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Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05

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Summary. The physiological requirements needed to enhance the production of laccases by the ascomycete *Botryosphaeria rhodina* MAMB-05 in submerged cultivation were examined under non-induced and induced (veratryl alcohol, VA) conditions. Under non-induced conditions (–VA), the initial pH, C:N ratio, and inorganic N source did not influence laccase production, in contrast to Tween 80, soybean oil, and copper, which significantly increased laccase production, and proline and urea, which suppressed laccase formation. In addition, Tween 60 could serve as the sole carbon source for the production of these enzymes. Under VA-induced conditions of fungal growth, factors such as inoculum type, time-point of addition of inducer, initial pH, C:N ratio, and type of N source, influenced the production of laccases; however, unlike the non-induced conditions, proline and urea did not act as suppressors. Each of these physiological conditions exerted different effects on biomass production. The nutritional conditions examined for *B. rhodina* MAMB-05 are discussed in relation to their influence on fungal growth and laccase production. [**Int Microbiol** 2007; 10(3):177-185]

Key words: *Botryosphaeria rhodina* · laccases · veratryl alcohol · C:N ratio and N sources · Tween and soybean oil · copper

Introduction

Fungi belonging to the genus *Botryosphaeria* are phytopathogens that attack a wide range of host plants of agricultural, forestry, ecological and economic importance, and cause dieback diseases in trees and rots in pre-harvested fruits. *Botryosphaeria* species primarily direct their attack on the plant cell wall, producing enzymes associated with cell-wall degradation [9]. An isolate of *Botryosphaeria* (MAMB-05), characterized at the species level as *B. rhodina* [Garcia JE, et al. (2004) GenBank accession no. AY612337] and found to be ligninolytic, produces only laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) [4]. Laccase is increased above basal levels when the fungus is grown on nutrient medium containing the laccase inducer veratryl alcohol (VA) [4,7], and culture conditions can be optimized for laccase production by the response surface method [46]. Under fermentation conditions in the presence (+VA; induced) or absence (–VA; non-induced) of inducer in the nutrient medium, it has been found that aeration [7], the type of carbohydrates used as C source [2], and the nature of the lignin-like phenolic compounds [8] included in the medium influence laccase production by *B. rhodina*.

Laccases (multi-copper oxidases) [27] are widely distributed among fungi associated with wood-decay (mainly basidiomycetes), but filamentous fungi, including the ascomycetes, such as *B. rhodina* [7], are also recognized as producing laccases [19]. In these fungi, as in their basidiomycete counterparts, several laccase isozymes encoded by multiple genes are expressed under different environmental

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conditions [24]. Consequently, in different fungi, the production of minor laccase isoforms can be enhanced under appropriate culture conditions [21].

Laccase production occurs during secondary metabolism and is subject to complex regulation by nutrients (C, N, inducers, and copper) in the culture medium during fungal growth. These regulators affect the transcription levels of laccase and other genes in various fungal taxa [17,25,34,35,41]. Furthermore, genes encoding laccase isozyme are differentially expressed in the presence of certain aromatic inducers, with total transcript levels differing markedly depending upon the nature of the aromatic compounds [44]. Fermentation variables also affect laccase production and are important in optimizing conditions for maximum enzyme production. Thus, the growth conditions play a significant role in the production of extracellular fungal laccases [36].

The expression of laccases in some fungi is regulated by N-limiting conditions [12], while in others N-sufficiency (low C:N) results in enhanced enzyme production [20]. The response is dependent upon the fungal genera and species within these genera [43]. The effect of aromatic inducers (e.g., VA and 2,5-xylidine) on laccase production has also been reported to be dependent upon the composition of the nutrient media [3,36]. Compounds that modify the fungal membrane (surfactants) are known to promote laccase secretion by diverse fungal strains [33]. As laccases are multi-copper-containing proteins, copper induces laccase formation [31].

In continuing our basic studies on the physiology of laccase production by *B. rhodina*, we examined the nutritional requirements of this ascomycete with the objective of enhancing laccase production. In *B. rhodina*, laccase induction by VA always occurred at enzyme titers higher than basal levels, and VA was always added prior to inoculation using agar plugs colonized with fungus. In these earlier studies, however, the biochemical parameters of laccase production were not examined. We now report the influence of inoculum type, time-point of VA addition, initial pH, C:N ratio, type of N source, the surfactants Tween 80 and Tween 60 as sole C source, a vegetable-seed (soybean) oil, and copper on the levels of laccase produced when *B. rhodina* MAMB-05 is cultivated under non-induced (–VA) and induced (+VA) conditions. Each of the different physiological conditions also has an effect on biomass production by the ascomycete.

