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Short communication

Spore production in solid-state fermentation of rice by *Clonostachys rosea*, a biopesticide for gray mold of strawberries

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Abstract

Gray mold caused by *Botrytis cinerea* is an important disease of strawberry. *Clonostachys rosea* is a mycoparasite of *B. cinerea* that reduces fruit losses when used as a biocontrol agent. Since spore production by *C. rosea* has not been optimized, we investigated factors affecting sporulation under aseptic conditions on white rice grains. The greatest spore production in glass flasks, 3.4×10^9 spores/g-dry-matter (gDM), occurred with an initial moisture content of 46% (w/w wet basis), inoculated with 1×10^6 spores/gDM and hand shaken every 15 days. However, a lower inoculum density (9×10^3 spores/gDM) and no shaking also gave acceptable sporulation. In plastic bags 1.1×10^8 spores/gDM were produced in 15 days, suggesting that larger scale production may be feasible: with this spore content, 24 m^2 of incubator space would produce sufficient spores for the continued treatment of 1 ha of strawberry plants.

Keywords: *Clonostachys rosea*; *Botrytis cinerea*; Biological control; Grey mould; Strawberry

1. Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is highly appreciated in many countries, including Brazil, where it is frequently produced in greenhouses. The conditions in greenhouses favor the growth of the ''gray mold'', *Botrytis cinerea* Pers.:Fr., which can cause pre- and post-harvest losses of 50% or more [1]. Control with fungicides is difficult because the plants have fruits at all stages of development and it is not possible to ensure a pre-harvest interval free of fungicide. In any case, chemical control has become difficult due to the development of resistant strains as well as increased concern of consumers towards pesticide use [2–4].

The non-pathogenic, saprophytic fungus *Gliocladium roseum* Bainer, now reclassified as *Clonostachys rosea* (Link:Fr.) Schroers, Samuels, Seifert & W. Gams [5], is a mycoparasite that has emerged as an effective antagonist of *B. cinerea* and promises to improve disease management in several crops [6–9]. In the specific case of strawberries, *C. rosea* is efficacious in reducing fruit losses due to *B. cinerea* [1,6,10,11].

An efficient, standardized process for mass production of *C. rosea* spores is needed, but studies have focused on the efficacy of spores in disease management rather than production of the spores themselves. Spores are generally produced by solid-state fermentation (SSF) on wheat grains [6,8,9,11]. SSF has two main advantages over submerged liquid fermentation. Firstly, fungal spores produced by SSF are typically more robust and have longer shelf-lives than those produced by liquid fermentation [12,13]. Secondly, SSF processes can be undertaken by relatively unskilled workers and it may therefore be possible to transfer the production technology to farmer cooperatives that work under the guidance of government institutions that provide support to farmers, like EMBRAPA in Brazil.

Solid substrates other than wheat have been tested, including rice, a mixture of sphagnum peat and wheat bran, potato dextrose agar, corn meal, poultry layer mash and oat bran [7,13–15]. The aim of the current work was to optimize the conditions for spore production by *C. rosea* in SSF of white rice.

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2. Materials and methods

2.1. Inoculum production

C. rosea was isolated at EMBRAPA-Grape and Wine (Bento Gonçalves, state of Rio Grande de Sul, Brazil) and supplied by EMBRAPA-Environment (Jaguariúna, State of São Paulo, Brazil). It was maintained on potato dextrose agar (PDA) (BioBRÁS Diagnósticos) slants at 4 °C. Two agar plugs (0.5 mm diameter) from PDA cultures were shaken in a test tube with 10 ml of 0.01% Tween 20 in sterile distilled water. An aliquot of 0.3 ml was spread across the surface of each of several PDA plates, which were incubated at 24 °C under constant fluorescent light (''daylight'' lamps, 20 W) for 8 days. After adding 40 ml sterile water to each plate, the surface was scraped with a sterile spatula. The spore concentration of this spore suspension was adjusted to 1×10^7 spores/ml.

2.2. Fermentation procedures for spore production

The control fermentations involved 250 ml Erlenmeyer flasks each with 10 g of dry white organic (i.e., pesticide-free) rice adjusted to 50% (w/w, wet basis) moisture. Flasks were plugged with cotton wool, autoclaved (121 °C, 15 min), inoculated with 0.5 ml spore suspension and shaken to distribute the spores. Flasks were incubated, without shaking, at 24 °C under constant light (''daylight'' lamps, 20 W) for 60 days. In the optimization of spore production described in the following paragraphs this standard procedure was used for control flasks while the stated modifications were made for test flasks.

