Tolerance to Ammonia in the Common Edible Mushroom Induced in Vitro

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Abstract. Commercial strains of *Agaricus bisporus* (Lange) Imbach that were grown in vitro at 32C for 4 to 5 weeks on a medium containing 250 µg amantadine/ml followed by hyphal tip-culturing showed an enhanced tolerance of ammonia in the compost during cropping trials. Yield of mushrooms by a treated off-white strain (GSP-18) and a treated white strain (Marlowe-503) was higher in composts with elevated levels of ammonia (0.11% to 0.27%) than that of the untreated cultures. Tolerance was related to the ability of the treated cultures to grow vegetatively in the ammonia-enriched compost. Tolerance was expressed by the cultures for at least 14 months following induction.

The common edible mushroom is grown commercially in a composted mixture of chopped-moistened straw, horse manure, poultry manure, gypsum, and other fibrous and nonfibrous plant materials (Schisler, 1982). Conversion of these raw ingredients into a nutritive substrate that will support the growth and development of *A. bisporus* to the exclusion of other microorganisms occurs in two biologically interrelated steps (Sinden and Hauser, 1950). The first step (Phase I) involves an outdoor aerobic fermentation whereby microorganisms assimilate readily available forms of carbohydrate and N in the compost. The second step (Phase II) occurs indoors and involves a pasteurization at 60C to destroy pathogens and insects followed by a final conditioning period at ≈ 50C to allow the formation of N-rich lignin-humus complexes that are used by *A. bisporus*. During composting, nitrogenous compounds undergo microbial conversion, liberating large amounts of ammonia. In the commercial cultivation of *A. bisporus*, care must be taken to insure that the ammonia is cleared from the compost, since it is highly inhibitory to the fungus and will reduce the yield of mushroom (Schisler, 1982; Sinden

and Hauser, 1950; Wuest et al., 1986). Here, we report that, while evaluating the phenotypic traits of *A. bisporus* cultures subjected to an antiviral regime in vitro, specifically thermal therapy (van Zaayen, 1979) in combination with the antiviral drug amantadine-HCl (Hoffmann, 1977), we discovered that treated cultures exhibit an enhanced tolerance of ammonia in the compost during cropping. We demonstrate that treated commercial strains display more rapid colonization of and mushroom productivity in compost with elevated levels of ammonia than their untreated counterparts.

Mushroom strains GSP-18 and Marlowe-406 (off-white strain), and Marlowe-503 (white strain) were subculture from commercial spawn. Cultures were maintained on potato dextrose yeast agar (PDYA) at 24C and transferred to fresh medium every 3 to 4 weeks. Cultures were heat-treated in the presence of amantadine as follows: 4-mm-diameter mycelial plugs were transferred to 3.5-cm-diameter petri plates containing corn meal agar (10 g corn meal and 10 g agar/liter) amended with 250 µg amantadine/ml (amantadine HCl, Sigma, St. Louis) and incubated for 4 to 5 weeks at 32C. Hyphal tips were taken from the margin of the cultures, using 30-fold magnification, and transferred to PDYA. Rye grain spawn for cropping trials was prepared by conventional methods (Fritsche, 1988).

Mushroom production trials were carried out essentially as described by Carroll and Schisler (1976). Briefly, compost was prepared according to the short method of Sinden and Hauser (1950) using wheat straw-bedded horse manure supplemented with

brewers' grain, poultry manure, and gypsum at 50, 28, and 50 kg·Mt⁻¹ of dry horse manure, respectively. A 7-day Phase I composting was used with turnings on days 0, 2, 5, and 7. During Phase II composting, a specific temperature profile between 48 and 57C was maintained in the compost for 7 days with a pasteurization period of 60C for 2 hr on day 3. Following Phase II, the N content of the compost ranged from 2.10% to 2.39% and the ammonia level from 0.02% to 0.08%. Unless otherwise stated, 110 g of rye grain spawn was mixed with 23 kg of compost and placed in a tray measuring 14 × 60 × 60 cm. The compost was maintained at 24C for 13 days for mycelial colonization and then overlaid with a 3.5-cm-deep layer of a mixture of sphagnum peat and ground limestone (casing). The compost was maintained at 24C for 10 days, at which time it was lowered to 18C to induce the formation of mushrooms. Throughout the cropping cycle, the air was maintained at 18C and the casing was routinely watered to field capacity. In full-scale cropping trials, six replicate trays for each treatment were arranged in an incomplete randomized block design within the growing room. The weight of mushrooms harvested from each tray was recorded daily for 56 days. Statistical analysis of the data was done using the Waller-Duncan K-ratio *t* test (Steel and Torrie, 1980).

