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ORIGINAL PAPER

Enhancement of the antioxidants ergothioneine and selenium in *Pleurotus eryngii* var. *eryngii* basidiomata through cultural practices

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Abstract Antioxidants are molecules that may reverse, prevent or slow cellular damage caused by free radicals. Increasing dietary intake of antioxidants is thought to reduce oxidative stress that may contribute to the development of several diseases. Mushrooms are known to contain antioxidants such as selenium, ergothioneine and phenolics that may serve this role. Here we sought to enhance selenium and ergothioneine concentration in *Pleurotus eryngii* var. *eryngii* basidiomata by modifying the techniques used for their commercial cultivation. To enhance selenium content in mushrooms, substrates were supplemented with sodium selenite (Na_2SeO_3) to reach selenium concentrations of 5 and 10 μg/g. Basidiomata of one commercial isolate (WC888) accumulated selenium up to 4.6 and 9.3 μg/g (d.w.), respectively. Therefore, a serving size (85 g) of fresh *P. eryngii* mushrooms produced on substrates supplemented with 5 and 10 μg/g of Na_2SeO_3 would supply 70.4 and 116.3% of the daily value of selenium (DV = 70 μg), respectively. Since selenium-enriched mushrooms would supply more than 20% of the DV, they could be considered an excellent source of selenium. Ergothioneine concentration was enhanced in mushrooms produced on low-moisture (55%) substrate compared to the commonly used 60% (high-moisture) in commercial cultivation. Mushrooms produced on low-moisture substrate had ergothioneine concentrations of 3.0 mg/g, while mushrooms produced on high-moisture substrate contained 2.2 mg/g or less. Use of a casing overlay for mushroom production resulted in significant yield increases on low-moisture substrate but not on high-moisture substrate.

Keywords *Pleurotus eryngii* var. *eryngii* · Antioxidants · Ergothioneine · Selenium · Cultural practices · Casing overlay · Mushroom yield

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Introduction

Mushrooms have powerful antioxidant properties derived from compounds such as selenium (Se), ergothioneine (ERGO), and phenolics. Selenium is an essential micronutrient in human nutrition because it is a component of the antioxidant enzymes called selenoproteins. These proteins aid in the prevention of cellular damage caused by free radicals. Other health benefits attributed to Se include anti-inflammatory and anti-carcinogenic properties (Ryan-Harshman and Aldoori 2005). Se is found in soil where it is absorbed by plants and fungi and later accumulated in animals. Important sources of Se are plants, meats, seafood and nuts (ODS 2004). The daily value (DV) of Se determined by the Food and Drug Administration (FDA) for the human diet is 70 μg. It has been shown that higher oral intakes of Se, in the order of 200 μg/day (Se-enriched yeast), are associated with a reduction in the risk of

colorectal, lung and prostate cancer (Clark et al. 1996; Combs et al. 1997). Intake of 200 μg/day is within the safe range since the tolerable upper intake levels (UL) of Se in adults is 400 μg/day (IOM 2000).

ERGO is a thiol compound and a natural antioxidant of biological origin. In animals, ERGO provides several physiological benefits such as enhancement of metabolic energy, protection against formation of cataracts, molecular regulation of anti-inflammatory mechanisms in lungs, etc. (Shukla et al. 1981; Kawano et al. 1982; Rahman et al. 2003). Synthesis of ERGO in nature is restricted to fungi and *Mycobacterium* (Melville et al. 1956; Genghof and Van Damme 1964; Hartman 1990; Akanmu et al. 1991). ERGO synthesized by these organisms is taken up by plants from the soil and then passed to animals and humans where it accumulates at different concentrations in tissues and blood (Melville 1958). Ey et al. (2007) analyzed several foods finding that *Boletus edulis* and *Pleurotus ostreatus* had the highest concentrations of ERGO (528.1 and 118.9 mg/kg wet weight, respectively). Black turtle bean, chicken and pork liver and pork kidney followed on the list with 13.5, 10.8, 8.7, and 7.7 mg/kg (w.w.), respectively. A DV has not been established for ERGO.

In recent years, general concerns regarding human health issues have resulted in increased consumption of foods or supplements rich in antioxidants. Therefore, research efforts have focused on developing cost-efficient methods to synthetically or naturally produce antioxidants for human consumption. Lately, mushroom scientists have investigated factors that influence ERGO and Se concentrations in edible and medicinal mushrooms since antioxidant-enriched mushrooms may be used as components in functional foods or as dietary supplements (Werner and Beelman 2002; Beelman and Royse 2006; Dubost et al. 2007).

Se is readily absorbed from the production substrate and accumulated in mushrooms, especially when Se is supplied in the form of sodium selenite (Werner and Beelman 2002; Stajic et al. 2005; Beelman and Royse 2006). On the other hand, Dubost et al. (2007) demonstrated that ERGO concentration in *Agaricus bisporus* increased when the fungus was grown in relatively dry substrate and when the substrate was fragmented and supplemented with the amino acid histidine. Therefore, we sought to evaluate selected cultural practices in an attempt to enhance concentration of Se and ERGO in basidiomata of *P. eryngii* var. *eryngii*. Se accumulated linearly in basidiomata in response to supplementation of substrate with sodium selenite; only low-moisture substrate effectively enhanced ERGO concentration in mushrooms. Use of a casing layer increased mushroom yield on low-moisture substrate (55%).

