Antibiotic Production of the Biocontrol Agents *Epicoccum nigrum* and *Candida sake*

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Abstract

In the framework of the study of the mode of action of biocontrol agents (BCAs) it is important to know if BCAs are antibiotic-producers. *Epicoccum nigrum* 282 and *Candida sake* CPA-1 are BCAs effective against post-harvest pathogens of stone and pome fruits. The antibiotics produced by these BCAs and the relationship to biocontrol were studied. Production of antibiotics by *E. nigrum* in *in vitro* cultures began at 5 days of incubation being maximal at different times depending on media used. However, no antibiotic was detected when *E. nigrum* was grown in a solid state-fermentation system or in peaches. In the case of *C. sake*, no antibiotic was detected either *in vitro,* in liquid fermentation cultures of the yeast, or in apples.

Keywords: apples; antibiotic; biocontrol; *Monilinia* spp.; *Penicillium expansum*; peaches; post-harvest

INTRODUCTION

One of the objectives of the Biopost-harvest EU project ("Development of biocontrol agents for commercial application against post-harvest diseases of perishable foods", www.biopostharvest.org) is the determination of the mode(s) of action of the BCAs. *Epicoccum nigrum* Link (strain 282) and *Candida sake* (Saito and Ota) van Uden and Buckley (strain CPA-1) are effective BCAs against post-harvest pathogens of stone and pome fruits such as *Monilinia* spp. and *Penicillium expansum*, involved in this project. The production of antibiotics by these two strains was studied in order to fulfil the registration procedure of these biopesticides.

MATERIALS AND METHODS

To test for optimal production of antibiotics by the two strains conical flasks (250 ml) containing 50 ml of different culture media (PDB: potato-dextrose broth, SCA: sucrose casamino acid, GAP: glucose ammonium nitrate and PE: peach extract, for *E. nigrum*; and NYDB: nutrient dextrose broth, MYE: malt yeast extract, and YMS: yeast malt sucrose, for *C. sake*) (BROWN *et al.* 1987; MADRIGAL *et al.* 1991) were each inoculated with three disks of 8 mm diameter mycelium in the case of *E. nigrum*, or with fresh cells of *C. sake* (10^8 cells/ml). Flasks were incubated at different conditions (stationary or 150 rpm – 25°C for *E. nigrum*, and 150 rpm – 20°C for *C. sake*) and times (5, 10, 15, 20 and 30 days for *E. nigrum*, and 1, 3, 7 days for *C. sake*). Then, media were separated from the fungal biomass and sterilized to obtain the crude filtrates. Cells-free culture filtrates of *C. sake* were also produced by liquid fermentation of the yeast in molasses (40g/l):urea (1.2 g/l). In addition conidia of *E. nigrum* were produced in solid-state fermentation bags containing a mixture of peat:vermiculite:lentilmeal (1:1:0.5; w:w:w) incubated at 20–25°C for 10 days. The content of bags was extracted with diethyl ether or methanol. Organic phases were evaporated and redisolved in water. The toxicity of these crude filtrates or the organic phases was bioassayed *in vitro* and *in*

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vivo towards the respective pathogens (*M. laxa*, and *P. expansum*). *In vitro* bioassays were made on the germination of spores and the growth of germ tubes of *M. laxa*, or *P. expansum* as described in DE CAL *et al*. (1988). *In vivo* bioassays were made with *C. sake* in apples artificially infected with *P. expansum*. Nine replicates were made and the complete experiment was repeated at least twice. Lesion diameters were measured after 7 days of incubation at 22°C and 75% HR in an air-conditioned room.

Production of antibiotics was also tested *in vivo*. Peaches and apples artificially inoculated with *M. laxa* (10^4 conidia/ml) or *P. expansum* (10^4 conidia/ml), respectively, were dipped in a water suspension of *E. nigrum* (10^6/ml) or *C. sake* (10^6/ml), respectively, for 1 min. After 7 days incubation peach tissues were extracted with diethyl ether and methanol, and apple tissues with water. Fractions were bioassayed against *M. laxa* or *P. expansum* as describe above.