To test the effect of initial moisture contents, the rice grains were adjusted to moisture contents of 37%, 46%, 53% and 64% (w/w, wet basis) with distilled water, before autoclaving.

To test the effect of the drying regime, control flasks were plugged with cotton wool, flasks in a second group were covered with tissue paper (Kleenex), while flasks in a third group were plugged with cotton wool during 15 days, these plugs then being replaced with tissue paper.

To test the effect of inoculum level, a spore suspension of 1.2×10^8 spores/ml was prepared by using the same 40 ml of solution to harvest spores from three PDA plates. It was diluted with distilled water to give suspensions of 1.8×10^5 to 1.2×10^8 spores/ml (giving inoculum levels of 9×10^3 to 6×10^6 spores/gDM after inoculation with 0.5 ml per flask).

To test the effect of the shaking regime, control flasks remained static during incubation. The other shaking regimes were: daily, every 3 days, every 7 days and every 15 days. Flasks were always hand-shaken in a standard manner (flask bottoms were swiveled backward and forth through an arc of 90°, for 1 min at a rate of 80 oscillations/min, being hit against the hand on the backswing).

2.3. Spore production in plastic bags

Twenty high-density polyethylene bags (25 cm × 20.5 cm, 1 l) each contained 50 g of white rice grains and 37.5 ml of distilled water. After autoclaving (121 °C, 15 min), each bag was inoculated with 2.5 ml spore suspension (1×10^7 spores/ml) and gently shaken to distribute the spores. The open end of each bag was plugged with a cotton wool plug of the same size as those that were used for the flasks, the plug being secured with a rubber band. The bags were incubated at 24 °C under constant light (''daylight'' lamps, 20 W) for 60 days.

2.4. Sample processing and statistical analysis

For each treatment, three flasks (or bags) were collected at 0, 15, 30, 45 and 60 days. Spores were extracted from 1 g samples in 50 ml of 0.01% Tween 20 solution in a 250 ml beaker using a magnetic stirrer (280 rpm) for 30 min. Appropriate dilutions were made and spores were counted in a Neubauer chamber. The moisture content was estimated by drying 5 g of sample to constant weight at 55 °C. All values are given as means ± standard error. The spore contents obtained at the end of each fermentation were compared by one-way ANOVA. Significant differences were accepted at the level of $p < 0.01$.

3. Results

3.1. Spore production on rice in glass flasks

Moisture content is a key process variable in SSF processes. Over the range of initial moisture contents of 37–64% (w/w wet basis), the highest spore content ($p < 0.01$), 1.3×10^9 spores/gDM, occurred at 46% initial moisture (Fig. 1a). Note that growth and sporulation depend on a related parameter, the water activity. The poor performance at 37% initial moisture is probably due to the water activity being too low. On the other hand, the substrate with 64% initial moisture was sticky and agglomerated, which would have restricted the diffusion of oxygen within the substrate bed.

Several authors have claimed that a decrease in water content during the process favors sporulation by *C. rosea* but did not present moisture content profiles [6,8,9,13]. In the present work, in all cases the moisture content fell significantly during the fermentation (Fig. 1b). After 30 days the moisture content of the substrate with 64% initial moisture had fallen to 38% while in all other cases it had fallen to less than 10%. An initial moisture content of 46% gave the best balance, allowing a high enough water activity for early growth, while also allowing the substrate to dry sufficiently for sporulation to be stimulated.

The drying rate depends on the manner in which the flask is closed and the relative humidity of the surrounding atmosphere.

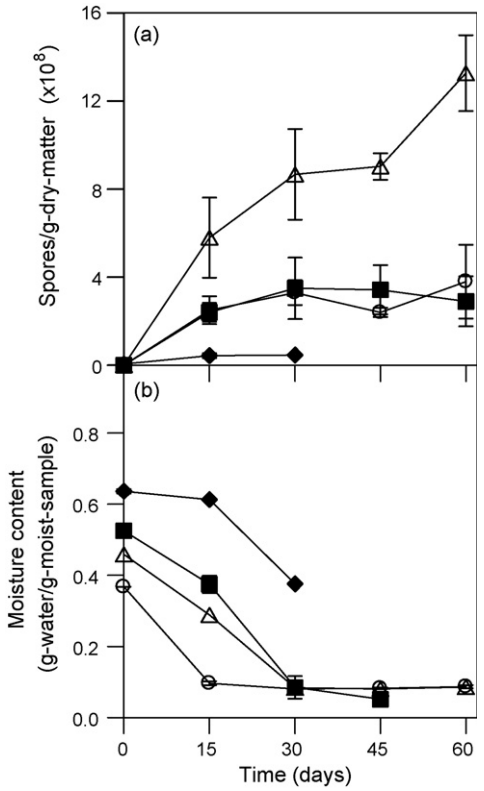

Fig. 1. Effect of initial moisture content on spore production and moisture content changes during the cultivation of *Clonostachys rosea* on white rice grains. (a) Spore production and (b) moisture content changes. Key: initial moisture contents—(○) 37%; (△) 46%; (■) 53%; (◆) 64% (w/w wet basis). The bars represent standard errors.