Materials and methods

Microorganism, culture media, and growth conditions. The fungus *Botryosphaeria rhodina* MAMB-05 (formerly described as *Botryosphaeria* sp. MAMB-05, [4]) was grown in submerged liquid cultivation in baffled Erlenmeyer flasks at 28°C on a rotary shaker (180 rpm) for 4.5 days on basal nutrient medium (VMSM, Vogel minimal salts medium [47], containing as C source 10 g glucose/l, pH 6.0) using three 7-mm agar plugs as the inoculum [7]. During induction experiments, VA (Aldrich, USA) was added to the basal medium prior to inoculation at a concentration of 30.4 mM [46]. All experiments were done in replicates of four. The results represent the mean value ± SD.

Effect of inoculum type. Two types of inocula were evaluated: fungal-colonized agar plugs and homogenized mycelium. To ensure that equivalent amounts of mycelium were used in these experiments, a correlation (r^2 = 0.9381) was established between the optical density (OD, at 400 nm) of the homogenized mycelium and the dry weight of mycelium (mg). A preparation containing homogenized mycelium diluted 20-fold gave an OD of 0.8 (equivalent to 0.82 mg mycelium), and three agar plugs (0.84 mg colonized mycelium) were used for inoculation in comparative studies.

Effect of time-point of VA addition. *B. rhodina* was cultured on basal medium and inoculated with three agar plugs. At 0, 12, 24, 36 and 48 h after inoculation, VA (30.4 mM) was added to the growing cultures, after which the fungi were left to grow for 4.5 days. A control, in which no VA was added to the nutrient medium, was included for comparison purposes.

Effect of initial pH. Basal medium was prepared with and without added 30.4 mM VA, and the initial pH adjusted within the range 3.0–8.0 using 1M HCl and 1M NaOH.

Effect of carbon/nitrogen (C:N) ratio. The C:N ratio was examined using NH_4NO_3 and L-asparagine as N sources in the presence and absence of 30.4 mM VA. Modified VMSM was prepared according to Vogel [47], but omitting the N source. A concentrated solution of NH_4NO_3 and L-asparagine (0.65–5.0 ml of 1.0 M and 1.71 M solutions for each N source to give a final C:N ratio of 3.29–25.33) was added to 80 ml of the modified VMSM solution. The pH was adjusted to 6.0, and water added to a final volume of 100 ml. In the presence of inducer, VA was added to the modified VMSM solution to a final concentration of 42.2 mM. Aliquots of 22.5 ml were dispensed into 100-ml baffled Erlenmeyer flasks, which were then sterilized. Steam-sterilized glucose solution (2.5 ml of 100 g/l solution) was added aseptically, and the flasks inoculated with agar plugs and cultivated as described above.

Effect of N sources. Two types of N sources were examined: inorganic ($NaNO_3$, NH_4Cl, $[NH_4]_2SO_4$, $[NH_4]H_2PO_4$, $[NH_4]_2HPO_4$, and NH_4NO_3, the N source of VMSM), and organic (L-asparagine, L-proline, and urea). Modified VMSM was prepared as above, and each of the aforementioned N sources was added separately to a final C:N ratio of 6.58 in the presence and absence of 30.4 mM VA. Sterile glucose was then added, and the flasks were inoculated and cultivated as described above.

Effect of Tween 60 and 80. *B. rhodina* was cultured on basal medium in the presence and absence of Tween 80 (1.0 g/l) under non-induced and induced (30.4 mM VA) conditions. At 4.5 days, the extracellular fluid (ECF) was recovered by centrifugation (1250 ×*g*, 30 min); the mycelium was removed and washed with distilled water, macerated under liquid N in 0.2 M Tris-HCl buffer (pH 6.8), and left at 4ºC for 5 h. Following centrifugation, the supernatant (intracellular fluid) was assayed for laccase activity. In another experiment, *B. rhodina* was grown on VMSM containing Tween 60 (1.0 g/l) as the sole C source.

Effect of soybean oil. Commercial food-grade soybean oil (Liza, Brazil) was added to VMSM at a concentration of 1% (v/v). In a separate experiment, soybean oil was added to basal medium. Both experiments were conducted in the absence of inducer. The nutrient medium was inoculated with agar plugs and cultivated as described above. Mycelium recovered by centrifugation was washed twice with water to remove residual oil and the biomass was determined.

Effect of copper. VMSM with and without added 30.4 mM VA was prepared as described above, with NH_4NO_3 as N source (C:N ratio 6.58, pH 6.0) but omitting copper from the trace-element solution. A solution of $CuSO_45H_2O$ (0.5 g/l or 10.0 g/l) was added to the VMSM to give final copper concentrations of 0–41 µg Cu/ml (i.e., 0–640 µM $CuSO_45H_2O$). Aliquots of 22.5 ml were dispensed into Erlenmeyer flasks and sterilized. Sterile glucose was then added and the flasks were inoculated and cultivated as described above.