Materials and methods

Fungal isolate and spawn (inoculum) preparation

A commercial isolate of *P. eryngii* var. *eryngii* (WC888) held in The Pennsylvania State University Mushroom Culture Collection (PSUMCC) was used in this study. Spawn was prepared with 91 g of rye grain (Hesco, Watertown, ND), 13 g of hardwood sawdust, 3 g of $CaSO_4$, and 120 ml of warm tap water as described by Rodriguez Estrada and Royse (2007).

Substrate preparation, inoculation and incubation

Substrate preparation followed the procedures described elsewhere with minor modifications (Rodriguez Estrada and Royse 2007). Mushrooms were produced on sterilized substrate composed of the following ingredients: cottonseed hulls (56%), corn distiller's waste (4%), calcium sulfate (1%), ground soybean (12%) and oak sawdust (27%). Dry weight (d.w.) of each component, with the exception of $CaSO_4$, was determined in triplicate with an Ohaus moisture analyzer (model MB35). Quantities of ingredients were adjusted to meet the desired percentages of dry matter and water. Dry ingredients were mixed for 2 min with a paddle mixer at the Mushroom Research Center (MRC). Warm tap water (ca. 40°C) was added to the substrate to reach the target moisture content. After the substrate was packed into either bags or bottles (depending on the intended production method), it was autoclaved for 90 min at 121°C. Cooled substrate was inoculated with spawn and transferred to an incubation room (9 m^2) at the MRC. Substrates were incubated at 21°C with a cycle of 8 h light/16 h dark using cool-white fluorescent bulbs. Final moisture contents of the substrates for each treatment were determined in triplicate with the moisture analyzer.

Selenium supplementation (Crop 1)

A completely randomized design was used to evaluate the effects of Se substrate supplementation on concentration of the element in *P. eryngii* basidiomata. Anhydrous sodium selenite (Na_2SeO_3) was added to the substrate to reach Se concentrations of 5 and 10 μg/g (d.w.). Na_2SeO_3 was dissolved in warm tap water prior to addition to the substrate and subsequently mixed for 10 min (Beelman and Royse 2006). Polypropylene (PP) bottles (1,050 ml capacity, 9 cm diameter × 15.5 cm deep) were used as containers. Bottles (8 per treatment) were filled with a machine that automatically stopped substrate flow when the container was full. Then, holes (top to bottom) were made with a rotating metallic shaft and residual substrate was removed mechanically from the neck of the bottles. Bottles were

capped manually with PP lids containing a filter. After autoclaving and cooling, the substrate was inoculated with 6.4 g (±0.1 g) grain spawn placed into each hole. Following incubation for 27 days, 2 mm of the colonized substrate and aerial mycelium were removed with a scratching machine (Rodriguez Estrada and Royse 2007). The bottles then were transferred to a production room (relative humidity-RH 90%, 16°C and 8 h light/16 h dark) where tap water (ca. 15 ml) was sprayed onto the exposed substrate in each bottle. A perforated plastic sheet (holes 7 mm diameter, spaced 44 mm × 94 mm) was placed on top of the bottles to prevent substrate desiccation. Mushrooms were harvested when the largest mushroom in each bottle reached maturity (approximately 42 days after inoculation). For Se analyses, two mushrooms harvested from one bottle comprised one replicate and two replicates were obtained per treatment. Two substrate samples were also obtained per treatment prior to sterilization. Mushrooms and substrates were processed to a fine powder as described in the sample preparation section (below).

Influence of moisture content (Crop 2) and fragmentation of the substrate (Crop 3) on ERGO concentration in basidiomata

Two crops were produced to investigate factors that might influence ERGO concentration in the basidiomata. Crop 2 was designed to determine the influence of moisture content of the substrate (55 and 60%) packed in PP bottles. Substrates were prepared, inoculated and incubated as explained above. Crop 3 examined the influence of substrate fragmentation on ERGO concentration in basidiomata. Three treatments were included in the completely randomized design: (1) substrate contained in bags without fragmentation; (2) substrate contained in bags during incubation, subsequently fragmented and transferred to plastic bins; and (3) same as treatment 2 with addition of a casing layer after substrate fragmentation. Polypropylene bags (20 cm × 48.5 cm) with medium porosity filters (Unicorn Import and Manufacturing, Commerce, TX) were used as containers. Each bag (4 bags/treatment) was filled with 2.5 kg moist substrate and autoclaved. After cooling overnight, substrates were inoculated with 30 g (±0.1 g) grain spawn and bags were heat-sealed and shaken by hand to distribute spawn evenly throughout the substrate. After incubation (27 days), bags were transferred to a production room. Bags for treatment 1 remained unopened until primordia formed (approximately 6 days after transfer to the production room). However, bags for treatments 2 and 3 were immediately opened; the substrate was manually fragmented and placed into 6.1 l plastic bins (33 cm long × 18.5 cm wide × 12 cm deep). Non-cased bins were covered with a plastic sheet that was removed after primordia formation. Cased substrates were covered with 1 kg of casing soil (2:1 d.w. peat moss and calcium carbonate at 78.7% moisture). The production room was maintained at 90% RH, 16°C, and a light/dark cycle of 8-h/16-h. Since mushrooms matured at slightly different times, they were harvested for a period of 2–3 days (approximately 40 days after inoculation). For ERGO analyses, three whole mushrooms obtained from one experimental unit comprised one replicate. Three replicates were obtained per treatment. Mushrooms were processed as described in the sample preparation section (below).

