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β-Adenosine, a bioactive compound in grass chaff stimulating mushroom production

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Abstract

Fructification and yield of the edible mushrooms *Pleurotus pulmonarius* and *Stropharia rugosoannulata* are clearly enhanced when wheat straw is supplemented with 30% *Lolium perenne* grass chaff. The bioactive compound in the methanol extract of grass chaff was identified as β-adenosine. In vitro biological activity tests showed that 0.012 mg of β-adenosine per ml of medium stimulated earlier fructification of *Pleurotus pulmonarius*. Mushroom fruiting trials showed that when 12 mg β-adenosine was added to 1 kg wet wheat straw, primordia of *Pleurotus pulmonarius* appeared two days earlier and primordia of *Stropharia rugosoannulata* appeared 18 days earlier when compared to pure wheat straw substrate. This concentration of β-adenosine had no impact on the mushroom yield of *Pleurotus*, but resulted in a 2.2 fold increase in yield for *Stropharia*. β-Adenosine at 25 mg per kg wet wheat straw increased the yield of *Pleurotus* with 52% and the yield of *Stropharia* with 258%, but this concentration delayed primordial formation in *Pleurotus*.

Keywords: *Lolium perenne*; *Pleurotus pulmonarius*; *Stropharia rugosoannulata*; Mushroom fruit body formation; Oyster mushroom; King Stropharia; Rye grass

1. Introduction

Mushroom growth and fruit body formation are greatly influenced by various environmental factors. Abiotic and biotic factors such as temperature, humidity, light, pH, gaseous exchange, nutrient status, minerals, sexuality and genetics, microbial interactions, hormones and enzymes can all have an influence on mushroom development (Ganny and Gooding, 1987; Stamets, 1993). The mushroom substrate, which is the main source of nutrients, is one of the crucial factors that greatly affect growth and fructification (Elliot, 1994; Moore, 1998).

Different species of cultivated mushrooms have different substrate requirements (Philippousis et al., 2000). Some species grow best in fermented pasteurized substrates while other species grow best in sterilized, pasteurized or simply moistened substrates. Suitable substrates lead to fast colonization and high density of mycelium, enhance primordial formation and fructification and increase mushroom yield (Stamets, 1993). The nutrient availability from the substrates and mycelium greatly contributes to the formation of the fruit bodies or carpophores (Chang and Miles, 1988; Ruiters and Wessels, 1989).

The addition of organic supplements to wheat or rice straw resulted in earlier fructification and increased the yield of *Pleurotus* mushrooms (Royse et al., 1991; Royse and Zaki, 1991; Krisnamoorthy, 1997; Chowdhury et al., 1998). Supplements can also influence the chemical composition and nutritional value of the *Pleurotus* mushroom (Tshinyangu, 1996; Krisnamoorthy, 1997). Furthermore, nitrogen-rich supplements influenced crop yield and fruit body size of shiitake (Kalberer, 2000).

Poppe and Sedeyn (1989) have performed production-stimulating tests with the king Stropharia, *Stropharia rugosoannulata* on straw substrates. In general, the

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indoor culture of this mushroom on straw is very capricious with late and low fructification. Poppe and Sedeyn (1989) added different types of hay to wheat straw and observed that ripe seed-setting *Lolium* grass hay, but not young *Lolium* grass hay, stimulated fruit body production. The addition of pure *Lolium* grass seed also gave good results, but was too expensive. Finally, they found that the addition of chaff from *Lolium* seeds even gave better results and resulted in earlier fructification and a very strong yield increase. Later on Domondon et al. (2000) showed that the addition of grass chaff to wheat straw also enhanced the yield of various *Pleurotus* species and stimulated earlier fructification. In addition, the carpophores harvested from wheat straw supplemented with 30% grass chaff had a significantly higher protein content than the fruit bodies harvested from pure wheat straw substrates.

Lolium perenne, or ryegrass, is an important forage species used mainly for pasture, hay crop or ground cover (Adams et al., 1998). In Europe, grasslands occupy about 52 million hectares. Of these 52 million, at least 12 million hectares use ryegrass cultivars for pasture (Prine, 1991). Maintenance of these pasture lands means that a continuous grass seed production is needed. Processing of grass seeds generates grass chaff that is left, as a waste, in mountain heaps in most grass seed-processing centers. The abundant availability of this waste and the fact that it stimulates earlier fructification makes it an interesting supplement to add to mushroom substrates. However, it is not known why grass chaff enhances fructification. In this study we detected, isolated and identified one of the bioactive substances in the grass chaff of *Lolium perenne* and proved that it enhanced fructification of *Pleurotus* and *Stropharia*.