Enzymatic assays. Cell-free culture fluid was obtained after removal of the mycelium by centrifugation (1250 ×*g*, 30 min) and was used as the source of enzyme. Laccase activity was assayed against the putative laccase substrate ABTS (2,2'-azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]) at pH 3.0 and 50°C [4], and monitored as the increase in A_{420} (ε = 36,000/M/cm). Laccase activity was expressed in units as µmol oxidized product formed/min/ml of enzyme under the assay conditions.

Analytical techniques. Reducing sugars were determined by the cuproarsenate method [42], and total sugars by the phenol-sulfuric acid method [11] using glucose as the standard. Protein was determined according to Bradford [5]. Fungal biomass was measured gravimetrically and dried to constant weight at 70°C.

Statistical analyses. Analysis of variance (ANOVA) and Tukey tests were done using STATISTICA, version 6 [www.statsoft.com] (StatSoft Inc., 2001).

Results and Discussion

Effect of inoculum type. The types of inoculum preparations for the production of laccases vary and include spore suspensions [12], mycelium [30], homogenized mycelium [33], and fungal-colonized agar plugs [41], but there are no general recommendations regarding which inoculation method is the best. However, we found that the type of inoculum used for the production of laccases by *B. rhodina* under induced conditions greatly affected fungal growth and significantly influenced laccase production. When basal medium was inoculated with agar plugs colonized with *B. rhodina*, the growing cultures tolerated high concentrations of VA (30.4 mM). Under these conditions, higher levels of inducible laccase (4.83 ± 0.38 U/ml) were produced compared to the control (–VA; 0.53 ± 0.01 U/ml), but growth was reduced (4.41 ± 0.40 g/l vs. 5.92 ± 0.28 g/l for the control). When freshly homogenized mycelium was used as inoculum in amounts equivalent to those of the agar plugs, cultures growing on basal medium containing the same level of inducer (30.4 mM) were severely affected. Specifically, the biomass was lower (1.12 ± 0.11 g/l) and laccase titers were reduced (0.15 ± 0.03 U/ml). In this case, the VA concentration needed to be significantly lowered (10 mM) to achieve similar laccase levels (4.20 ± 0.03 and 3.25 ± 0.04 U/ml) at equivalent amounts of mycelium as inoculum.

The agar plug inoculum appears to tolerate far higher levels of VA [7,46] than the homogenized mycelium. One reason for the disparity in growth and laccase titers depending on the type of inoculum may be related to the toxicity of VA and to cell damage, such that disrupted mycelium arising from homogenization are more sensitive to the inducer. This sensitivity of different inocula may explain why inducers must be added at low concentrations to effectively stimulate the production of laccases in some basidiomycetes [12,33,41]. However, this is the first time that this effect has been reported in ascomycetes.

Table 1. Effect of the time-point of addition of veratryl alcohol (30.4 mM) on growth and laccase production by *Botryosphaeria rhodina* MAMB-05

Time of addition (h)	Laccase activity (U/ml)	Biomass (g/l)
Control[a]	2.99 ± 0.13	7.51 ± 0.29
0	5.18 ± 0.86	4.61 ± 0.25
12	4.56 ± 0.55	5.37 ± 0.73
24	3.72 ± 0.43	6.71 ± 0.38
36	4.12 ± 0.59	7.74 ± 0.67
48	1.57 ± 0.28	7.47 ± 0.32

[a]Non-induced conditions (–VA).

Effect of time-point of VA addition. There is wide-ranging variation in the optimal time-point at which inducing compounds should be added to nutrient medium to effectively produce laccases when agar plugs are used as inoculum, i.e., prior to inoculation [6], during the exponential phase of growth (after 24 h [12] or 48 h [29]), or later, during stationary phase (6th day [41]). In our study, the time-point of VA addition resulted in important differences in the amount of laccase produced by *B. rhodina* (Table 1). Highest laccase titers occurred only when VA was added to the nutrient medium at the beginning of fermentation, i.e., prior to inoculation, and they decreased when the inducer was added later in the experiment. Fungal growth, however, appeared to favor the later addition of VA following inoculation, yielding amounts of biomass comparable to those of non-induced cultures (control value in Table 1).