Influence of moisture content, histidine supplementation and casing layer on ERGO concentration and yield (Crop 4)

Crop 4 was designed as a 2 × 2 × 2 factorial experiment with four replicates per treatment. Three factors were evaluated at two levels: (1) histidine supplementation at 0 and 10 mM (H10), (2) moisture content of the substrate at 55 and 60%, and (3) cased (C) and non-cased (NC) substrates. Therefore, the experiment was composed of 8 treatments as follows: 1. NC55H10, 2. C55H10, 3. NC60H10, 4. C60H10, 5. NC55, 6. C55, 7. NC60, and 8. C60. Two batches of substrate were prepared. Quantities of raw material and water were adjusted to meet the requirements for 55 and 60% moisture content in substrate. Each batch was divided in half and each half was supplemented with 10 mM w.w. of histidine ($C_6H_9N_3O_2$, Sigma Chemical Co.). Histidine was dissolved in warm tap water prior addition to the substrate (Dubost et al. 2007). The substrate was packed, sterilized and inoculated as explained above. Four bags per treatment were randomly selected for casing after incubation; therefore, each treatment had four replicates. Bags for non-cased treatments (1, 3, 5 and 7) remained closed until primordia were formed. Bags for treatments 2, 4, 6 and 8 were opened (25 days after spawning) and cased with 550 g of casing soil. For ERGO analyses, three whole mushrooms, obtained from one experimental unit comprised one replicate. Three replicates were obtained per treatment. Mushrooms were processed as described in the sample preparation section (below).

Sample preparation and solids

Fresh, whole, mature mushrooms were weighed, chopped (10–15 mm^3 cubes), placed in polystyrene weigh boats, and frozen at −80°C for 24 h in a ThermoForma freezer (model 721). Then, mushrooms were freeze-dried (lyophilizer model 15 SRC-X; Virtis Genesis Co., Gardiner, NY) for 48 h. Freeze-dried mushrooms were transferred to new weigh boats and weighed immediately to minimize moisture absorption. Percentage solids in basidiomata were

calculated by dividing dry and fresh mushroom weight and multiplying by 100. Dry tissue was ground in a food processor and then sieved through a mesh screen (size 16; 1.18 mm, Fisher Scientific Co.). Mushroom powders (~10 g) were placed in sample bags and stored at room temperature in a glass desiccator until analyzed (Dubost et al. 2006). Substrate samples obtained from crop 1 (Se supplementation) were prepared in a similar manner.

Se and ERGO analyses

Chemical analyses of Se were performed at the Pennsylvania State University Agricultural Analytical Laboratory. Mushroom and substrate powders were digested with nitric acid (HNO_3), and then subject to inductively coupled plasma-atomic emission spectrometry (ICP-MS) according to the Environmental Protection Agency protocols 3051A and 6010 for Se analysis of solid waste (USEPA 1986). ERGO detection and quantification was performed through high performance liquid chromatography (HPLC) in the Department of Food Science at PSU following protocols of Dubost et al. (2006). Bagged mushroom powders were stored in a desiccator for a maximum of 8 days before ERGO was quantified. ERGO was extracted from 1 g of freeze-dried sample with an HPLC graded ethanol solution (10 mM dithiotreitol, 100 μM betaine, 100 μM 2-mercapto-1-methyl-imidazole). Compound separation was carried out with two Econosphere C18 columns (Alltech) mounted on HPLC equipment (1050 system, HP). A 50 mM sodium phosphate solution with 3% acetonitrile and 0.1% triethylamine (pH 7.3) was used as a mobile phase with an isocratic gradient. A DAD detector (HP) with a wavelength of 254 nm was used for compound identification.

Estimation of solids, Se and ERGO

Estimates for total solids, Se and ERGO produced per bottle or bag (production units) and ERGO per kg of dry substrate were calculated with formulas 1, 2 and 3, respectively.

$$\text{Solids per unit (g)} = \frac{\text{solids/basidioma (\%)} \times \text{mushroom fresh weight (g)}}{100} \quad (1)$$

$$\text{Se (μg) or ERGO (mg) per unit} = \text{solids/unit (g)} \times \text{Se (μg/g) or ERGO/mush (mg/g)} \quad (2)$$

$$\text{ERGO (mg) per kg of dry substrate} = \frac{\text{ERGO/bag}}{\text{Dry substrate (kg)}} \quad (3)$$

where

Unit	Bottles or bags
Fresh weight	Yield (g) per unit
Mush	Individual measurements obtained from a replicate composed of two or three mushrooms
Se or ERGO per unit	Se or ERGO obtained from mushrooms grown in ca. 500 g (bottles) or 2.5 kg (bags) of wet substrate
ERGO/bag	Obtained from formula 2 (estimation of ERGO per unit)
Dry substrate	Oven dry weight

Production performance

Yield, biological efficiency (BE) and number of mushrooms were determined for each crop. Yield is expressed as fresh mushroom weight (g) per bottle, bag or bin. Biological efficiency is the ratio of fresh mushroom weight to substrate dry weight expressed as a percentage (Royse 1992). Production length was determined in Crops 2, 3 and 4. Production length is the number of days elapsed from inoculation to harvest. Mushrooms produced in bottles (Crop 2) were harvested in 1 day. However, mushrooms grown in bags or bins were typically harvested within 2–4 days (Crop 3 and 4). In the latter case, the mean point between the first and last day of harvest was used as a reference to determine production length.