2. Results and discussion

2.1. In vitro trials with boiled grass chaff extracts

Boiled extracts obtained from grass chaff and wheat straw were combined at different concentrations and were tested for bioactivity with two mushroom species, *Pleurotus pulmonarius* and *Stropharia rugosoannulata*. The mean mycelial diameter of *P. pulmonarius* on the agar plates with different combinations of boiled extracts showed no significant difference between 100% wheat straw, 90:10 wheat straw/grass chaff and 70:30 wheat straw/grass chaff extract (data not shown). For *S. rugosoannulata*, the diameter of mycelial growth in 70:30 wheat straw/grass chaff boiled extract (74.7 mm) was significantly different from the 90:10 wheat straw/grass chaff (57.7 mm), 100% grass chaff extract (71.0 mm), 100% wheat straw extract (52.7 mm) and plain agar (56.3 mm). Also the mycelial growth was denser with the 70:30 wheat straw/grass chaff extract when compared with the other treatments.

Plates were maintained in fruiting conditions for further observations of possible primordial formation. Pinhead formation was observed on the plates inoculated with *P. pulmonarius* 20 days after inoculation (Table 1). Tiny primordia were plentiful on the agar plates with 70:30 ratio of wheat straw/boiled grass chaff extract as compared to the agar plates with extracts of 100% wheat straw. This indicates that the bioactive compound in the grass chaff is thermo stable as shown by the development of more primordia on the agar plates with boiled grass chaff extract. Pinhead formation was not observed, however, with *Stropharia* (data not shown).

2.2. Isolation of the bioactive compound

Grass chaff of ryegrass, *Lolium multiflorum*, was extracted until exhaustion with hexane, dichloromethane, ethyl acetate, methanol and water as shown in Fig. 1. The crude extracts obtained were evaluated for their efficacy in stimulating mycelial growth and primordial formation. Only the methanol extract added to agar resulted in denser mycelium and induced primordial formation. Therefore, this extract was subjected to RP vacuum layer chromatography (VLC). Each fraction was tested for bioactivity using agar plate test (plain water agar for mycelial growth, fruiting medium for primordial formation). Fractions F5 and F6 that stimulated primordial formation were then subjected to centrifugal counter current partition chromatography (CPC) to obtain 12 fractions (Fig. 1). Crystallization of the most active fraction of this series afforded finally a pure compound which was identified as β-adenosine **1** by MS and NMR spectroscopy, including ^{1}H NMR, ^{13}C NMR, COSY, HETCOR and DEPT spectra. The basic data are identical with those published for β-adenosine (Arpalahti et al., 1998; Trifonova et al., 1999) and with our data obtained from authentic β-adenosine.

Table 1
Primordia formation by *P. pulmonarius* on fruiting medium supplemented with boiled extracts from wheat straw (WS) and grass chaff (GC)

Substrates[a]	Number of primordia[b]
100% WS	5±1.2 C
90:10 WS/GC	10±0.3 B
70:30 WS/GC	20±2.3 A
100% GC	8±1.4 B

[a] Substrates: WS = wheat straw, GC = grass chaff.
[b] Mean of four replicates. Primordia formation was observed for a period of 30 days. Values followed by the same letter are not significantly different for $P = 0.05$.