These effects were tested by closing the flasks in three different ways (Fig. 2). The highest spore production ($p < 0.01$), 7.8×10^8 spores/gDM, occurred in the flasks that were covered with tissue paper throughout (Fig. 2a). In this case the moisture content decreased rapidly from an initial value of 52% to 8% at 15 days (Fig. 2b). Final spore contents were lower in the other two treatments, in which the moisture content remained around 50% for the first 15 days. The moisture content then decreased to 7% by day 30 in those flasks in which the cotton wool plugs were replaced with tissue paper on day 15. In flasks that were covered with cotton wool plugs during the whole 60 days, the moisture content decreased more slowly, reaching 15% at day 30.

Drying of the substrate promotes production of penicillate conidiophores: these conidiophores yield more spores than the verticillate conidiophores that predominate when *C. rosea* is cultured under persistent high humidity [6]. Tissue paper presents less of a barrier to the diffusion of water vapor than does a cotton wool plug and therefore allows faster drying and higher spore production. However, tissue paper is problematic since it does not resist autoclaving and provides a poor barrier against contamination. Therefore cotton wool plugs were maintained for the remaining experiments.

Inoculum densities around 10^5 spores/gDM have been used for SSF with *C. rosea* [9,11,12] but there are no reports about the optimization of inoculum density. With inoculum densities from 9×10^3 to 1×10^6 spores/gDM, spore production levels were of the order of 3×10^9 spores/gDM (Fig. 3). At higher inoculum levels, spore production decreased slightly to around 2×10^9 spores/gDM, however, the difference was not statistically significant ($p > 0.1$). Clearly, a lower inoculum level could be used than that used in the current work. This would be advantageous at production scale since the required volume of inoculum would be smaller. However, we maintained the inoculum concentration at 1×10^6 spores/gDM since it fell within the range that gave optimum spore production.

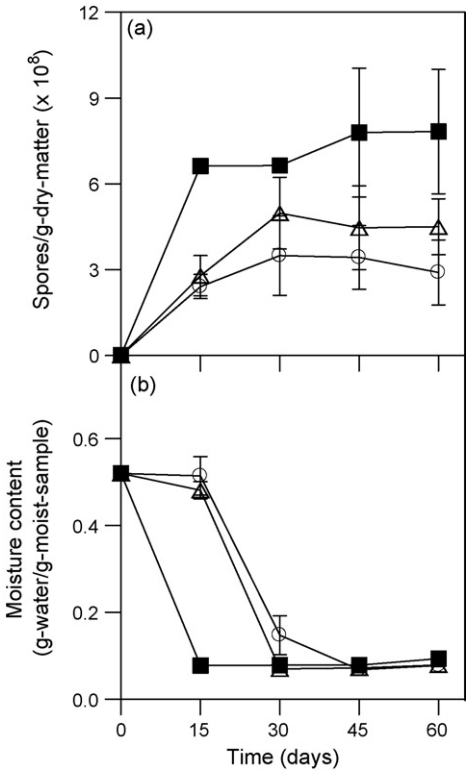

Fig. 2. Effect of drying regime on spore production and moisture content changes during the cultivation of *C. rosea* on white rice grains. (a) Spore production and (b) moisture content. Key: (○) flasks covered with cotton wool plugs during 60 days; (■) flasks covered with tissue paper during 60 days; (△) flasks covered with cotton wool plugs during 15 days and than replaced with tissue paper until day 60. The bars represent standard errors.

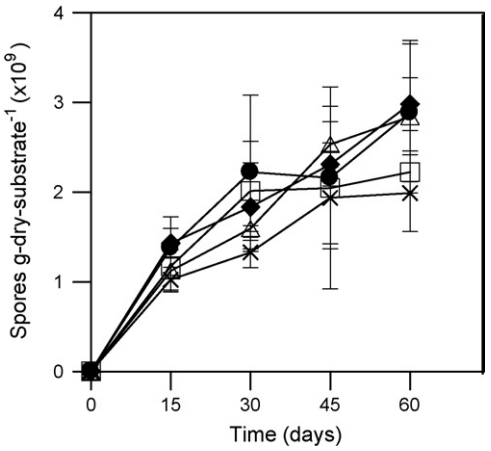

Fig. 3. Effect of inoculum density on spore production during the cultivation of *C. rosea* on white rice grains. Key: spore production using inoculum levels of (×) 6×10^6, (□) 2.4×10^6, (△) 1×10^6 (control), (◆) 9×10^4, and (●) 9×10^3 spores/gDM. The bars represent standard errors.