Statistical analyses

Analyses of variance and mean separations were performed with the SAS statistical software program JMP® (version 7, 2007). A one-way analysis of variance (ANOVA) was used to examine yield, biological efficiency, number of mushrooms, production length, Se, and ERGO concentration in the completely randomized experiments where only one factor was evaluated at a time (Crops 1, 2 and 3). A two-way analysis of variance for the two-level factorial experiment (Crop 4) was performed by the standard least squares procedure. Individual sources of variation (casing, histidine and substrate moisture) and their interactions were included in the model. Mean separations were performed using the Tukey–Kramer Honestly Significant Difference (HSD) test. The statistical software program Minitab (version 14, 2003) was used to construct a correlation matrix based on Pearson coefficients among ERGO, solids, substrate moisture and yield in Crop 4.

Results

Selenium supplementation

Selenium concentration in basidiomata (Crop 1)

Supplementation of substrate with Na_2SeO_3 significantly enhanced Se content in basidiomata. Selenium concentration in mushrooms harvested from non-supplemented substrates was below the detection level (<1.5 μg/g), while Se concentrations in mushrooms harvested from substrates containing 5 and 10 μg/g of Se were 4.6 and 9.3 μg/g (d.w.), respectively (Table 1). A significant correlation ($r = 0.982$) was observed between Se concentration in the substrate and Se content in the basidiomata. Se accumulation was approximately linear in relation to levels of this element added to the substrate (Fig. 1).

Yield, BE, number of mushrooms and solids (Crop 1)

Selenium supplementation affected yield, BE and solids (%). However, variations in these responses were not negative when compared to the non-supplemented treatment (Table 2). For example, yield and BE per bottle in treatment Se10 ppm were comparable to the non-supplemented treatment.

ERGO and mushroom production influenced by moisture content (Crop 2) and fragmentation of substrate (Crop 3)

Moisture content of substrate—Crop 2

ERGO concentrations in mushrooms were significantly influenced by moisture content of the substrate. ERGO concentration in basidiomata produced on low-moisture substrate was significantly higher than in mushrooms harvested from high-moisture substrate (Table 3). Conversely, yield, BE, number of mushrooms and production cycle length were significantly higher for the 60%-moisture treatment (Table 4). Solids were higher in mushrooms harvested from low-moisture substrates.

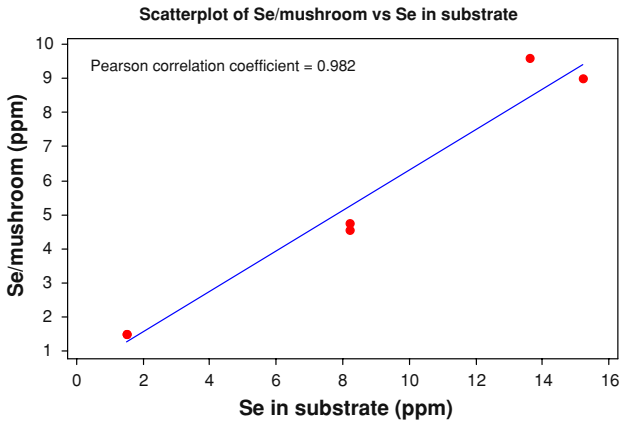

Fig. 1 Scatter plot showing significant positive correlation between Se concentration in substrates and Se content in *P. eryngii* var. *eryngii* basidiomata

Fragmentation of substrate—Crop 3

Substrate fragmentation did not significantly influence ERGO concentration in mushrooms, yield or BE (Tables 3, 4). However, number of mushrooms, solids and production cycle length were significantly different among treatments. The highest number of mushrooms and the shortest production cycle was obtained from substrate contained in bags. Mushrooms with the highest percentage of solids were obtained from non-cased substrates.

ERGO and mushroom yield affected by substrate moisture content, histidine supplementation, and casing layer application (Crop 4)

Ergothioneine

Moisture content of the substrate and addition of a casing layer significantly influenced ERGO concentration in

Table 1 Selenium concentration in substrate, *Pleurotus eryngii* var. *eryngii* basidiomata, Se per bottle, per serving size of raw mushrooms (85 g) and percentage of the DV (daily value) as result of substrate supplementation with sodium selenite

Treatment	Se in substrate (μg/g, d.w.)[b]	Se in basidiomata (μg/g, d.w.)[b]	Se/bottle (μg)	Se/serving (μg)[c]	DV (%)[d]
Se (0ppm)[a]	<1.5 c	<1.5 c	26.94	N/A	N/A
Se (5 ppm)	8.1 b	4.6 b	74.41	49.3	70.4
Se (10 ppm)	14.4 a	9.3 a	134.20	81.4	116.3

N/A not applicable

[a] Control

[b] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$)

[c] Se in a serving of fresh mushrooms (85 g). Amounts were calculated from solids content

[d] Daily value (DV) of Se according to the Food and Drug Administration (FDA) is 70 μg

Table 2 Effects and mean comparisons (Tukey–Kramer HSD test) for yield, BE, number of mushrooms and solids content of basidiomata of *Pleurotus eryngii* var. *eryngii* as affected by supplementation of substrate with sodium selenite

Treatment	Yield (g)[a,c]	BE (%)[a,c]	Number mushrooms[a,c]	Solids (%)[b,c]
Se (0 ppm)	144.9 ± 9.1 a	68.7 ± 3.9 ab	4.3 ± 1.8	12.4 ± 0.1 a
Se (5 ppm)	128.4 ± 10.5 b	65.1 ± 5.7 b	3.1 ± 2.2	12.6 ± 0.4 a
Se (10 ppm)	140.1 ± 9.2 ab	71.9 ± 4.7 a	3.0 ± 0.9	10.3 ± 0.4 b

