Trichoderma spores and 6-pentyl-alpha-pyrone production in solid state culture for biological control

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Abstract:

Many *Trichoderma* species are able to produce spores and secondary metabolites like 6-pentyl-alpha-pyrone (6-PP), a lactone with antibiotic properties and coconut aroma. The aim of this work was to compare the ability of three strains of filamentous fungi: *Trichoderma harzianum G18, Trichoderma viride G19, Trichoderma asperellum G17* to produce spores and 6-PP in solid state culture (SSC). Sugarcane bagasse added to nutriment solution was used as support material for the culture. The maximum concentration of spores and 6-PP obtained by *T. harzianum G18*, were respectively $5.2x10^9$ spores/ g of carbone source and 0.08mg of 6-PP /g of Dry Matter (DM).

Key words: *Trichoderma species*, solid state fermentation, 6-pentyl-alpha-pyrones, spores, sugarcane bagasse.

Introduction:

Currently, the production of biopesticides has gained great attention because is an important alternative to replace the chemical pesticides used on the field crops (Glare *et al.*, 2012). Fungal lytic enzymes, secondary metabolites and spores play a very important role in the biological control of plant diseases. The fungi genus *Trichoderma* known since 1887 for their antagonistic properties (Reyes *et al.*, 2012), have been used as a biological control organism against several plant pathogens. During their development, *Trichoderma* produce biomass, primary metabolites (enzymes, organic acids), spores and secondary metabolites like 6 pentyl-alpha-pyrone (6-PP) which has a characteristic coconut aroma. Fungicide properties of this compound were also reported (Etschmann *et al.*,2015). The microbial fermentation seems to be a good route for spores and 6-PP production since its chemical synthesis is a difficult and costly process. It was reported that 6-PP production is apparently related to antagonism response of some *Trichoderma* species (Ladeira *et al.*,2010), and to culture conditions (De Souza Ramos *et al.*, 2008). Furthermore the solid state fermentation (SSF) give higher yields of 6-PP than submerged fermentation (Sarhy-Bagnon *et al.*, 2000). Agro-industrial residues are generally considered as the best substrates for SSF, mainly due both nutritional quality and low cost. Nowadays, world concerns about the environment enhanced the importance of the use of such materials.

The aim of this work is to present (i) the results obtained from 3 strains of *Trichoderma* that produce a large quantity of spores and 6-PP; (ii) the kinetics of spore and 6-PP production in order to study the relationship between secondary metabolism and fungal sporulation.

2. Materials and methods

2.1. Microorganisms:

Three filamentous fungi; *Trichoderma harzianum* G18, *Trichoderma viride* G19, and *Trichoderma asperellum* G17 from the IRD/IMBE fungi collection were used in present study. For the colony description, all strains were maintained on potato dextrose agar (PDA) medium, incubated at 30°C and stored at 4°C.

2.2. Culture medium for 6-PP production in SSC:

For 6-PP production the culture medium composition was (g/l): Glucose (30,0), KH_2PO_4 (7,0), Na_2HPO_4 (2,0), $NaNO_3$ (2), $MgSO_4*7H_2O$ (1,5), $CaCl_2*6H_2O$ (0,1) and 2ml/l trace elements solution containing (mg/l in distilled water): $FeCl_3*6H_2O$ (8,0), $ZnSO_4*7H_2O$ (1,0). The carbon/nitrogen ration (C/N) was 60 and the pH was adjusted to 6,0. Sugarcane bagasse was used both as carbon source and as a support for solid state culture. It was impregnated to 75% humidity, with these mineral solution medium and 16g of the solid culture medium were placed into 100 ml flasks. The flasks were autoclaved (sterilized at 120°C for 20 min and then inoculated with a concentrated spore suspension (2.10^7 spores/g dry matter). Cultures were incubated at 25°C for 10 days.

2.4. Extraction of secondary metabolites:

Volatiles compounds were recovered by soxhlet extraction system from solid fermented materia using pure hexane. Samples (10 g of the fermented materia) were co-distilled at 60°C with 100ml hexane during 45 min. γ - undecalactone (0.08 mg) was added as the internal standard before extraction.

2.5. Quantitative analysis of secondary metabolites:

6-PP analysis were performed on an Agilent Technology gas chromatograph 7890A (GC) equipped with a split/splitless injector (T=200°C) and a flame ionization detector, (T=260°C). Aroma constituents were separated on a Supelcowax capillary column (internal diameter: 0.25 mm, length: 60 m, film thickness: 0.25µm). The carrier gas was dihydrogen (column flow 1ml/min) and the split ratio was 1:2. The oven temperature was as follows: 30min at 180°C, from 180 to 230°C at 10°C/min, 10min at 230°C.