The addition of inducers during the exponential phase can generally reduce undesirable effects of toxic compounds on fungal growth [36]. VA has been demonstrated to be a powerful inducer of laccases in many basidiomycetes [29], but there are few comparable studies for ascomycetes. In most of those, inducer was added to the culture medium after established growth of the fungus, resulting in enhanced laccase production at low inducer concentrations (e.g., <0.3 mM, [12]). In *B. rhodina*, the induction of laccases by VA occurs at higher concentrations [7,46] when added prior to inocula-

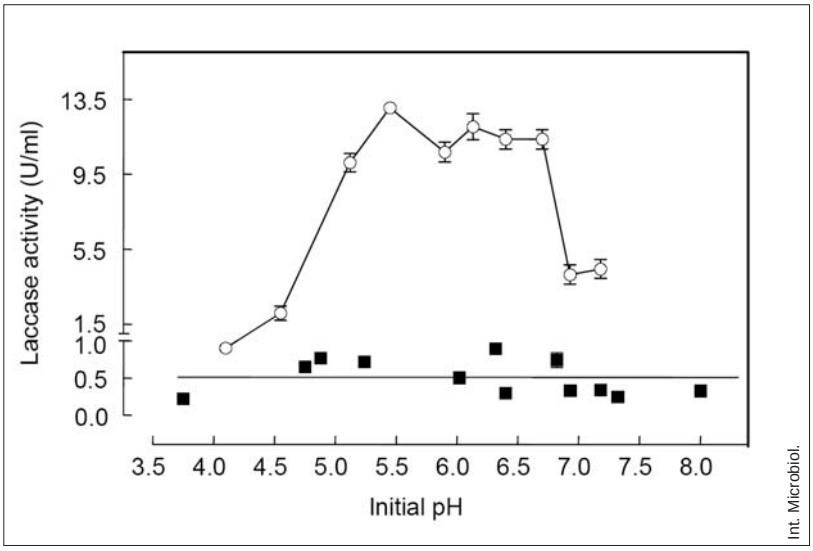

Fig. 1. The effect of initial pH on laccase production by *Botryosphaeria rhodina* MAMB-05 under non-induced (–VA) and induced (+VA) conditions of growth. NH_4NO_3 was used as the N source at a C:N ratio of 6.58. Closed squares, –VA; open circles, +VA.

tion with agar plugs. Under these conditions, the fungus adapts to the presence of the inducer during growth and tolerates the higher concentrations of VA presented. As previously reported, VA only becomes toxic to *B. rhodina* at concentrations >40 mM [7].

Effect of initial pH. Linear regression analysis of laccase production by *B. rhodina* within the initial pH range of 3.5–8.0 indicated no general effect ($P > 0.05$) of initial pH on laccase production under non-induced conditions (Fig. 1). In the presence of inducer, however, there was a pronounced effect of initial pH, resulting in a 15-fold increase in laccase titers as the initial pH increased from 4.0 to 5.5. Moreover, laccase production was 25-fold higher in induced cultures than in non-induced cultures. The optimal initial pH of 5.5–6.5 is similar to that reported for other fungi [26,29,41]. ANOVA and regression analysis of initial pH with respect to the growth of *B. rhodina* showed a significant effect ($P < 0.001$), with increasing biomass production as the pH increased in the range of 3.5–7.5 for both non-induced (range: 4.6 ± 1.05 g/l to 7.26 ± 0.09 g/l) and induced (1.60 ± 0.40 g/l to 4.42 ± 0.55 g/l) cultures. The amount of biomass produced was always higher in non-induced fungal cultures, in agreement with previous findings [46].

Effect of C:N ratio. The C:N ratio of the basal medium was 6.58. At this ratio, the amount of laccase produced under non-induced conditions on either N source, inorganic NH_4NO_3 or organic: L-asparagine, was similar (3.21 ± 0.36 and 3.37 ± 0.76 U/ml, respectively). The effect of increased N levels at a constant C (glucose) level (C:N ratios in descending order: 25.3–3.3) on laccase production by *B. rhodina* likewise did not appear to be influenced by either of the N sources used under non-induced conditions. ANOVA analysis confirmed that neither N source had an effect ($P > 0.05$) on laccase production over the range examined. Consequently, the C:N ratio of the VMSM was kept constant at 6.58. Similar observations regarding the lack of influence of nutrient N levels on laccase production were described for *P. sajor-caju* [13,41]. However, in *Pycnoporus cinnabarinus* [12] and *Pycnoporus sanguineus* [33], the C:N ratio is an important factor in laccase production, while laccase production in cultures of *Trametes trogii* [22] and *Fomes scleroderm-meus* [32] grown on high N is higher than when the fungi are grown on low N medium (high C:N).