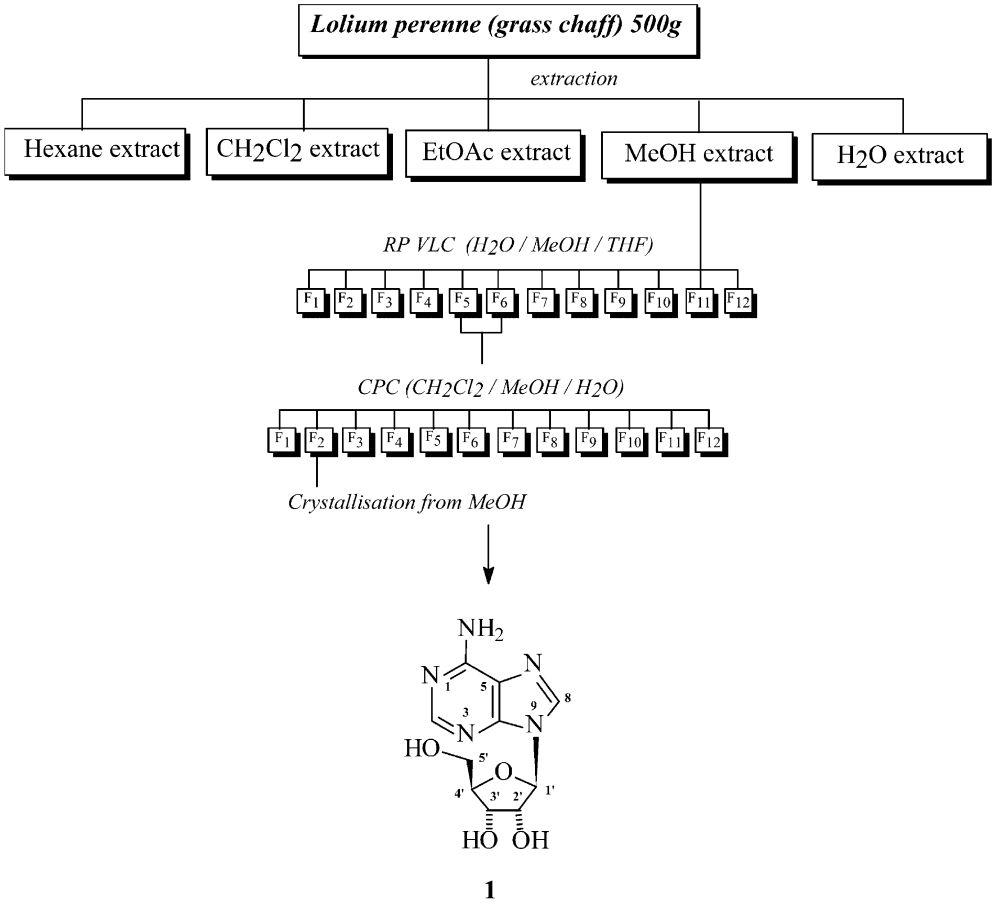

Fig. 1. Isolation scheme for β-adenosine 1.

2.3. In vitro trials with β-adenosine

The isolated β-adenosine **1** was further tested for stimulation of primordial formation in comparison with commercial β-adenosine in agar plate tests. The results showed that the purified compound and β-adenosine at a concentration of 0.012 mg/ml induced primordial formation 5 days after exposure to fruiting conditions. These results coincide with our previous experimental in vivo trials where wheat straw substrates supplemented with grass chaff also showed *P. pulmonarius* primordia formation after 5 days. Interestingly, plates supplemented with β-adenosine or the isolated compound **1** showed a prominent formation of rhizomorphs. When isolated compound **1** or commercial β-adenosine was added at concentrations of 0.025 or 0.05 mg per ml of medium however, primordia only appeared after 10 days.

2.4. Fruiting trials with β-adenosine

Table 2 shows the number of days to primordial formation, the harvest period and the mean of total yield for *Pleurotus pulmonarius* and *Stropharia rugosoannulata* grown on wheat straw supplemented with grass chaff or β-adenosine. Full mycelial colonization took 14 days for *P. pulmonarius* and 21 days for *S. rugosoannulata* irrespective of the type of substrate. When the fully colonized substrates were opened and exposed to fruiting conditions, the first primordium of *Pleurotus* appeared after 5 days on the substrates supplemented with 30% grass chaff. Primordia appeared 2 days earlier on wheat straw substrates supplemented with 12 mg β-adenosine kg^{-1} wet substrate when compared to pure wheat straw substrate. This concentration of β-adenosine however, had no effect on mushroom yield. β-Adenosine at 25 mg/kg wet substrate delayed primordial formation, but increased *Pleurotus* mushroom yield with 52%. In the fruiting tests carried out by Domondon et al. (2000) primordial formation was delayed when pure grass chaff was used as a substrate in comparison with pure wheat straw or wheat straw supplemented with 10 or 30% grass chaff. It is possible that the abundance of adenosine in pure grass chaff is the factor that delays fruit body formation.

Stropharia primordial formation occurred after 24 days on wheat straw supplemented with 30% grass chaff and after 27 days on wheat stray with 12 mg β-adenosine. Here, however, also 25 mg β-adenosine enhanced primordial formation (primordia appeared after 37

Table 2
Number of days to primordia formation, harvest period and total yield of *P. pulmonarius* and *S. rugoso-annulata* on wheat straw (WS) substrate supplemented with grass chaff (GC) or β-adenosine (β-adn)