[a] Based on eight replicates per treatment

[b] Based on two replicates per treatment

[c] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$). Standard deviation (estimation of variance) follows ±

Table 3 Effects of moisture content (Crop 2) and fragmentation of substrate (Crop 3) on ergothioneine (ERGO) concentration in basidiomata of *Pleurotus eryngii* var. *eryngii*

Treatment (Crop 2) (%)	ERGO (mg/g, d.w.)[a]	Treatment (Crop 3)	ERGO (mg/g, d.w.)[b]
Bottles 55	2.57 ± 0.22 a	Bags[c]	2.20 ± 0.21
Bottles 60	2.21 ± 0.10 b	Bins[d]	2.18 ± 0.24
		Cased bins[d]	2.04 ± 0.23

[a] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$)

[b] Standard deviation (estimation of variance) follows ±

[c] Non-fragmented

[d] Fragmented

mushrooms (Table 5). Histidine supplementation and factor interactions were not significant (Table 5). However, slightly higher ERGO concentration was obtained for supplemented treatments. For instance, average ERGO concentration in mushrooms for histidine supplemented treatments 1–4 was 2.46 mg/g, while concentration for non-supplemented treatments 5–8 was 2.29 mg/g (Table 6). The average for low-moisture, non-supplemented treatments (5 and 6) was 2.62 versus 2.92 mg/g for low-moisture, histidine-supplemented treatments (1 and 2).

Yield, BE, solids, and production cycle length

Yield, BE, solids, and production cycle length were significantly affected by casing layer but not by histidine supplementation (Table 7; Fig. 2). In general, highest yields, BEs and lowest solids/mushroom were obtained from cased treatments (2, 4, 6, and 8). While lowest yields, BEs and highest solids/mushrooms were found in non-cased treatments (1, 3, 5 and 7). Production cycle length was affected only by a casing overlay. Mushrooms from cased treatments were harvested at 37–41 days after spawning while mushrooms produced on non-cased

Table 4 Effects of moisture content (Crop 2) and fragmentation of substrate (Crop 3) on yield, BE, number of mushrooms, solids, and production cycle length for *Pleurotus eryngii* var. *eryngii*

Treatment	Yield (g)[a,d]	BE (%)[a,d]	Number mushrooms[a,d]	Solids (%)[b,d]	Production cycle length (days)[a,c,d]
Crop 2					
Bottles 55%	102.8 ± 14.1 b	51.3 ± 6.5 b	2.3 ± 0.9 b	12.7 ± 0.9 a	41.7 ± 0.8 b
Bottles 60%	148.3 ± 11.4 a	70.9 ± 5.7 a	4.3 ± 1.5 a	10.3 ± 0.6 b	43.1 ± 0.7 a
Crop 3					
Bag[e]	631.3 ± 37.1	63.1 ± 3.7	23.7 ± 1.2 a	12.3 ± 1.2 a	37.0 ± 0.0 c
Bins[f]	512.8 ± 96.3	51.3 ± 9.6	15.0 ± 5.9 ab	12.6 ± 1.2 a	40.5 ± 1.0 b
Cased bins[f]	711.5 ± 172.8	71.2 ± 17.3	13.0 ± 3.5 b	8.7 ± 0.3 b	42.5 ± 0.6 a

[a] Based on twelve replicates in Crop 2 and four replicates in Crop 3

[b] Based on three replicates per treatment

[c] Number of days from inoculation to harvest peak

[d] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$). Standard deviation (estimation of variance) follows ±

[e] Non-fragmented

[f] Fragmented

Table 5 *P* values obtained from analysis of variance for influence of three factors (casing, moisture content of substrate, and histidine supplementation of substrate) and their interactions on ergothioneine (ERGO) concentration, ERGO/bag and ERGO/kg of dry substrate for *Pleurotus eryngii* var. *eryngii* (Crop 4)

Source	DF	ERGO/Mush	ERGO/bag	ERGO/kg
Model	7	0.0033*	0.0001*	0.0017*
Effects tested				
Casing (C)	1	0.0278*	0.1237	0.0749
Histidine (H)	1	0.2661	0.7989	0.1283
Moisture (M)	1	0.0002*	<.0001*	0.0004*
C × H	1	0.9777	0.1742	0.1239
C × M	1	0.4579	0.0023*	0.0034*
H × M	1	0.3739	0.0326*	0.0598
H × C × M	1	0.5265	0.2404	0.2200

* P values < 0.05 were considered significant

treatments were picked 40–45 days after spawning (Fig. 2c; Table 8).

Correlation of ERGO, moisture content of substrate, yield and solids

The significant negative correlation (−0.799) between substrate moisture content and ERGO/mushroom indicated that higher ERGO concentrations were found in mushrooms produced on low-moisture substrates (Table 9). Results also indicated that higher ERGO/bag (mg) was obtained from treatments that produced more solids/bag and ERGO concentration was higher in mushrooms that had a high percentage of solids.

