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SCREENING OF *METARHIZIUM* SPP. STRAINS FOR ANTICANCER INDOLIZIDINE ALKALOID PRODUCTION AND ITS RAPID DETECTION BY MS ANALYSIS

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ABSTRACT

Six fungi strains (*M. anisopliae* 3935, 4516, 4819, PL57, PL43 and *M. flavoviride* CG291) were studied regarding their ability to produce an anticancer indolizidine alkaloid. The culture process was carried out in Shaken flask at 26ºC and 200 rpm using three different culture medium containing oat meal extract supplemented with glucose and DL-lysine or Czapek culture medium. The mycelial extracts produced by *Metarhizium spp.* cultures were directly submitted to electrospray ionization mass spectrometry (ESI-MS) analysis and the highest alkaloid concentration (approximately, 6 $mg.L^{-1}$) was reached when *M. anisopliae* 3935 was tested.

Key words: *M. anisopliae*, *M. flavoviride*, anticancer, alkaloid, fermentation

INTRODUCTION

Nowadays, the cancer is the second largest death cause after the cardiovascular diseases. However, according to recent estimates of the World Health Organization (WHO) up to 2015 this disease can be the first death cause in the world since that more than 10 million people in the world develop the cancer every year and approximately 6 million die because of this disease. In this context, is well-known the need to develop new products and processes to complement the used procedures in this disease treatment.

In the present study, several *Metarhizium* ssp. strains were evaluated with respect to its ability to produce an anticancer alkaloid commonly known as swainsonine because it was firstly isolated from the Australian legume *Swainsona canescens* (Fig. 1).

Swainsonine is a polyhydroxylated indolizidine alkaloid that has been isolated from plants (1-3) and produced, as a secondary metabolite, by cultures of *Rhizoctonia leguminocola* (4), *M. anisopliae* (5-7) and more recently by fungal endophytes of locoweed (8). On the other hand, this alkaloid and its analogues have been chemically synthesized (9) which is very expensive due to the difficulties created by their four chiral centers resulting in more than twenty reaction steps and in racemic mixtures.

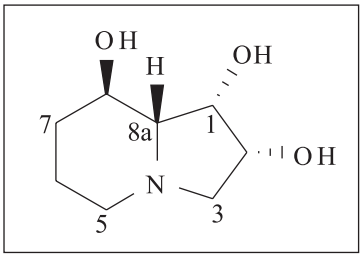

Figure 1. Polyhydroxylated indolizine alkaloid ((1S, 2R, 8R, 8aR)-1,2,8-trihydroxyocta-hydroindolizidine).

A number of the polyhydroxylated alkaloids have been reported with anticancer activity (10), however swainsonine has attracted significant attention in recent years because of its antimetastatic, antiproliferative and immunomodulatory activity, finding applications in the therapeutic treatment of the cancer and AIDS (10-14). In despite of, extraction by plant and chemical synthesis, the swainsonine production by fungal cultures has received a little attention. Consequently, in this work we

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investigated the preliminary swainsonine production in a laboratory scale in Erlenmeyer shaken flask by six strains of *Metarhizium ssp*. isolates from Brazilian soil using different culture media.

MATERIALS AND METHODS

Microorganisms and reagents

M. anisopliae 3935, 4516 and 4819 were obtained from Tropical Research Foundation "André Tosello" culture collection, Campinas, Brazil. *M. anisopliae* PL57, PL43 and *M. flavoviride* CG291 were kindly provided by Doctor João Lúcio de Azevedo from College of Agriculture "Luiz de Queiroz" (ESALQ/USP), Brazil. Stock cultures were maintained on oatmeal agar at 4ºC. Swainsonine from *Rhizoctonia leguminocola* was purchased from Sigma-Aldrich, USA and dissolved in water to yield a standard solution of 0.1% w/v. The analytical grade water was purified in a Milli-Q system (Millipore, Bedford, USA). All chemical reagents and culture media used in this work were purchased from Merck and Sigma-Aldrich.