Substrate	Days to primordia formation[a]		Harvest period (d)[a]		Total yield ± SD (g fresh weight)[b]	
	Pleurotus	*Stropharia*	*Pleurotus*	*Stropharia*	*Pleurotus*	*Stropharia*
100% WS	9	45	25	40	210 ± 10 C	34 ± 15 C
WS/30% GC	5	24	44	60	395 ± 9 A	205 ± 10 A
WS + 12 mg β-adn	7	27	30	40	230 ± 14 C	109 ± 12 B
WS + 25 mg β-adn	14	37	30	45	320 ± 17 B	122 ± 17 B

[a] The spawn run for full mycelial colonization of the inoculated bags were 14 and 21 days respectively, for *Pleurotus* and *Stropharia*. Standard deviations for days to primordia formation and harvest period were for each value smaller than 1 day.
[b] Values are the means of three replicates. SD indicated standard deviation. Treatment means were compared by Duncans Multiple Range Test. Within each column, values followed by the same letter are not significantly different from each other for $P = 0.05$.

days) when compared to pure wheat straw substrate (primordia appeared after 45 days), but this concentration was less effective when compared with 12 mg β-adenosine. For *Stropharia*, both concentrations of β-adenosine significantly increased the total mushroom yield with 220 to 258%.

Ohga (1989) showed that adenosine, adenine and adenosine triphosphate were the most important growth-activating substances for shiitake in hot water extracts of Welsh onion. In addition, Yoshikawa and Ooga (1989) conducted an in vitro study on adenine and its derivatives as mushroom growth enhancers. Their results showed that addition of adenine or its derivatives significantly increased the weight of *Agrocybe aegerita* carpophores. Addition of 15 mg/l of adenine, adenosine or adenosine monophosphate to substrates produced fruit bodies with a total weight of 13.96 g/dish versus 9.3 g/dish for the control.

Adenosine is a precursor of cyclic-adenosine monophosphate (cylic-AMP), synthesized in the presence of the enzyme, adenyl cyclase (Griffin, 1993). Cyclic-AMP is one of the first recognized secondary messengers, which modulates various eukaryotic cellular processes (Laychock, 1989). In fungi, it was postulated to be involved in nutrition, reproduction and morphogenesis and dimorphism (Pall, 1981; Griffin, 1993). In *Coprinus comatus*, the level of cyclic-AMP was increased only during fruiting, hence, it was surmised that it acts as a fruit body-inducing substance. Likewise, cyclic AMP at 200 mg/l enhanced mycelial growth of *Calvatia gigantea* by 24% (Alexander and Lippert, 1989). Thus, it is possible that the ready availability of adenosine in grass chaff induces the synthesis of cyclic-AMP and consequentially induces primordial formation and enhances fruit body production.

Moreover, Lou and Montag (1994) showed that the dominant fractions of nucleosides in mushrooms are adenosine, guanosine and uridine. A reversed phase-HPLC analysis of the nucleoside profiles of medicinal mushrooms showed that *Ganoderma lucidum*, *Lentinus edodes* and *Cordyceps sinensis* contained large amounts of adenosines in their fruiting bodies (Shiao et al., 1994). These previous findings of adenosine abundance in mushroom fruit bodies may further give light to the present results that β-adenosine enhances in vitro primordial formation in *P. pulmonarius*. Apparently, the availability of adenosine in the grass chaff, which probably served as a precursor for cyclic-AMP, enhances cellular processes, such as morphogenesis, resulting in an earlier primordial formation and enhanced fruit body production. Likewise, there is a ready source for the synthesis of adenosine nucleosides for cellular synthesis. Therefore, it can be concluded that the availability of adenosine in substrates could be an important factor in mushroom fructification.

However, primordial formation occurred earlier and mushroom yield was much higher in wheat straw supplemented with 30% grass chaff compared to wheat straw supplemented with β-adenosine. This indicates that grass chaff contains other components, aside from β-adenosine, that enhance fructification and mushroom yield. Accordingly, it can be concluded that fructification is not caused by a single factor or substance but it is interplay of several compounds and several factors.

3. Experimental

3.1. General experimental procedures

The ^{1}H, ^{13}C, DEPT and COSY NMR spectra were obtained with a JNM-EX270 FT NMR apparatus (Jeol, Japan). Mass spectra were measured with a LCT (Micromass) consisting of an Alliance 2690 LC (Waters) and an orthogonal accelerated time-of-flight MS analyzer using a Z-spray interface and operated in positive ion electrospray mode and controlled with Mass lynx 3.3 software. Melting points were determined on a

Büchi 535 apparatus (Büchi, Switzerland). CPC was carried out with a CCC-1000 High Speed Countercurrent Chromatograph (Pharma-Tech Research Corp. USA), equipped with a SSI 300 pump, a Pharmacia LKB Uvicord S II detector (254 and 280 nm) and a Retriever II fraction collector. All precoated TLC plates (200×200×0.25 mm) for both normal (Silica gel 60 F_{254}) and reversed phase (RP-18 F_{254s}), and the solvents used for isolation and purification, were from Merck, Germany.

