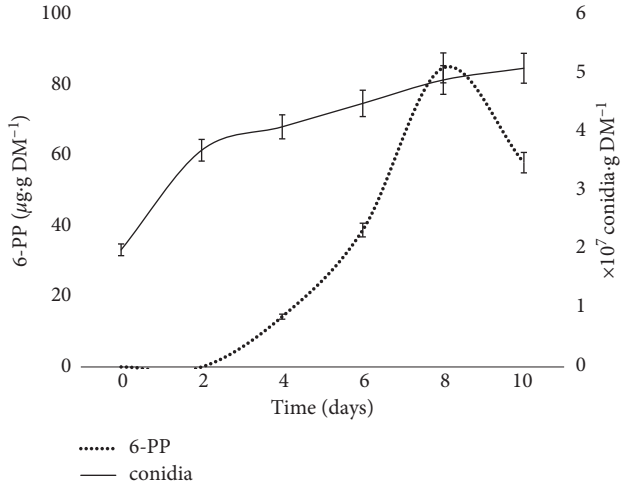

Figure 2: Evolution of conidia and 6-PP production in the solid medium, during the culture of *T. asperellum* G7 on solid medium.

When comparing the two types of cultures (solid and liquid culture), a significant difference on 6-PP biosynthesis was observed. The maximum 6-PP concentration in SSF was 80 times higher than the one obtained in LC. These results confirm those previously obtained by Sarhy-Bagnon et al. [15] and Galindo et al. [30]; these authors explain that the inhibitory effect of 6-PP and the acidification observed in LC appeared to be responsible for the low concentrations obtained in the liquid medium. These results underline the differences between SSF and LC already observed in the literature. Indeed, for many secondary metabolites, SSF exhibits increased production when compared to its liquid counterpart [31].

On the contrary, a very marked pattern was observed on the relationship between conidiogenesis and 6-PP production: growth on the solid medium showed that conidia and 6-PP production followed a similar trend at the beginning of cultivation for *T. asperellum* G7. This does mean that the production of conidia and secondary metabolites from the genus *Trichoderma* is more related to the culture conditions which cause an increase in the concentration of 6-PP and conidia. This effect has a direct impact on the technical feasibility of conidia production because under these culture conditions, it is possible to use reactors such as trays, bags, or bioreactors to scale up the fermentation process for 6-PP and conidia production.

T. asperellum G7 selected by this screening is a promising strain, thanks to its ability to produce considerable quantities of 6-PP. The behavior of this strain should be investigated against phytopathogenic fungi such as, for example, *Fusarium oxysporum*, *Botrytis cinerea*, *Crinipellis perniciosa*, and *Rhizoctonia solani* [12, 13, 32, 33]. For this reason, it is important to produce 6-PP, conidia, or biomass in large amounts to meet field requirements. In addition, the opportunity for local agroindustrial by-products of low commercial value to be used in a biotechnological process makes this process an important valorization approach.

5. Conclusion

Trichoderma strains isolated from different natural sources and derived from different laboratories (Mexico, Tunisia, Greece, and France) were screened for 6-PP production. 6-PP production could be detected on solid media in the case of 7 *Trichoderma* strains. Among those, five belonged to the species *T. asperellum* (G7, G18, G3, G11, and G9). However, strains belonging to *longibrachiatum* species were unable to significantly produce 6-PP. The present study shows for the first time a new strain "*T. asperellum* G7" able to produce large amount of coconut aroma. Comparison between SSF and LC revealed that, for this strain, 6-PP production was continuous during 10 days of incubation on the solid medium, whereas in LC, 6-PP production decrease was observed in addition to being 80 times inferior compared to SSF. This indicates a clear advantage of SSF over LC for this process.

Abbreviations

SSF: Solid-state fermentation
LC: Liquid cultivation
PDA: Potato dextrose agar
6-PP: 6-pentyl-alpha-pyrone.

Data Availability

The 6-PP analysis data used to support the findings of this study are currently under embargo while the research findings are commercialized. Requests for data, 12 months after publication of this article, will be considered by the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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