Culture media preparation

Three different culture media were employed: *M1*- medium based on oatmeal extract 2% w/v was prepared by cooking oatmeal in distilled water for approximately 10 minutes followed by filtration. After, this solution was supplemented with 10 $g.L^{-1}$ D-glucose and 0.1 $g.L^{-1}$ DL-lysine solution. *M2* - similar to *M1* medium based on oatmeal extract 2% w/v, supplemented with 10 $g.L^{-1}$ D-glucose solution and 1.8 $g.L^{-1}$ DL-lysine solution. *M3* - Czapek medium with the addition of 1.8 $g.L^{-1}$ of DL-lysine. All media were sterilised at 121ºC, 15 psi for 15 min.

Fermentation

Spore suspensions were prepared from previously sporulated cultures on oatmeal agar slopes by vigorous vortexing in sterile water. Inoculums, of approximately 10^7 $spores.mL^{-1}$ counted in a Neubauer camera, were aseptically added to each Erlenmeyer flask containing sterile medium. Thus, fermentations were carried out in shaken flasks (250 mL) at 26ºC and 200 rpm for 10 days.

Biomass determination

The biomass was determined by dry mass analysis. Fermented medium samples were filtered through pre-weighed filter paper (Whatman No.1) and the harvested biomass was washed twice with distilled water and then dried until constant weight at 65ºC and 8 kPa in a vacuum oven GST-920 model (SUPRILAB, Brazil).

Glucose assay

The glucose concentration was measured at 500 nm, using a spectrophotometer model 4800 (Hach Company, USA), according to the glucose oxidase method using a quantitative Glucose Enzyme Color Reagent kit from Bio Diagnóstica (Curitiba, PR, Brazil).

Identification and alkaloid concentration quantification

The alkaloid determination was carried out by Electrospray ionization mass espectrometric (ESI-MS) analysis in a positive mode using a Q-TOF mass spectrometer (Micromass, Manchester, UK). The general conditions were established according to Moller *et al*. (16): source temperature at 80ºC, capillary voltage of 3 kV and cone voltage of 40 V. For detection and alkaloid quantification a standard curve was prepared adding 0.5 µL of formic acid and methanol solution (1:1 v/v) to the standard swainsonine solution (500 µL) to yield 0.1% as the final concentration whose solution was conveniently diluted. The fermented medium samples were prepared by similar form and the ESI(+)-MS analysis was carried out by direct infusion with a flow rate of 10 $\mu Lmin^{-1}$ using a syringe pump (Harvard apparatus). Mass spectra was acquired and accumulated over 60 s and scanned over 50 to 800 m/z range. The ion of interest was judged by visual inspection and compared with the standard and then ESI-MS/MS was performed to acquire its mass spectrum and verify the alcaloide production.

RESULTS AND DISCUSSION

The fermented media at the end process were filtrated at vacuum, centrifuged at 2000´g in an EPPENDORF refrigerated centrifuge (model 5804R) and the supernatants were analyzed for determination produced alkaloid by ESI-MS. Previously, the standard swainsonine sample was examined by ESI(+)-MS yielded a intense protonated molecular ion ($[M+H]^+$) as the most abundant ionization product, as is shown in the Fig. 2A, at m/z 174 which is characteristic of this molecule. After, direct infusion of fermented culture medium under ESI(+)-MS yielded spectra with a number of ions between 50 to 800 m/z scanned range shown as illustrative way for fermented culture by 3935 strain in the Fig. 2B and 2C. Nevertheless, similarly to the standard analysis the mass spectrum shows an protonated molecular ion at m/z 174, suggesting the tryhydroxylated alkaloid swainsonine presence, although numerous other peak occur in the ESI-MS analysis, possibly corresponding to other metabolites, residual culture medium and several extracellular compounds excreted by cell and consequently present in the sample analysis. Then, ESI-MS/MS was performed by mass selecting of ion at m/z 174 (Fig. 2B and 2C) and its spectra, correspondents to the are shown, for the standard and fermented culture by 3935 strain, in Fig. 3A to 3C. The resulting MS/MS product ion spectra exhibited successive losses of 18 a.m.u, suggesting losses of hydroxyl groups as water corresponding to the ions m/z 156, 138 and 120, respectively, which allowed to confirm the swainsonine alkaloid production. Although a large number of compounds are present in fermented