Quantitative analysis of 6-PP was carried out using the internal calibration method, with γ -undecanolactone (99%, Aldrich) as internal standard.

3. Results

The culture on PDA reveals that the growth rate is rapid and colonies become wooly and compact. The surface colony color is white and becomes greenish in time, visible when the conidia is formed for *T.viride*, and *T.harzianum*. The color is yellow to green for *T.asperellum*. Microscopic appearance reveals that *T.viride* may also produces clamydospores and for all these *Trichoderma* species the conidia were unicellular, round or ellipsoidal, green in color, smooth walled or rough, with an average diameter of 3 µm, and are grouped in sticky heads at the tips of the phialides. The apical growth of *T.harzianum* on PDA at 30°C was 17.02mm/day, 16.01mm/day for *T.asperrellum* and 16.52mm/day for *T.viride*. This allows to deduce that majority of *Trichoderma* species cultures grow up rapidly at 25°C to 30°C.

3.1. Spores production by *Trichoderma* species cultivated in SSC:

The results show that all these strains have a difference of sporulation. Indeed, at the beginning of the cultures, the solid material show a white color due to the development of the mycelium and after 7 days of fermentation, the color becomes green (Fig.1). The reproduction follows out stage by the development of the fungus and on the environmental conditions. The sporulation process, covering conidiogenesis and conidia maturation, is regulated by a complex set of genes (Park and Yu 2012). Environmental stimuli such as light or a wound on the mycelium can also initiate the process of conidiogenesis (Flodman and Noureddini 2013).

Indeed, conidiogenesis leads to the production of propagules during asexual reproduction. These propagules are naturally elements of dispersion but also constitute a form of resistance (linked in particular to the phenomenon of dormancy) to hostile environment conditions (Wang *et al*., 2013).

A sporulation based on direct counting of the spores using the cell of Malassez was carried out for the *Trichoderma* species (Table 1) strains in order to evaluate their sporulation index (SI; represent the number of conidia produced by one gram of carbon source initial present in culture medium (Roussos, 1985).

Spores production by these three strains with 2 methods culture (PDA and SSF) revels that *T.harzianum* is a very sporulating strain (in both culture modalities) compared to other filamentous fungi, *T. viride* and *T. asperellum*, which show sporulation indices respectively 0.13 and $1.4*10^9$ sp/g of carbon source respectively (Table1). This important production of spores in SSC with respect to the synthetic medium can be explained by this mode of culture (SSC culture). However, SSC makes it possible to get as close as possible to the natural conditions of development of the fungi.

3.2. 6-PP production by *Trichoderma* species using SSC:

Results of the isolation of volatile compounds from different tested cultures of fungi applying soxhlet extraction are presented in the Table 2. We identified 6-PP only in the culture of *T.viride* and *T.harzianum* (0.08 and 0.03 mg/g.DM respectively). In cultures of *T.asperellum* this lactone was not detected. The production of 6-PP prove to be dependent of both composition of the environment, especially which relates to the source of carbon and nitrogen, or the control of thermodynamic parameters such as temperature or

Figure 1. Growth of *Trichoderma harzianum* in SSC at 27°C: after 2days (a); after 7 days (b).

Table1. The amount of spores production during the culture of *Trichoderma* species on PDA and SSC.

Strains	Number of spores (10^9 sp/g of carbon source)	
	PDA	**SSC**
T.harzianum	2.80	5.20
T.viride	0.11	0.13
T.asperellum	1.40	1.40

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Table2. 6-PP production by *Trichoderma* species using SSF.