Discussion

Addition of Na_2SeO_3 at 5 and 10 μg/g to the substrate resulted in basidiomata with Se concentrations of 4.6 and 9.3 μg/g (d.w.), respectively. Therefore, a serving size of raw mushrooms (85 g) produced from substrates enriched with Se at 5 ppm would provide 49.3 μg of the 70 μg DV. Mushrooms grown on substrates supplemented at 10 ppm would provide 81.4 μg of Se, representing 116.3% of the DV (Table 1). Hence, according to the FDA, a serving size of fresh *P. eryngii* mushrooms produced in Na_2SeO_3-supplemented substrates should be considered an excellent source of Se because it provides more than 20% of the DV. No significant variations in yield, BE and number of mushrooms were observed as a result of substrate supplementation with Na_2SeO_3. However, fewer solids were produced in treatment Se10 ppm compared to the non-supplemented (Se0 ppm) and Se5 ppm treatments. Differences in solids content of the mushrooms may be due, in part, to differences in dry substrate and water availability since substrate wet weights for this treatment were also the lowest. Variation in substrate weight from bottle to bottle and batch to batch was observed (data not shown). The first treatment prepared for the Se experiment was the non-supplemented substrate, followed by 5 ppm- and 10 ppm-treatments. Bottles prepared for the latter treatments contained slightly lower substrate matter than bottles prepared at the beginning of the trial. Yields and BEs from Se0 ppm and Se10 ppm treatments were similar, confirming that less substrate in Se10 ppm did not affect performance of the fungus. Since BE is defined as the ratio between mushroom yield and substrate dry weight, a higher BE means that the

Table 6 Ergothioneine (ERGO) concentration in basidiomata and estimated amount of ERGO per bag and ERGO per kg of dry substrate as influenced by casing overlay, substrate moisture content and histidine supplementation of substrate

Treatment[a]	ERGO (mg/g)[b,e]	ERGO (mg/bag)[c,e]	ERGO (mg/kg)[d,e]
1. NC55H10	3.00 ± 0.31 a	179.67 ± 21.20 ab	159.71 ± 18.84 ab
2. C55H10	2.84 ± 0.57 a	175.81 ± 16.63 ab	156.28 ± 14.79 ab
3. NC60H10	2.29 ± 0.33 ab	157.98 ± 30.64 abc	157.98 ± 30.64 ab
4. C60H10	1.72 ± 0.18 b	111.45 ± 13.17 cd	111.44 ± 13.18 bc
5. NC55	2.79 ± 0.32 ab	175.41 ± 8.15 abc	159.46 ± 7.41 ab
6. C55	2.45 ± 0.24 ab	215.10 ± 6.76 a	195.54 ± 6.15 a
7. NC60	2.16 ± 0.12 ab	134.53 ± 10.18 bcd	144.47 ± 19.84 abc
8. C60	1.78 ± 0.13 b	91.40 ± 21.46 d	93.74 ± 22.01 c

[a] NC55H10: non-cased, 55% moisture,10 mM histidine supplementation; C55H10: cased, 55% moisture, 10 mM histidine supplementation; NC60H10: non-cased, 60% moisture, 10 mM histidine supplementation; C60H10: cased, 60% moisture, 10 mM histidine supplementation; NC55: non-cased, 55% moisture; C55: cased, 55% moisture; NC60: non-cased, 60% moisture; C60: cased, 60% moisture

[b] ERGO concentration

[c] Estimated amount of ERGO (mg) produced per bag (substrate unit)

[d] Estimated amount of ERGO (mg) produced per kg of dry substrate

[e] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$). Standard deviation (estimation of variance) follows ±

Table 7 *P* values from the analysis of variance for three factors (casing, moisture of substrate and histidine supplementation of substrate) and their interactions influencing yield, BE, number of mushrooms, solids/mushroom, solids/bag, and production cycle length for *Pleurotus eryngii* var. *eryngii* (Crop 4)

Source of variation	DF	Yield (g)	BE (%)	Number mushrooms	Solids/mushroom[2] (%)	Solids/bag	Production cycle length (days)
Model	7	0.0080*	0.0076*	0.2272	<.0001*	0.0115*	0.0248*
Effects tested							
Casing (C)	1	0.0005*	0.0009*	0.0560	<.0001*	0.3474	0.0008*
Histidine (H)	1	0.3912	0.2383	0.2003	0.1360	0.6087	0.6286
Moisture (M)	1	0.5845	0.1296	0.1682	<.0001*	0.0808	0.2166
C × H	1	0.3893	0.3858	0.6196	0.6866	0.3422	0.1209
C × M	1	0.0072*	0.0147*	0.9316	0.0054*	0.0093*	0.5608
H × M	1	0.2579	0.3116	0.5272	0.1169	0.0038*	0.6286
H × C × M	1	0.8132	0.8347	0.2501	0.0060*	0.0485*	0.7743