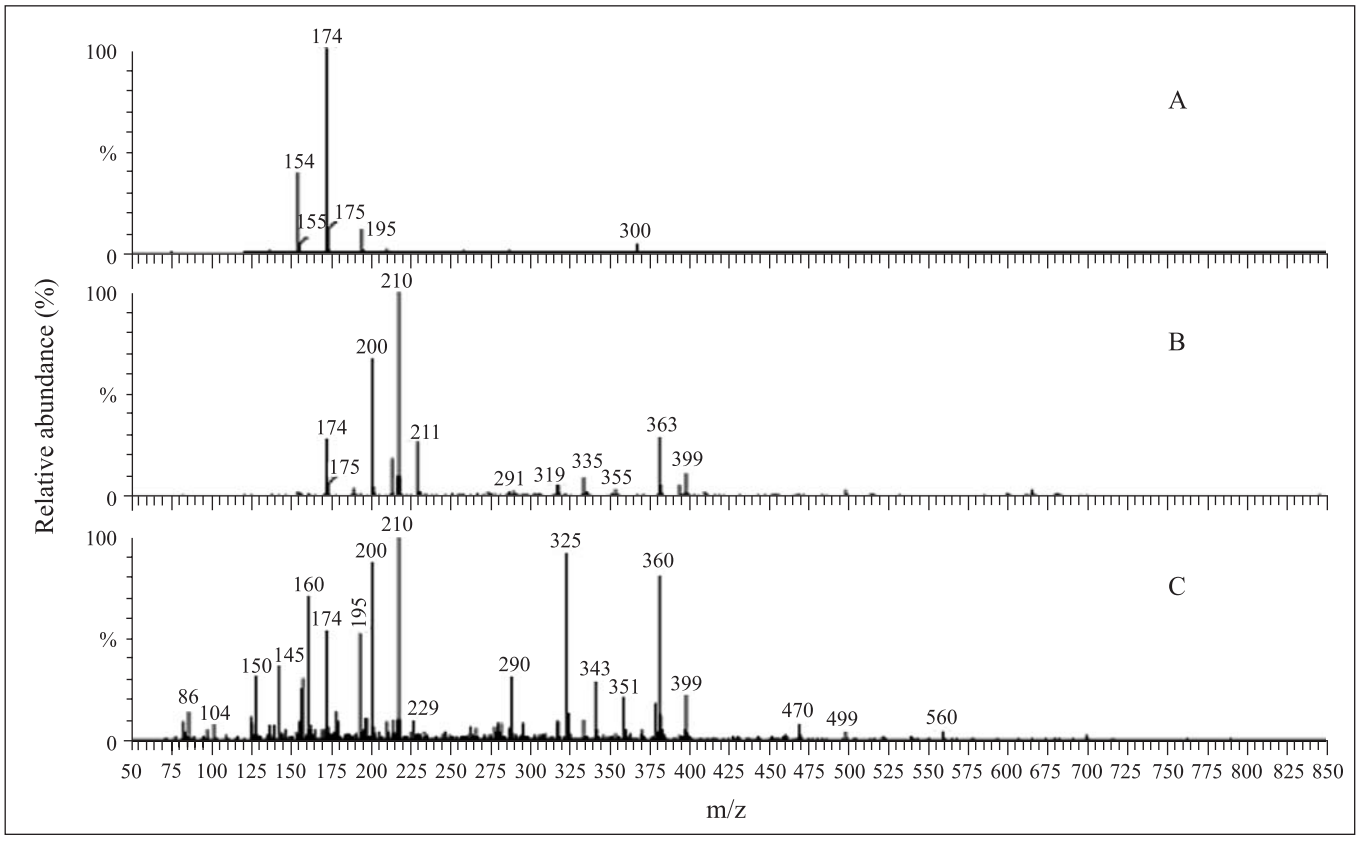

Figure 2. Electro-Spray Ionisation Mass Spectra obtained in a positive mode (ESI(+)-MS) of swainsonine standard obtained from Sigma (A), alkaloid obtained by *Metarhizium anisopliae* 3935 in oatmeal culture fermented medium (B) and in a Czapek-Dox culture fermented medium (C).

culture medium, fluctuation in the relative intensities of the signal ions was not found by direct infusion of the samples. In addition, with this technique derivatization is avoided and the extended analysis time used in gas chromatographic e.g. is drastically reduced.