Strains	6-PP (mg/g.DM)
T.harzianum	0.08
T.viride	0.03
T.asperellum	0

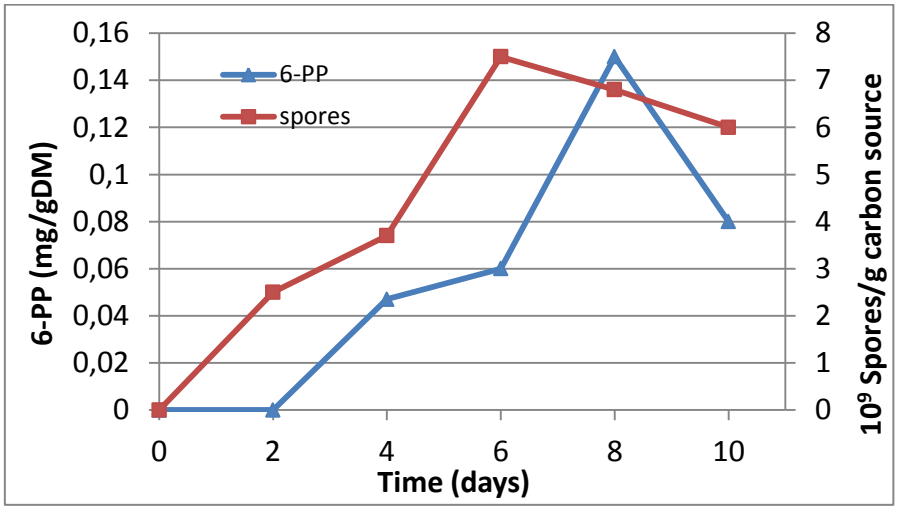

Figure 2: Evolution of the number of spores and 6-PP concentration in the medium during the culture of *Trichoderma harzianum*.

humidity. Similar results have been described by Hölker *et al*. (2004)

3.3. Kinetic of 6-PP and spores production during the SSC of *T.harzianum:*

6-PP concentration and spores production were determined during 10 days of SSC of *T. harzianum* (Fig.2). Spores production was initiated after 1 day of incubation. A high number of spores (7.5 10^9 spores/g carbon source) from the fourth to the sixth day of culture and then a lower rate (6.10^9 spores/g carbon source) was noticed (Fig. 2). After a one day lag phase, 6PP production started. The maximum rate of 6-PP production was observed after 8 days (0.15mg/.g.DM). After that, the 6-PP production decrease progressively to attain values of 0.08 mg./ g.DM at 10 days. Cooney *et al*. (1997) reported a maximum 6-PP yield of 0.9 mg (g dry matter) by a *Trichoderma* isolate after 19 days of SSC. Results reported in the present study indicated that *T. harzianum* cultured on sugarcane bagasse impregnated with nutriment solution produced 0.15 mg (g DM) after 10 days of SSC (Fig. 2) and 6-PP production started at day 2. The kinetic study show that conidiogenesis and the production of 6-PP follow a similar trend at the beginning of cultivation and the production of 6-PP begins after the growth phase, which is a common characteristic of secondary metabolites (Calvo *et al*., 2002). Spores production by *T.harzianum* in SSC as a function of time, showed a considerable increase of the latter as a function of incubation time. The kinetics of the sporulation allows us to deduce that during its life the filamentous fungus pass through two important stages: a phase of vegetative multiplication of 2 to 3 days in fact it is this vegetative multiplication which leads later to an important biomass production and a sporulation phase which is the most important step in the process of vegetative propagation of the fungi. Therefore a normal development of the mycelium is a prerequisite for the important production of conidiospores. This production of conidia is related to the quantity and nature of the source of carbon and nitrogen present in the culture medium. In this case it is favored by a depletion of nutritive resources. Also the proliferation of the mycelium is stopped and allocates a part of the energy in the production of propagules able to resist in time for this change of environment unfavorable. It is for this reason that conidiospores are called resistance structures (Etxebeste *et al*., 2010).

The production of 6-PP prove to be dependent on incubation time. *Trichoderma* strain may produce different metabolites at different stages of growth (Archer *et al*., 2008). Moreover, production of 6-PP was related to carbon and nitrogen sources (Berry.,1988). These results agree with those of Sarhy-Bagnon *et al* (Sarhy-Bagnon *et al*.,2000).

The antifungal properties of this compound, synthesized by *T. harzianum*, were demonstrated by Horace *et al*. (1986).

Conclusion: Under the present culture conditions, the *Trichoderma* species have differences in the production of spores and 6-PP. All this *Trichoderma* species produce spores and little between them that produce 6-PP (*T.harzianum*). 6-PP and spores production by *T.harzianum* were continuous during 10 days of incubation in SSC at 25°C. Nevertheless, the maximum rate of 6-PP was obtained after 8 days of cultivation in SSC. Indeed, the maximum 6-PP obtained in SSC was (0.15mg/g DM). The bagasse was chosen as solid support and as carbon source because *Trichoderma* are lingo-cellulolytic fungi able to

produce a variety of molecules in SSF (Roussos, 1985). Due to the important effect of *Trichoderma* in biological control, mainly through the production of spores and 6-PP (molecule with antifungal properties), optimization of 6-PP production must also be carried out. Several factors may interact 6-PP production like the type of inoculum (mycelium or conidia).

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