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ENHANCEMENT OF GROWTH OF *LENTINUS CRINITUS* IN SOIL USING BENOMYL AND VEGETABLE OIL

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ABSTRACT

In order to identify alternatives for soil fumigation in bioremediation process, the addition of benomyl and vegetable oil on the growth of *L. crinitus* and mitosporic fungi were evaluated. Benomyl can be used as an alternative to methyl bromide. Addition of vegetable oil favors the growth of *L. crinitus*.

Key words: benomyl, basidiomycetes, fungicide, soil bioremediation, laccase

Lentinus crinitus CCB274 has been selected for bioremediation of HCB-contaminated soils in São Vicente, São Paulo, Brazil. The growth of this fungus has been studied in bioreactors (400 kg of soil), where soil sterilization is achieved by fumigation with methyl bromide (7) and the addition of vegetable oil at the time of inoculation has been shown to increase hexachlorobenzene biodegradation (5). The use of methyl bromide poses an environmental risk (2), and its replacement is therefore desirable. The addition of growth inhibitors of mitosporic fungi may thus be an alternative to fumigation. Benomyl (Benlate) exerts a fungitoxic effect on most ascomycetous fungi (3) and has been added to culture media for the isolation of basidiomycetes because of the tolerance of this fungal group to this substance (10). In order to identify alternatives to fumigation, we evaluated the effect of the systemic fungicide benomyl and addition of vegetable oil on the growth of *L. crinitus* and mitosporic fungi.

L. crinitus CCB274 is deposited at the Basidiomycetes Culture Collection (CCB, Instituto de Botânica/SMA), São Paulo, Brazil. This fungus was isolated from decaying wood (8) and the culture has been maintained on 2% malt extract agar at 4ºC. Mitosporic fungi were isolated on 2% MEA during the growth of *L. crinitus* in bioreactors containing non-sterilized soils in a previous assay. Three morphologically distinct cultures were obtained and were identified as *Aspergillus* sp., *Penicillium* sp. and *Pestalotiopsis guepinii* by standard methods based on microscopy and identification keys. For the fungal growth on solid media, disks (5 mm) were cut from the plates where the fungus had grown, inoculated in the center of a Petri dish containing 2% MEA plus different concentrations of benomyl (5, 15, and 50 mg L^{-1}), and incubated at 28 ± 1ºC in triplicate. Fungal growth was determined by daily measurement of the radius (cm) in each quadrant of the plate. Fungi grown in medium with acetone and without benomyl were used as control. Soil from São Vicente, S.P., Brasil (98.0% sand, 2.3% organic matter, 0.06% nitrogen, 1.0 µg g^{-1} phosphorus and 0.01 mEq 100 mL^{-1} potassium, pH 3.65, with a cationic exchange capacity of 5.5 mEq 100 g^{-1} soil) was used. Growth of mitosporic fungi was analyzed on rose bengal medium (1% glucose, 0.5% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.0035% Rose bengal, 0.004% streptomycin sulfate, and 2% agar). Fifty grams of a mixture of soil and $CaSO_4$ (90:2.5, dry weight) containing benomyl (0, 5 or 10 mg kg^{-1} soil) was placed in 200-mL flasks in triplicate. *L. crinitus* grown in supplemented sugar-cane bagasse (6) was inoculated (2.5% soil dry weight). During inoculation, 5% of a suspension of vegetable oil and Tween 20 (1:0.1) was added and the cultures were incubated at 28 ± 1ºC. At 20 and 47 days samples were removed for the measurement of fungal biomass and laccase activity. The same parameters were evaluated in non-sterilized soil inoculated with inactivated *L. crinitus* biomass (sterilized at 120ºC for 1 hour). *L. crinitus* inoculated into soil sterilized by tyndallization (100ºC, 1 hour, 3 days) without benomyl

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was used as control. To determine the influence of vegetable oil on the establishment of *L. crinitus* in non-sterilized soil, oil and Tween 20 suspension was added 20 days after inoculation of the basidiomycete, in one treatment only. The flasks receiving the suspension at the time of inoculation were used as controls. All experiments were carried out in triplicate. Fungal biomass was estimated based on the measurement of ergosterol (9). The enzymatic extract was obtained from soil samples using 50 mM sodium acetate buffer (1:5), pH 4.5, after shaking (1 hour, 90 rpm) and filtration (45 µm). Laccase activity was determined by monitoring the increase in absorbance at 420 nm (4). One enzyme unit was defined as an activity producing 1 µmol oxidized ABTS per minute using an extinction coefficient of 43.2 $mM^{-1}cm^{-1}$ (2).