ESI(+)-MS was performed by direct infusions to determine the produced alkaloid, according to the standard curve attained for several concentration values of standard alkaloid. In this context, the produced alkaloid for all experiment are shown in the Fig. 4 together with the formed biomass, concentration glucose and pH. As observed, four strains produced the indolizidine alkaloid, *M. anisopliae* 3935, PL57 e PL43 and *M. flavoviride* CG291. However, the highest alkaloid concentration, approximately, 6.0 $mg.L^{-1}$ was attained when oatmeal extract supplemented with 1.8 $g.L^{-1}$ DL-lysine (*M2* medium) was used in the *M. anisopliae* 3935 culture. This culture medium was also the most favorable for the alkaloid production by PL57 and PL43 strains. When lysine and glucose were not used or when a low lysine concentration was used in the culture medium, extracellular alkaloid production was not detected. Therefore, we verified that lysine was a precursor for alkaloid production. On the other hand, the alkaloid biosynthetic pathway, which proceeds by lysine catabolism via Saccharopine, has been investigate by Harris (3) and Sim and Perry (15), but has not been completely explained.

A morphological analysis of the fermented culture medium showed a combination of both hyphal and pelleted growth. However, during the fermentation the biomass migrated and accumulated at the surface of the cultures. Probably, the extracellular alkaloid concentration was influenced by this fact. However, intracellular amounts of this alkaloid were not verified.

M. anisopliae 3935 presented a satisfactory growth in all culture media after 10 days of fermentation, not showing a significant variation in the relative biomass value (Fig. 4). On the other hand, the PL43 strain presented more higher growth on Czapek medium, but the alkaloid concentration was one of the smallest (0.9 $mg.L^{-1}$), i.e., the alkaloid production was inversely proportional to the fungus growth. The same results were observed for the PL57 strain, suggesting that the conditions which favor the cell growth were different from those that