Two methods were used to increase the ammonia level of the compost. In expt. 1, the composting process was terminated immediately following the pasteurization step in Phase II. This procedure raised the ammonia content of the compost, although it allowed little control of the final level. For this reason, the preferred method was to spray a standard compost with an appropriately diluted aqueous solution of reagent grade ammonium hydroxide at the rate of 5.6 liter/340 kg of compost as it was mechanically turned just before spawning (expts. 2 and 3). Control compost was sprayed with an equal volume of water.

Table 1. Mycelial growth of four heat- and amantadine-treated cultures of commercial strain GSP-18 with 0.11% ammonia in the compost.

Culture	Colonization of the compost ¹
G S P - 1 8	1.0
GSP-18A2 ²	3.1
GSP-18A3 ²	2.7
GSP-18A4 ²	4.0
GSP-18B2 ²	3.0

¹Relative mycelial growth in the compost at 13 days post-spawning using the scale: 0 = 0%, 1 = 1%-10%, 2 = 11%-50%, 3 = 51%-75%, and 4 = 76%-100% of the compost being densely colonized. Each mean is based on six replicates.

²Heat- and amantadine-treated culture.

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Fig. 1. Production of mushrooms by untreated and treated cultures of GSP-18 in compost with 0.27% ammonia. Untreated parental culture (GSP-18) and treated cultures (GSP-18B3, GSP-18B2, GSP-18A4, and GSP-18X2). Note that the mushrooms shown are in the 3rd week of production and that GSP-18 and GSP-18B3 have not yielded mushrooms.

Table 2. Productivity of heat- and amantadine-treated cultures of commercial strains Marlowe-503 and Marlowe-406 with 0.11% ammonia in the compost.^a

Culture	Yield (kg·m ⁻²) ^b					
	After 21 days of production		Yield difference (%)	After 56 days of production		Yield difference (%)
	0.02% NH ₃	0.11% NH ₃		0.02% NH ₃	0.11% NH ₃	
Marlowe-503	9.30 ab	7.70 c	-17	14.80 abc	14.65 abc	-1
Marlowe-503A6*	8.85 b	9.70 ab	+10	13.90 cd	15.60 a	+12
Marlowe-406	10.00 a	9.50 ab	-5	15.25 ab	15.15 ab	-1
Marlowe-406C3*	8.90 b	9.85 ab	+11	14.30 bcd	14.60 bcd	+4

^aThe productivity of each culture was evaluated in composts with 0.02% (control) and 0.11% ammonia at spawning.

^bMean separation within a production period by the Wailer-Duncan K-ratio *t* test, = 0.05. Each mean is based on six replicates.

*Heat- and amantadine- treated culture.

Using this method, the experimental level of ammonia varied within 8% of the predicted level as determined by the steam distillation method with MgO (Keeney and Nelson, 1982). In different experiments, the pH of the ammonia-enriched compost ranged from 7.3 to 8.3 but was always within 0.4 pH units of the control compost.

During the course of a study to assess the vegetative and reproductive characteristics of heat- and amantadine-treated strains of *A. bisporus*, four treated cultures of GSP-18 grew more extensively in a compost with a moderately elevated level of ammonia (0.11%) than the untreated parental culture (Table 1). After 21 days of cropping, no significant differences in yield of mushrooms existed among the cultures, although there was a trend for the treated ones to be more productive. Yield of the treated cultures ranged from 9.30 to 10.00 kg·m⁻² and that of the untreated culture was 8.65 kg·m⁻².