L. crinitus was inhibited by about 34% at a benomyl concentration of 5 mg L^{-1} when cultured on solid medium. Inhibition of about 66% and 92% was observed when 15 and 50 mg L^{-1} of the fungicide, respectively, were added. The mitosporic fungi *Penicillium* sp., *Aspergillus* sp. and *Pestalotiopsis guepinii* only grew on media without benomyl. These results confirm the semi-selective effect of benomyl which is toxic to most ascomycetes. Mitosporic fungal counts in soil were 1.28×10^7 CFU. The efficiency of 5 and 10 mg benomyl kg^{-1} soil to control the native mitosporic fungal microbiota and to favor the growth of basidiomycetes in non-sterilized soil was evaluated (Table 1). In treatments in which inactivated *L. crinitus* inoculum was used, benomyl significantly inhibited the growth of mitosporic fungi. Laccase activity was not detected in the treatments that received the inactivated *L. crinitus* inoculum. A direct relationship between laccase activity and benomyl concentration was observed, supporting the role of this fungicide in favoring the basidiomycete. The determination of fungal biomass based on ergosterol production did not permit the distinction between basidiomycetes and other fungi. However, when the effect of benomyl on the growth of mitosporic fungi is considered, the increase in ergosterol in the presence of 5 mg benomyl kg^{-1} may reflect the growth of *L. crinitus*. On the other hand, the biomass reduction observed in the presence of 10 mg benomyl kg^{-1} soil may be due to the inhibitory effect of this fungicide on *L. crinitus* growth as observed on solid media. Colonization of non-sterilized soil by *L. crinitus* was favored when oil was added 20 days after inoculation of the basidiomycete (Table 2). Vegetable oil is an easily assimilated energy source. When applied at the beginning of incubation, the oil favored the growth of mitosporic fungi evidenced by ausence of laccase activity. Laccase activity was found to be the best indicator for the presence and activity of basidiomycetes in soil. Benomyl at the concentration of 10 mg kg^{-1} can be used as an alternative to methyl bromide in soil bioremediation processes mediated by *L. crinitus*.

Table 1. Fungal biomass and laccase activity in non-sterilized soil inoculated with *Lentinus crinitus* CCB274 in the absence and presence of 5 and 10 mg benomyl kg^{-1} soil at 28ºC ± 1.

	Ergosterol content (µg g^{-1} of wet mass) after 47 days of incubation		Laccase activity (U L^{-1})	
Benomyl (mg kg^{-1} soil)	with heat -inactivated *L. crinitus*	with lived *L. crinitus*	20 days*	47days*
0	7.07±0.039	8.25±1.426	4.22 (a)	1.67 (c)
5	1.93±0.209	11.21±0.339	20.00 (a)	27.59 (bc)
10	3.64±0.183	4.81±0.448	27.04 (a)	70.37 (ab)
control	nd	10.48±1.293	19.07 (a)	133.33 (a)

Control: colonization of *L. crinitus* in sterilized soil in the absence of benomyl, nd: not determinated, * equal letters indicate statistycal equality in each colunm (Tukey test, $\alpha \leq 0.05$).

Table 2. Influence of the addition of vegetable oil on the growth of *Lentinus crinitus* CCB274 in non-sterilized soil after 47 days of incubation at 28 ± 1ºC.

Parameter	Inoculation of *Lentinus crinitus*	Addition of vegetable oil	
		1st day	20th day
Visual analysis of soil colonization	+	73%	80%
Ergosterol content	+ (a)	79%±13.6	59%±3.1
	- (b)	68%±0.3	60%±1.9
Laccase activity	+	1%±0.1	33%±2.1
	-	0%	0%

(a) The percentages were calculated considering the growth of *L. crinitus* in sterilized soil in the absence of benomyl as 100%.
(b) The percentages were calculated considering the growth of fungi in soil with heat-inactivated *L. crinitus* in the absence of benomyl as 100%.
Control laccase activity = 133.3 U L^{-1}.

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RESUMO

Aumento do crescimento de *Lentinus crinitus* em solo usando benomil e óleo vegetal

Para identificar alternativas para a fumigação de solo em processos de biorremediação, foi avaliada a adição de benomil

e de óleo vegetal no crescimento de *Lentinus crinitus* e fungos mitospóricos. Benomil pode ser usado como alternativa ao brometo de metila. A adição de óleo vegetal favoreceu o crescimento de *L. crinitus*.

Palavras-chave: benomil, basidiomicetos, fungicida, biorremediação de solos, lacase

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