To evaluate the effect of shaking on spore production by *C. rosea*, five shaking regimes were tested: every day, every 3 days, every 7 days, every 15 days and static. Spore production was similar in flasks that were shaken every 15 days, every 7 days and in static flasks (3.4, 2.6 and 2.7×10^9 spores/gDM, respectively) (Fig. 4). Spore production decreased significantly ($p < 0.01$) when flasks were shaken more often, with a final spore content of 1.8×10^9 spores/gDM in flasks shaken every 3 days and a final spore content of 1.3×10^9 spores/gDM in flasks shaken every day.

Shaking can alleviate some of the problems encountered with solid substrate beds. It can improve the accessibility of oxygen to the substrate surface by disrupting aerial fungal

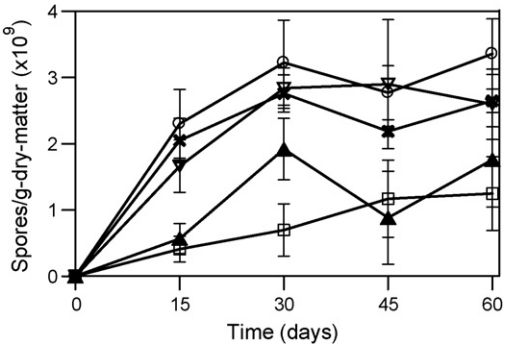

Fig. 4. Effect of agitation regime on spore production during the cultivation of *C. rosea* on white rice grains. Key: (×) static flasks; (○) agitation every 15 days; (▽) agitation every 7 days; (▲) agitation every 3 days; (□) agitation every day. The bars represent standard errors.

hyphae, which grow into and fill up the inter-particle spaces. However, shaking also has deleterious effects. In particular, shear forces can damage conidiophores, leading to decreased spore production.

3.2. Spore production in plastic bags

In the plastic bags the spore content increased to 1.1×10^8 spores/gDM over the first 15 days, and then increased slowly to 1.2×10^8 spores/gDM at 45 days. Note that spore contents were about 10-fold lower than those obtained in the flask-based studies.

4. Discussion

Recent studies have shown that *C. rosea* is an effective biofungicide for management of gray mold, caused by *B. cinerea* [1,6–11]. However, these studies focused on evaluating the efficacy of the product and made no effort to establish optimized methods for spore production. We have contributed the first study of the factors affecting the production of spores by a strain of *C. rosea* that is effective in the control of *B. cinerea* on strawberries. Our long-term aim is to develop a simple but reliable process for the production of spores of *C. rosea*, using technology that can readily be transferred to strawberry farmer cooperatives supported by governmental institutions.

Based on the results of the current paper, we can make a preliminary analysis of the feasibility of such a process. We took as our starting point a commercial product available in Brazil, Clonosnat®. This product contains 1×10^6 spores/ml and the recommended application rate varies from 200 to 600 l/ha, depending on the weather. An application rate of 600 l/ha means that each application requires 6×10^{11} spores/ha. If we harvest our bags at 15 days we will have 1×10^8 spores/gDM and, since each bag contains 50 gDM, we have 5×10^9 spores per bag. Therefore we need 120 bags per hectare. Each bag occupies an area of 0.05 m^2, so the total incubation area for one application is 6 m^2. The manufacturers recommend applications every 4 days and, given a 15-day fermentation time, we need to have four batches (with starting times staggered by 4 days) in the incubator at any one time. Therefore we need approximately 24 m^2 of illuminated shelf space in the incubator in order to service one hectare of strawberries. This is quite feasible and would not require large capital investments. Further, given that spore contents of 2×10^9 spores/gDM were obtained in 15 days in static flasks (Fig. 4), there is potential to reduce the required shelf space to the order of 1 m^2/ha. Such a process would be simplified by the fact that shaking is not necessary.

We have taken the first steps towards the establishment of a well-characterized and reproducible process for the production of spores of *C. rosea*. Once such a process is established, it will be possible to use *C. rosea* as part of the strategy for managing *B. cinerea* on strawberries. Of course, studies must proceed to determining the efficacy of the product in biological control in the field because virulence, survival and germinability of spores can vary with different production techniques.

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