In a pilot experiment (expt. 1) that was designed to explore the putative induced ammonia tolerance in GSP-18, an untreated culture and four treated cultures, GSP-18A2, GSP-18A4, GSP-18B2, and GSP-18B3, were grown in composts with 0.04% and 0.27% ammonia. For each culture, 1.8 kg of each compost was mixed with 10 g of spawn and placed in a 26-cm-diam × 13-cm-deep cylindrical plastic pot in replicate. All cultures showed a similar rate of vegetative growth and development of mushrooms in the control compost. Mycelial growth of the cultures was markedly inhibited at 0.27% ammonia. However, whereas GSP-18A2, GSP-18A4, and GSP-18B2 completely ramified the compost, GSP-18 and GSP-18B3 only grew sparsely along the surface. The three treated cultures that grew throughout the ammonia-enriched compost developed mushrooms ≈ 3 weeks earlier than those showing limited growth (Fig. 1). Yield data

were not recorded in this experiment because of the small number and size of the replications.

In a full-scale cropping trial (expt. 2), the productivity of treated cultures derived from commercial strains Marlowe-503 and Marlowe-406 was evaluated in composts containing 0.02% and 0.1% ammonia. During the 13-day spawn growth period, both untreated and treated cultures densely colonized the compost with 0.02% ammonia. At 0.11% ammonia, Marlowe-406, treated cultures of Marlowe-406C3 and Marlowe-503A6, and, to a lesser extent, Marlowe-503 developed sparse mycelial growth throughout the compost. At 7 days after casing, all cultures had grown extensively into the casing layer from the control compost (data not shown). Also, some growth into the casing layer from the ammonia-enriched compost was observed for Marlowe-406 and treated cultures Marlowe-406C3 and Marlowe-503A6. After 21 days of production from the compost with 0.11% ammonia, the yield of Marlowe-503 was reduced by 17% in comparison with the low ammonia treatment, whereas that of the treated culture Marlowe-503A6 was not significantly affected (Table 2). After 56 days of cropping, Marlowe-503A6 yielded 12% more on the compost with the elevated level of ammonia compared to the control compost. Treated and untreated cultures of Marlowe-406 yielded similarly on 0.11% ammonia compost.

The ability of treated cultures to yield well on compost with elevated ammonia was observed in a second full-scale cropping trial (expt. 3) using composts with 0.08% and 0.24% ammonia. As in the previous experiment, growth of the cultures through the compost and, subsequently, into the peat-moss casing layer was inhibited by the elevated level of ammonia. However, at 7 days postcasing, the underlying ammonia-enriched compost had become colonized with mycelium, particularly by the two treated cultures and Marlowe-406 (data not shown). Yield from each of the cultures after 21 days of production was significantly reduced by a 0.24% level of ammonia in the compost, but treated cultures tended to be higher-yielding than those untreated at the elevated ammonia level (Table 3). After 56 days, the yield for each culture was similar for the two regimes of ammonia. The decreased production observed for the treated cultures in the ammonia-enriched compost in this study compared to the increased yield observed in expt. 2 was probably due to the higher level of ammonia used. In expt. 2, the moderately elevated level of 0.11% proved inhibitory to the untreated cultures but not the treated cultures. In expt. 3, the 0.24% level used apparently exceeded the threshold of tolerance of the treated cultures.