All the experiments were repeated at least twice.

RESULTS

Results obtained with *C. sake* vs *P. expansum* showed no inhibition of the spore germination or the germ tube growth of *P. expansum* with any culture filtrate of *C. sake*. In addition, no inhibition of diameter lesions induced by *P. expansum* in apples cv. Golden was obtained by application of any culture filtrate. Extracts of apple tissues did not inhibited *P. expansum*. From these results we can conclude that no antifungal substances were produced by *Candida sake*.

In the case of *E. nigrum* 282, this fungus incubated in both stationary and shaking regime in PDB, produced antifungal compound(s) that inhibited the germination of spores of *M. laxa*. The concentration of this (these) compound(s) in the filtrates was (were) maximal at 10 days of incubation, when crude filtrates strongly inhibited *M. laxa*. At 20 days inhibition of *M. laxa* by the crude filtrate decreased, being non-existent at 30 days in stationary regime (Table 1). Inhibition of *M. laxa* by crude filtrates of *E. nigrum* produced in GAP and SCA are shown in Figure 1. Maximal inhibition was obtained by crude filtrates produced in GAP between 10 and 15 days. Crude filtrates produced in SCA inhibited less *M. laxa*. Inhibition of *M. laxa* by crude filtrates of *E. nigrum* produced in peach extract medium was maximal at 20–25 days of incubation (Table 2).

Table 1. Inhibition (%) of *Monilinia laxa* by crude filtrates of *Epicoccum nigrum* produced in Potato Dextrose Broth at different incubation days and incubation systems*

Incubation days	Incubation system	Germination	Sporelings
10	Stationary	100 a	100 a
	Shaking	97 a	92 b
20	Stationary	54 b	51 c
	Shaking	47 bc	41 d
30	Stationary	–	23 e
	Shaking	41 c	44 d

*Data are the mean of 3 replicates. Means followed by the same letter in each column are not significantly different by protected LSD test ($P = 0.05$)

Antifungal substances were extracted from media used in solid-state fermentation in absence of *E. nigrum* that inhibited germination of *M. laxa* (Figure 2). When *E. nigrum* was grown in this media no antifungal substances were extracted from it (Figure 2).

Antifungal substances were not produced by *E. nigrum* in peaches treated with *E. nigrum* and non inoculated with *M. laxa* (Table 3). Antifungal substances were extracted with ether and methanol from peaches inoculated with *M. laxa* and treated or non-treated with *E. nigrum* (Table 3).

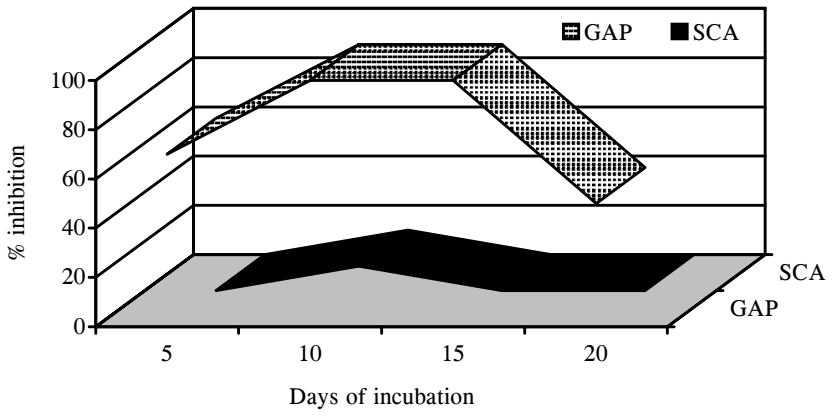

Figure 1. Inhibition (%) of *Monilinia laxa* by crude filtrates of *Epicoccum nigrum* produced in GAP and SCA media at different incubation times

Table 2. Inhibition (%) of *Monilinia laxa* by crude filtrates of *Epicoccum nigrum* produced in peach extract media at different incubation times*