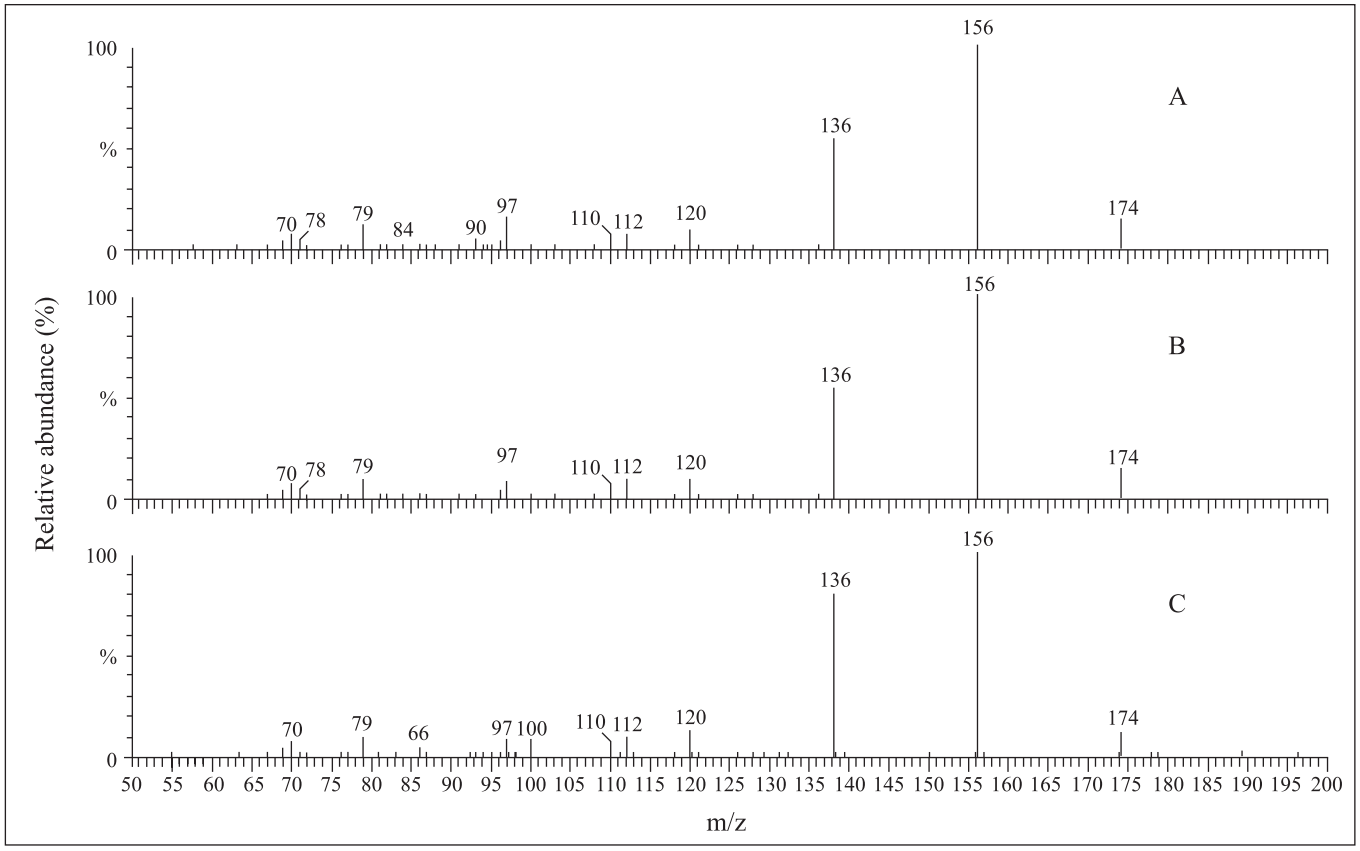

Figure 3. ESI(+)-MS/MS of swainsonine standard obtained from Sigma (A), swainsonine obtained by *Metarhizium anisopliae* 3935 in oatmeal culture fermented medium (B) and in a Czapek-Dox culture fermented medium (C).

influenced the alkaloid production, e.g., low pH values showed a positive effect on the alkaloid production (Fig. 4).

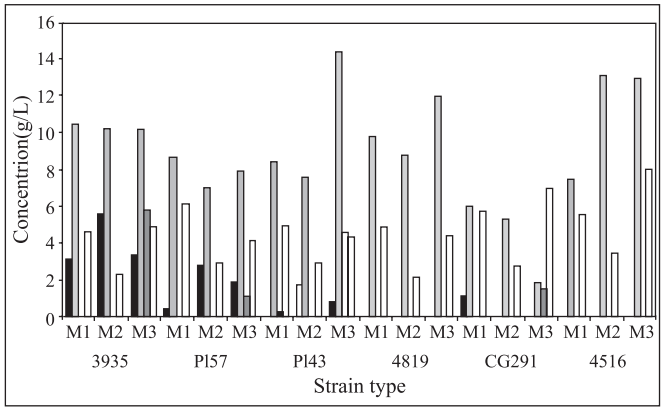

Figure 4. Obtained results for all fermented cultures in Erlenmeyer flask at 26ºC and 200 rpm, during 10 days, using the M1, M2 e M3 culture media. ■ - Alkaloid (mg.L^{-1}); ▒ - Biomass (g.L^{-1}); ▓ Glucose (g.L^{-1}); □ pH.

CONCLUSION

The highest indolizidine alkaloid concentration was reached when oatmeal extract supplemented with 1.8 g.L^{-1} DL-lysine (*M2* medium) was fermented by *M. anisopliae* 3539 and further studies to understand the alkaloid biosynthesis in order to optimize the fermentation process are in progress. The ESI-MS/MS provides a direct, simple, rapid and sensitive method for the formed alkaloid analysis of direct infusion of fermented culture medium by cultures of *Metarhizium spp.* strain.

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RESUMO

Seleção de linhagens de *Metarhizium* spp. para a produção de alcalóide indolizidínico anticancerígeno e sua rápida detecção por MS análise

O presente trabalho teve como objetivo avaliar diferentes cepas dos fungos *M. anisopliae* e *M. flavoviride* ao respeito da sua capacidade de produzir um alcalóide anticancerígeno, por fermentação em frascos erlenmeyers usando três meios de cultura distintos. De seis cepas testadas, quatro foram capazes de produzir o composto de interesse, *M. anisopliae* 3935, PL57 e PL43 e *M. flavoviride* CG291, sendo que a maior concentração de alcalóide (aproximadamente, 6 mg.L^{-1}) foi produzida pelo *M. anisopliae* 3935, contendo um meio constituído de extrato de farinha de aveia, glicose e DL-lisina a 26ºC e 200 rpm.

Palavras-chave: *M. anisopliae*, *M. flavoviride*, metabolito secundário, alcalóide anticancerígeno

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