* *P* values < 0.05 were considered statistically significant

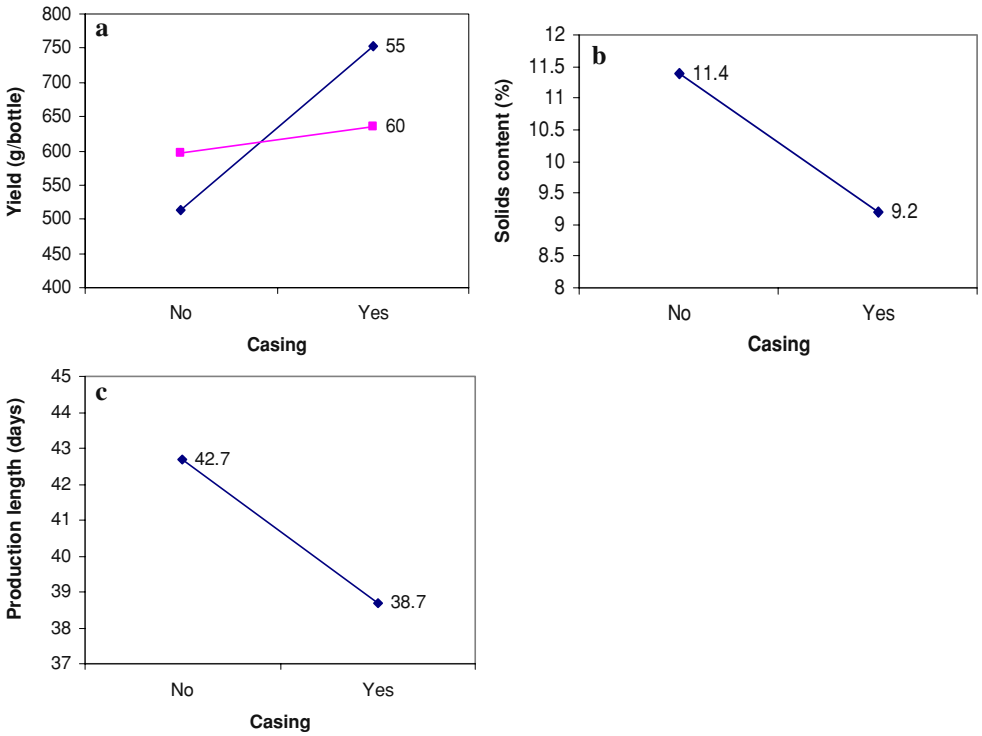

Fig. 2 Influence of a casing overlay on yield, production cycle length and solids content of *Pleurotus eryngii* var. *eryngii*. **a** Interaction plot showing significant increases in yield when the casing layer was applied to low-moisture substrate, **b** main effect plot showing decreased solids content in mushrooms harvested from cased substrate, **c** main effect plot showing use of a casing layer shortened production cycle length

fungus was able to use the nutrients present in the substrate more efficiently. In fact, BE was highest from substrate supplemented with 10 ppm of Se (71.9%). Although Se5 ppm treatment also showed significant differences in dry and wet weight substrate compared to the control (Se0 ppm), mushroom solids were not significantly different. Therefore, our hypothesis is that lower percentages of mushroom solids in treatment Se10 ppm might also be the result of a metabolic response of the fungus to substrate supplementation. It is known that Se, in certain concentrations, is toxic to fungi. Thuong et al. (1995) for example, observed that biomass in submerged cultures of *Saccharomyces cerevisiae* decreased when Se was added to the production media at 30–100 μg/ml. Even though Se10 ppm resulted in less solids/mushroom, the estimated overall amount of Se obtained in mushroom tissue per bottle was 134 μg, the highest among the treatments (Table 2). Accumulation of selenium in basidiomata as result of substrate supplementation is in agreement with studies performed on *P. cornucopiae*, *Grifola frondosa* and *A. bisporus* where Se accumulated at different rates (Werner and Beelman 2002; Beelman and Royse 2006). Although information regarding Se absorption and accumulation is available for the above mentioned species of

Table 8 Treatments and mean comparisons for yield, BE, number of mushrooms, solids content of mushrooms, and production cycle length of *Pleurotus eryngii* var. *eryngii* as affected by casing, substrate moisture content, and histidine supplementation of substrate

Treatment[a]	Yield (g)[b]	BE (%)[b]	Number mushrooms[b]	Solids content (%)[b]	Solids content (g/bag)[b]	Production cycle length (first to last day)[c]
1. NC55H10	498.5 ± 36.67 b	44.3 ± 3.26 b	17.0 ± 7.4	12.23 ± 0.61 a	59.90 ± 4.75 b	43–44
2. C55H10	700.0 ± 45.17 ab	62.2 ± 4.02 ab	16.0 ± 2.9	8.97 ± 0.78 cd	62.95 ± 9.76 b	37–39
3. NC60H10	611.5 ± 88.18 ab	61.2 ± 8.82 ab	17.8 ± 4.9	10.23 ± 0.33 bc	68.76 ± 3.60 ab	44–45
4. C60H10	628.3 ± 165.18 ab	62.8 ± 16.52 ab	11.5 ± 4.2	9.13 ± 0.39 cd	65.63 ± 14.47 ab	38–39
5. NC55	529.8 ± 38.72 b	48.2 ± 3.52 b	25.8 ± 9.0	12.21 ± 0.67 a	63.07 ± 4.20 ab	40–41
6. C55	807.3 ± 140.27 a	73.4 ± 12.75 a	16.7 ± 6.4	9.98 ± 0.13 bcd	88.08 ± 5.93 a	38–39
7. NC60	580.0 ± 35.75 ab	59.5 ± 3.67 ab	17.8 ± 8.7	10.85 ± 0.38 ab	62.39 ± 2.43 b	42–44
8. C60	640.3 ± 118.33 ab	65.7 ± 12.14 ab	14.8 ± 8.8	8.51 ± 0.12 d	50.94 ± 8.99 b	39–41

[a] NC55H10: non-cased, 55% moisture, 10 mM histidine supplementation; C55H10: cased, 55% moisture, 10 mM histidine supplementation; NC60H10: non-cased, 60% moisture, 10 mM histidine supplementation; C60H10: cased, 60% moisture, 10 mM histidine supplementation; NC55: non-cased, 55% moisture; C55: cased, 55% moisture; NC60: non-cased, 60% moisture; C60: cased, 60% moisture