Fungal growth was likewise not influenced by the C:N ratio in *B. rhodina*, as biomass levels did not statistically differ ($P > 0.05$) over the range of C:N ratios examined when inorganic N under non-induced and induced conditions (mean values of 7.41 ± 0.59 g/l and 2.98 ± 0.61 g/l, respectively) was used. A similar effect was also observed for *P. cinnabarinus* [12].

In the case of organic N (asparagine) at C:N = 6.58, the levels of laccase produced (10.56 ± 0.56 U/ml) were significantly higher than those arising from NH_4NO_3 (6.09 ± 0.52 U/ml) under induced conditions. A C:N ratio within the range 3.29–25.33 appeared to influence laccase production in induced cultures of *B. rhodina* and resulted in a ca. 30% increase in enzyme titers as the N levels increased ($P < 0.05$) within the range studied (Fig. 2). At a C:N ratio of 6.58 with asparagine as the N source, fungal growth was similar to that with NH_4NO_3 (mean values for both 6.39 ± 0.60 g/l) under non-induced conditions, but biomass production was adversely affected under induced conditions (7.78 ± 1.28 g/l

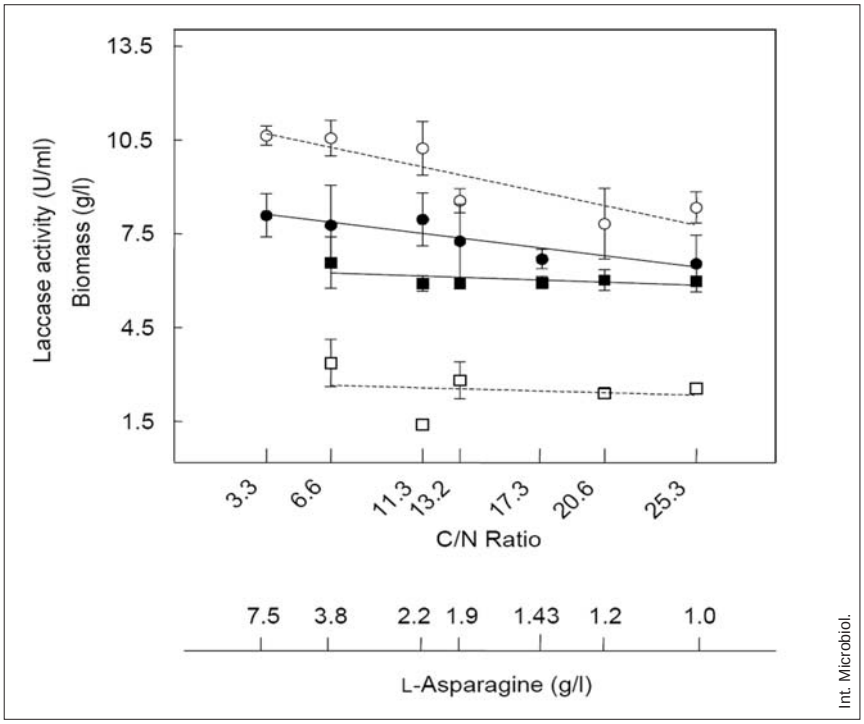

Fig. 2. The effect of increasing the C:N ratio on the production of laccase by *Botryosphaeria rhodina* MAMB-05 grown under non-induced (–VA) and induced (+VA) conditions. Solid and dashed lines represent lines of regression analysis. The concentrations of organic N (asparagine) are indicated in the reverse order. Open squares, laccase –VA; open circles, laccase +VA; closed squares, biomass –VA; closed circles, biomass +VA.

and 2.5 ± 0.32 g/l, for the respective N sources). This effect was also generally observed with proline and urea as N sources (see below). The growth of *B. rhodina* was positively affected by the C:N ratio when the N source was asparagine under induced—but not under non-induced—conditions. An inverse relationship existed for biomass production, which decreased as the C:N ratio increased (Fig. 2).

Effect of N sources. Another factor reported to be essential for efficient laccase production is the nature of the N sources for fungal cultivation [12,20,43]. We examined the ability of six inorganic N sources to stimulate laccase production by *B. rhodina*. Table 2 shows the results for the non-induced cultures. The N source did not significantly influence biomass production by *B. rhodina* when the fungus was grown under non-induced conditions, with similar amounts of enzyme produced in each case. Different inorganic N sources have been reported [18] to increase laccase activities in *P. ostreatus* strain 32, depending upon the species and strains of the genus *Pleurotus* studied [43] as well as the cultivation conditions.