Incubation days	Germination	Sporelings
5	49 a	44 b
10	–	34 b
15	43 a	29 b
20	77 b	24 b
25	70 b	22 b
30	58 b	17 b

*Data are the mean of 3 replicates. Means followed by the same letter in each column are not significantly different by protected LSD test ($P = 0.05$)

Table 3. Germination of *Monilinia laxa* spores in the presence of diethyl ether or methanol from peaches treated with *Epicoccum nigrum* and inoculated with *M. laxa**

Treatment	Diethyl ether	Methanol
Uninoculated/Untreated	91 a	93 a
M. laxa/Untreated	35 c	17 c
Uninoculated/*E. nigrum*	89 a	82 a
M. laxa/*E. nigrum*	67 b	31 b

*Data are the mean of 3 replicates. Means followed by the same letter in each column are not significantly different by protected LSD test

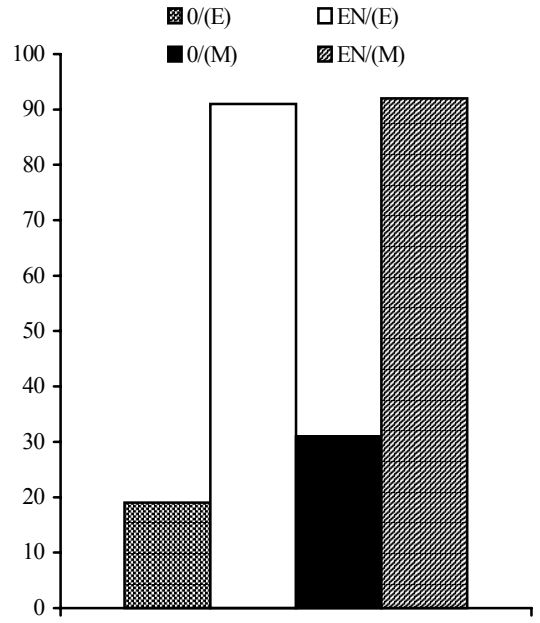

Figure 2. Percentage of germination of *Monilinia laxa* spores in the presence of extracts of diethyl ether (E) or methanol (M) from production media inoculated with *Epicoccum nigrum* (EN) or non-inoculated (0) in two experiments

DISCUSSION

The species *E. nigrum* is already known to produce various antifungal compounds *in vitro*, depending on the media it grows (BROWN *et al*. 1987). Some of them have not yet been identified but others have been characterized: epicorazine A, epicorazine B, epirodin A and B and flavipin. We demonstrate previously that *E. nigrum* 282 produced flavipin on potato dextrose agar (MADRIGAL *et al*. 1991). Here we demonstrated that production of antifungal compounds by *E. nigrum* 282 began at 5 days of incubation being maximal at different times depending on media used.

In the Biopost-harvest project a semi-industrial process to produce conidia of *E. nigrum* was developed (DE CAL *et al*. 2001), and production of antibiotics by *E. nigrum* was tested in this media. Although antifungal compounds toxic to *M. laxa* were extracted from this media non-inoculated with *E. nigrum*, no antifungal compound was detected in the media inoculated with *E. nigrum* and incubated for 10 days after the conidia production process. These experiments demonstrated that *E. nigrum* did not produced antibiotics in the solid-state fermentation media.

Experiments carried out in fruits picked at pre-harvest, at harvest and at post-harvest shown that antifungal compounds were extracted from fruits inoculated with *M. laxa*, treated or non-treated with *E. nigrum*, but not in fruits only treated with *E. nigrum*. Antifungal compounds should be extracted from rot peach tissues due to the infection of *M. laxa* since inhibition of germination is greater in the case of peaches only inoculated with *M. laxa* than in peaches inoculated with *M. laxa* and treated with *E. nigrum*.

From these results, we can conclude that although *E. nigrum* produced antibiotics when grown in some synthetic media, no antibiotic was produced by *E. nigrum* in the semi-industrial production media in solid-state fermentation or when it is applied to peaches. Also, we can conclude that *C. sake* does not produced antibiotics in the media tested, in the semi-industrial production media and in apples.

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