[b] Means followed by the same letter are not significantly different according to the Tukey–Kramer HSD test ($P = 0.05$). Standard deviation (estimation of variance) follows ±

[c] Production length is expressed as a range between the first and last day of mushroom harvest

Table 9 Pearson correlation coefficients among moisture, ergothioneine (ERGO), ERGO/bag, yield, solids/mushroom and solids/bag

	Moisture	ERGO	ERGO/bag	Yield	Solids/Mush
ERGO[a]	−0.799*				
ERGO/bag[b]	−0.777*	0.776*			
Yield	−0.027	−0.243	0.298		
Solids/mushroom	−0.372	0.461*	0.436	−0.501*	
Solids/bag[b]	−0.260	0.025	0.639*	0.790*	0.129

[a] Concentration

[b] Estimated amount of ERGO/bag

[*] Statistically significant values ($P < 0.05$)

mushrooms, studies on Se decreases or conversion during processing and cooking, Se speciation and bioavailability in mushrooms are especially scarce (Mutanen 1986; Van Elteren et al. 1998; Tham et al. 1999; Ogra et al. 2004). Such information is needed in order to thoroughly evaluate the benefits of Se-enriched mushrooms for the human diet.

Dubost et al. (2007) examined ERGO concentration enhancement in *A. bisporus* basidiomata as result of various factors such as moisture content, fragmentation and histidine supplementation of the compost. Dubost observed that *A. bisporus* mushrooms grown on low-moisture content compost (66%) yielded a higher concentration of ERGO than mushrooms harvested from high-moisture compost (72%). Fragmentation of the substrate also appeared to increase ERGO concentration in the mushrooms. Dubost et al. (2007) suggested that ERGO might be a stress-induced metabolite that is enhanced when the mycelium is exposed to low substrate moisture content and fragmentation of the production media. The same authors also found that histidine supplementation of the compost resulted in an increase of ERGO in mushrooms harvested during the second and third flushes. Based on that information, we sought to evaluate the effects of moisture content, fragmentation of the substrate and histidine supplementation on ERGO concentration in *P. eryngii* basidiomata. Moisture content of the substrate significantly influenced ERGO concentration in basidiomata, with lower moisture contents producing higher ERGO concentrations. However, substrate fragmentation did not affect concentration of ERGO. Initially we hypothesized that high concentrations of ERGO in mushrooms produced on low-moisture substrate might be result of a concentration artifact. Since lower yields were produced on low-moisture substrate, it seems likely that ERGO is more concentrated in less fungal tissue. The use of a casing layer provided an additional water source (besides the substrate) for the developing mushrooms resulting in increases in yield and BE. Therefore, we sought to determine if a casing layer placed over low-moisture substrates would result in mushrooms with high ERGO concentration. Thus, we designed an experiment that simultaneously evaluated moisture content, histidine supplementation and use of a casing layer on yield and ERGO concentration in basidiomata (Crop 4). Indeed, low-moisture content substrate produced basidiomata with a higher ERGO concentration than high-moisture substrates regardless of the yields obtained. Hence, high concentrations of ERGO in mushrooms were a result of a relatively dry substrate used to produce the basidiomata. Mushrooms with the highest ERGO concentration were produced on low-moisture, histidine-supplemented substrates (NC55H10 and C55H10). However, when ERGO/bag and ERGO/kg of dry substrate were calculated, the effect of histidine supplementation was

not significant. Quantities of ERGO/bag and ERGO/kg are estimated from yields and mushroom solids, and histidine supplementation did not affect either response. Supplementation of substrate with histidine significantly affected solids/mushrooms only in a three-way interaction (hisitidine × casing × substrate moisture content). Thus, the influence of histidine on solids/mushroom might be dependent on moisture, either supplied by the substrate or by the casing layer. Further experimentation would be necessary to confirm this hypothesis. Dubost et al. (2007) demonstrated that histidine supplementation increased ERGO concentration in the second and third breaks of *A. bisporus*. Because *P. eryngii* is usually harvested for only one break before the substrate is discarded, we restricted our analysis to a single flush. Use of a casing layer, as practiced by some growers in Italy and China, may allow production of second and third flushes (Rodriguez Estrada and Royse 2008). In future research, ERGO concentration in *P. eryngii* basidiomata may be determined in multiple flushes. In contrast to findings of Dubost et al. (2007) for *A. bisporus*, fragmentation of the substrate did not have a significant effect on ERGO concentration in *P. eryngii* basidiomata.

A casing overlay dramatically affected yield, BE, solids/mushroom and production length. It is generally assumed that moisture content of the substrate plays an important role in mushroom solids and yield. However, in the experiment where a casing overlay was used, substrate moisture did not significantly influence yield and BE. In fact, yield and moisture content of the substrate were not correlated. Highest yields were obtained from cased treatments (C55, C55H10, C60, and C60H10) regardless of the moisture content of the substrate. Since number of mushrooms was not significantly different among treatments we suggest that increases in yields might be due to formation of larger and heavier mushrooms.

In conclusion, our results indicate that presence of the antioxidants Se and ERGO in *P. eryngii* var. *eryngii* basidiomata may be enhanced through selected cultural practices. Supplementation of substrate with sodium selenite and use of relatively low moisture content in the substrate are cultural practices that would be relatively easy to implement on a commercial scale. Potential yield reduction by lower moisture content in the substrate may be overcome by the use of a casing layer. This practice would also shorten the production cycle from 43 to 39 days when non-fragmented substrate is cased.

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