In the induced cultures of *B. rhodina*, all inorganic N sources examined, with the notable exception of NH_4Cl, increased laccase titers (Table 2). Unconsumed NH_4Cl in the ECF may have affected laccase activity, as chloride ion is a potent inhibitor of this enzyme in *B. rhodina* [7]. The induced laccase titers were about five-fold higher than the corresponding laccase levels for the best respective inorganic N source incorporated into the nutrient medium under non-induced conditions. Biomass production when *B. rhodina* was cultured under induced conditions was similar, with the exception of NH_4Cl, but was generally lower than when the fungus was grown in the absence of VA. Among the organic N sources asparagine, L-proline, and urea, the latter two, but not asparagine, strongly suppressed laccase production in *B. rhodina* when added to the basal medium under non-inducing conditions (Table 2). In fact, barely measurable laccase activity could be detected with proline as the N source. A suppressive effect of some amino acids, including proline, was reported for the production of manganese peroxidase in *Phanerochaete chrysosporium* [1] and laccase in *Cyathus bulleri* [10]. Many secondary metabolic pathways are negatively affected by N sources (e.g., proline and urea) favorable for growth, and high N concentrations can alter the synthesis of sensitive enzymes involved in secondary metabolism during fermentation [39]. This may well be the case with proline and urea in *B. rhodina*. The amount of fungal biomass increased two-fold when *B. rhodina* was grown on proline under non-induced conditions (Table 2).

Under induced conditions, proline did not suppress laccase production in *B. rhodina*, although enzyme titers decreased ca. 24 % compared to the control. This trend was also seen with urea and asparagine as N sources (Table 2). Proline enhanced biomass production under induced condi-

Table 2. Laccase production by *Botryosphaeria rhodina* MAMB-05 and growth of the fungus in the presence of different N sources added to non-induced (–VA) and induced (+VA) cultures. N sources were added to yield a final C:N ratio of 6.58

	–VA[a]		+VA	
Nitrogen source	Laccase activity (U/ml)	Biomass (g/l)	Laccase activity (U/ml)	Biomass (g/l)
Inorganic				
NH_4NO_3	0.44 ± 0.60	6.06 ± 1.92	12.69 ± 0.46	5.50 ± 0.35
$NaNO_3$	2.89 ± 0.30	5.15 ± 0.37	5.39 ± 1.45	3.59 ± 1.55
NH_4Cl	0.35 ± 0.04	5.50 ± 0.80	0.13 ± 0.02	3.20 ± 0.47
$(NH_4)_2SO_4$	0.39 ±0.15	5.13 ± 0.23	14.86 ± 4.09	4.48 ± 0.50
$NH_4H_2PO_4$	0.20 ± 0.05	4.75 ± 0.27	15.26 ± 1.26	5.14 ± 0.69
$(NH_4)_2HPO_4$	3.12 ± 0.45	5.38 ± 0.26	9.86 ± 1.01	4.91 ± 0.37
Organic[b]				
No asparagine	0.50 ± 0.06	6.44 ± 0.53	11.35 ± 0.98	3.98 ± 0.48
Asparagine	3.37 ± 0.76	6.58 ± 0.82	10.56 ± 1.38	7.78 ± 1.28
No proline	0.55 ± 0.10	6.41 ± 0.18	8.64 ± 1.71	3.62 ± 0.31
Proline	0.0002 ± 0.00003	12.24 ± 0.96	6.98 ± 1.01	14.68 ± 0.16
No urea	0.98 ± 0.13	5.16 ± 0.27	4.56 ± 1.84	1.52 ± 0.36
Urea	0.08 ± 0.02	5.11 ± 0.38	3.65 ± 0.16	3.36 ± 0.17

[a]VA, veratryl alcohol.
[b]In the absence of organic N sources, the N source used in the minimal salts medium was replaced by NH_4NO_3.

tions (3.5-fold) compared to fungal cultures grown in the absence of proline. This trend also occurred with urea and asparagine as N sources, and it appeared that in the presence of organic N sources VA was no longer able to reduce fungal growth. However, the mechanism behind this response is unclear. In contrast to our observations with *B. rhodina*, urea was reported to stimulate laccase production in *P. ostreatus* strain 32 grown under static conditions [18]. Similarly, the inducer 2,5-xylidine did not inhibit laccase production in *P. ostreatus* when grown on urea as N source [36]. In *B. rhodina*, replacing the inorganic N source by asparagine increased laccase production some two-fold under induced conditions (Table 2) and biomass likewise increased. High concentrations (80 mM) of asparagine, as N source, also enhanced laccase production in *F. sclerodermeus* [32].