It is well-recognized that levels of ammonia in the compost at spawning approaching 0.07% are inhibitory to *A. bisporus* (Wuest et al., 1986). Hence, one goal of the composting process is to reduce the ammonia concentration below the threshold level that restricts mycelial invasion of the compost and

Table 3. Productivity of heat- and amantadine-treated cultures of commercial strains Marlowe-503 and Marlowe-406 with 0.24% ammonia in the compost.^a

Culture	Yield (kg·m ⁻²) ^b					
	After 21 days of production		Yield difference (%)	After 56 days of production		Yield difference (%)
	0.08% NH ₃	0.24% NH ₃		0.08% NH ₃	0.24% NH ₃	
Marlowe-503	9.30 ab	4.80 f	-48	14.15 bc	13.00 cd	-8
Marlowe-503A6*	10.25 ab	7.70 ed	-25	15.95 ab	15.65 ab	0
Marlowe-406	11.80 a	7.30 e	-38	16.90 a	14.70 abc	-13
Marlowe-406C3*	10.90 ab	8.25 cde	-24	16.00 ab	14.05 bc	-12

^aThe productivity of each culture was evaluated in composts with 0.08% (control) and 0.24% ammonia at spawning.

^bMean separation within a production period by the Wailer-Duncan K-ratio *t* test; *P* = 0.05. Each mean is based on six replicates.

*Heat- and amantadine-treated culture.

reduces the yield of mushrooms. Serendipitously, we discovered that strains of *A. bisporus* subjected to thermal treatment in the presence of amantadine in vitro followed by hyphal-tip culturing may exhibit an improved tolerance of ammonia in the compost. Cultures of *A. bisporus* treated in this manner typically show an increased rate of vegetative growth on nutrient media and rye grain (data not shown). Additionally, tolerance of ammonia, expressed in terms of yield, seems to be related to an enhanced ability of the culture to grow vegetatively in the ammonia-enriched compost. This result is consistent with the finding that tolerance was expressed during a shorter cropping cycle (i.e., 21 days vs. 56 days). Presumably, a tolerant culture, owing to a higher rate of colonization, can effectively obtain nutrients from the compost for the formation of mushrooms early in the cropping cycle. During longer cropping cycles, the interstitial ammonia slowly dissipates from the compost, thereby enabling more sensitive cultures to grow and produce mushrooms late in the cycle. Additionally, cultures of *A. bisporus* subjected to thermal treatment alone respond with an improved vigor (van Zaayen, 1979). This finding raises the question of whether amantadine and hyphal tip-culture are essential to the induction of ammonia tolerance.

The results of this study do not bear on the mechanism underlying the enhanced ammonia tolerance. One possibility is that the heat and amantadine treatment followed by hyphal tip-culturing eliminates from the fungus viruses that effect the utilization of ammonia. However, using a broad-spectrum assay (Romaine and Schlagnhauser, 1989),

we were unable to demonstrate the presence of viruses in the cultures either before or after treatment. This finding does not preclude the idea that the cultures were cured of viruses that had escaped detection. Interestingly, virus infection in the fungus *Leucostoma persoonii* is associated with an altered N metabolism (Pazur and Adams, 1988).

An alternative explanation for the enhanced ammonia tolerance is that it reflects an altered N metabolism directly attributed to the treatment itself. For example, it is well-known that heat treatment of organisms, including fungi (Plesofsky-Vig and Brambl, 1985), elicits the formation of heat-shock proteins that are associated with an improved thermotolerance and resistance to other physical and chemical stresses. If heat-shock proteins are somehow involved in the enhanced ammonia resistance, they are unlikely to be operational at the level of transport across membranes, since the movement of ammonia into fungal cells occurs by passive diffusion (Macmillan, 1954).

Finally, it remains to be determined if the improved tolerance of ammonia is a general response by all strains of *A. bisporus*. In the present study, an off-white strain (GSP-18) and a white strain (Marlowe-503) were rendered more tolerant, whereas a second off-white strain (Marlowe-406) was not. If additional cultures of Marlowe-406 had been evaluated, it is conceivable that a tolerant one would have been obtained. This notion is supported by the observation that one of the five GSP-18 cultures failed to respond to the treatment. However, Marlowe-406 was more tolerant of ammonia than Marlowe-503, as judged by its significantly higher produc-

tivity in the ammonia-amended composts before heat and amantadine treatment. How the inherent tolerance of a strain relates to its response to treatment has yet to be determined. Regardless, it seems that the tolerance becomes a relatively stable trait of a culture, since it was expressed during the 14 months following induction that was required to perform this study.

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