Effect of surfactants. The use of surfactants in enzyme production has been well-documented. Surfactants increase the permeability of the membrane's lipid bilayer, which facilitates the secretion of intracellular enzymes [28]. However, in *B. rhodina* cultured under non-induced or induced conditions, Tween 80 behaved differently, increasing laccase titers by significantly increasing laccase production (from 0.77 ± 0.02 to 1.03 ± 0.12 U/ml for –VA; and from 6.01 ± 0.099 to 9.14 ± 0.102 U/ml for +VA, in the absence and presence of Tween 80, respectively). The low intracellular laccase levels (–VA: 0.024 and 0.033 U/ml; +VA: 0.053 and 0.092 U/ml) measured under either growth condition did not account for the increases in laccase activity in the ECF when the fungus was grown in the presence of Tween 80. These observations suggested that Tween 80 induced laccase production in *B. rhodina*, and that cellular secretion of laccase into the ECF was not promoted by the presence of the surfactant. Tween 80 was reported to enhance the production of laccases in *P. sanguineus* [33] and *T. trogii* [22], while in *Ganoderma* sp. various Tween surfactants enhance laccase production [40] under non-induced conditions. By contrast, the presence of Tween 80 in cultures of *P. sanguineus* grown on 2,5-xylidine as inducer resulted in decreased laccase production [33].

The addition of Tween 80 to induced cultures of *B. rhodina* greatly influenced the amount of biomass produced, raising the biomass levels (mean 6.37 ± 0.32 g/l) to those of non-induced grown-cultures (6.49 ± 0.42 g/l). This increase

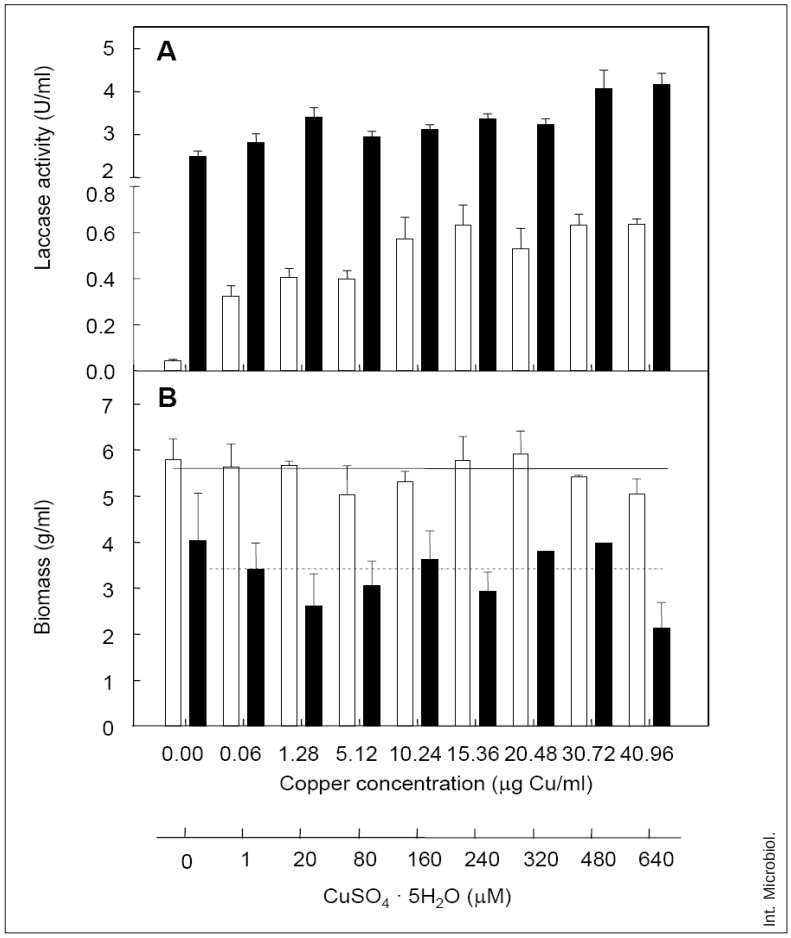

Fig. 3. The effect of copper on (**A**) laccase and (**B**) biomass production by *Botryosphaeria rhodina* MAMB-05 grown under non-induced (–VA) and induced (+VA) conditions. The solid and dashed horizontal lines of (B) represent lines of regression analysis. The amount of copper contained in Vogel minimal salts medium was 0.064 μg/ml. The copper concentration is shown as μg Cu/ml and μM $CuSO_4.5H_2O$. Open bars, –VA; closed bars, +VA.

in biomass might be attributable to the ability of *B. rhodina* to use Tween 80 as a C source. It was therefore of interest to examine the effect of Tween as sole C source. Tween 60 behaves similarly to Tween 80 in promoting laccase and biomass production [16]. As sole C source, Tween 60 supported both fungal growth (2.22 ± 0.08 g mycelium/l), and extracellular laccase production (0.30 ± 0.04 U/ml) albeit, for the latter, at a low level. Although growth was three-fold lower on Tween 60 than on basal medium and laccase production was low compared to the control, the results confirmed that *B. rhodina* is able to grow on Tween 60 as sole C source and to produce laccase. *Acinetobacter radioresistens* has also been reported to use Tween 80 as sole C source [23], but it produces a lipase.

During the cultivation of *B. rhodina* on the different Tween surfactants, fine oil-like globules appeared in the ECF, resulting in a milky appearance. This observation suggested that Tween is degraded by *B. rhodina*, presumably through the action of lipases. Recently, we determined that *B. rhodina* and several other strains of this fungus as well as *B. ribis* are lipolytic in nature, producing lipases when cultured on basal medium containing various vegetable seed oils (unpublished data). The recent report that *B. ribis* is pathogenic to olive trees, including the fruit [37], is consistent with the lipolytic activity expressed by this fungus.

Effects of soybean oil. In our studies on lipase production by *B. rhodina* grown on vegetable oils (unpublished results), soybean oil alone supported the growth of *B. rhodina* when incorporated into the VMSM. This result suggested that vegetable oil served as a carbon source, presumably hydrolyzed by fungal lipases as described above. The amount of biomass produced by *B. rhodina* grown on 1% soybean oil as sole C source was 21.88 ± 1.11 g/l, compared to 6.25 ± 0.51 g/l when the VMSM contained 1% glucose, but no oil. The addition of soybean oil (1% v/v) to VMSM also promoted the production of laccase by some five-fold (0.36 ± 0.15 to 1.74 ± 0.09 U/ml) compared to basal medium (no added oil) under non-induced conditions. When soybean oil, however, was incorporated into the basal medium, laccase levels decreased from 1.74 ± 0.09 to 1.04 ± 0.85 U/ml and appeared to be repressed. Glucose has often been found to repress genes that

are used in the metabolism of alternative C sources, and this is thought to be an energy-saving response [38]. To our knowledge, this is the first report of vegetable oils influencing laccase production.

Effect of copper. The induction of laccase synthesis by copper is widespread among fungi in which copper is necessary for laccase synthesis rather than for the activation of a pre-existing protein (apoenzyme) [14]. According to the literature, the amount of copper required to enhance laccase production varies greatly among fungi—generally within the range 0.003–40 μg Cu/ml. There are also reports that higher concentrations (5 mM $CuSO_4$ or 320 μg Cu/ml) effectively stimulate laccase synthesis [14].

Cultures of *B. rhodina* growing on basal medium to which no copper was added produced low levels of laccases. This was probably due to the presence of trace amounts of copper in the mineral salts of VMSM and in the water source, neither of which was pretreated with EDTA. Basal medium contains 0.064 μg Cu/ml, which was sufficient to greatly stimulate laccase production compared to medium lacking added copper (Fig. 3). The results obtained with the nutrient medium (C:N 6.58) used to grow *B. rhodina* indicated that copper enhanced laccase production under N-enriched conditions. Similar copper-mediated increases in laccase were reported for *P. ostreatus* [30] and *T. pubescens* [14] grown on N-rich medium. Exogenously added copper (within the range of 0–41 μg/ml) affected laccase production in non-induced cultures of *B. rhodina* (Fig. 3), resulting in a 15-fold increase in enzyme titers, with a maximum at 10.24 μg Cu/ml. Higher concentrations (up to 41 μg Cu/ml) did not further increase laccase production. A similar effect was also reported in *T. versicolor* [6] and *Hortaea acidophila* [45] when copper was added to nutrient medium. In the absence of copper, there was almost no detectable laccase activity [45].

Copper did not affect fungal biomass production over the copper concentration range examined, as linear regression analysis showed no significant effect. However, at the highest concentration (41μg Cu/ml), 16% less biomass was produced (Fig. 3). A concentration of 0.1–1.0 mM copper has been reported to be a requirement for optimal fungal growth [14], but at higher concentrations the metal becomes toxic, as demonstrated in growing cultures of *H. acidophila*, which are totally inhibited at 10 mM $CuSO_4$ [45].

In the presence of the inducer VA, copper only slightly enhanced laccase levels in *B. rhodina*, resulting in a ca.1.7-fold increase (Fig. 3). A similar observation regarding the effect of copper on laccase production was reported for *T. pubescens* [15] when several putative laccase inducers were added to growing cultures in the presence of copper. Biomass production by *B. rhodina* under induced conditions of growth was not affected by copper over the concentration range studied. In fact, regression analysis showed no significant effect. However, at higher copper loading, biomass was reduced by 47%.

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