Grass chaff (500 g) was successively extracted until exhaustion with *n*-hexane (*n*-C_6H_{12}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), methanol (MeOH) and water (H_2O). The extracts were filtered over a filter paper (Scheiler & Schuell, 595/2 folded filters, ∅ 240 mm) and concentrated or dried *in vacuo* in a rotavapor at a temperature of 40 °C. The residues of the crude extracts were tested for their efficacy in stimulating mycelial growth and primordial formation of *Pleurotus* in agar plates. The MeOH extract (30.81 g), which showed the best mycelial growth and stimulated earlier primordial formation, was selected for further study.

Twenty-two grams of the methanol extract were adsorbed by Lichroprep C_{18} and fractionated with RP VLC (P_4 glass-sintered funnel packed with 30 g of Lichroprep C_{18}, 40–63 μm, Merck). The elution was performed under vacuum by stepwise gradient starting with 500 ml each of water (two times), water/methanol 97.5:2.5, 95:5, 92.5:7.5, 90:10, 85:15, 80:20, 75:25, 70:30, 100% methanol, and then THF. These filtrates were monitored by RP-TLC, of which, 12 fractions (F1-F12) were obtained. Active fractions (F5 and F6, 320 mg) were combined and subjected to CPC with the solvent system CH_2Cl_2/MeOH/H_2O (7:13:8): the lower phase (LP) as the stationary phase and the upper phase as mobile phase (MP); elution configuration, tail to head; sample dissolved in nine ml UP and nine ml LP, filtered with glass fiber filter (Acridose, 0.45 μm, Gelman Science) and injected into the coiled columns (350 ml) with a sample loop of 14 ml; flow rate, 1 ml/min; fractions collected in 14 ml; revolution speed, 1000 rpm with a pressure at 140 psi; equilibrium, 90 ml; run time, 340 min; push out with a flow rate of four ml/min, and fraction in 8 ml; detection, 254 and 280 nm. Based on the TLC monitoring, 12 fractions were obtained (F1-F12). The most active fraction F2 (39 mg) was purified by crystallization with MeOH to obtain the pure compound **1** (20 mg) as a fine white solid (MeOH): mp 234.4–235.4 °C [lit. Mp 234–235 °C (Budavari et al., 1989)]; ^{1}H NMR δ (270 MHz, DMSO-d_6) 3.47 (2H, *s*, NH_2), 3.59 (2H, *m*, H-5′), 3.97 (1H, *q*, J=3.3 Hz, H-4′), 4.15 (1H, *dd*, J=5.0, J=3.3 Hz, H-3′), 4.62 (1H, dd, J=5.9, J=6.3 Hz, H-2′), 5.24 (1H, *d*, J=4.6 Hz, OH-3′), 5.49 (1H, *d*, J=3.0 Hz, OH-2′), 5.51 (1H, *d*, J=4.0 Hz, OH-5′), 5.88 (1H, *d*, J=6.33 Hz, H-1′), 8.14 (1H, *s*, H-2), 8.36 (1H, *s*, H-8) ppm; ^{13}C NMR δ (67.5 MHz, DMSO-d_6) 61.8 (C-5′), 70.8 (C-3′), 73.6 (C-2′), 86.1 (C-4′), 88.1 (C-1′), 119.4 (C-5), 140.0 (C-8), 149.1 (C-4), 152.5 (C-2), 156.2 (C-6) ppm; ES$^+$MS (LCMS): *m*/*z* 268 (M+1, 5%), 137 (7), 136 (100), 108 (20).

3.2. Microorganisms and plant material

Two mushroom species, *Pleurotus pulmonarius* (Phytopathology Laboratory collection, Ghent University) and *Stropharia rugosoannulata* (cap tissue isolate from Merelbeke, Belgium) were used to test the efficacy of grass chaff as a stimulant for fructification or primordial formation. Mother cultures were prepared in polystyrene Petri plates with malt extract agar and were used as sources of mycelial discs for bioassays. Mushroom spawn and mushroom substrate (chopped and hammermilled wheat straw) were obtained from the mushroom spawn laboratory Mycelia (St. Amandsberg, Ghent, Belgium). *Lolium perenne* grass chaff was collected at the grass seed-production center in Merelbeke, Belgium.

3.3. In vitro trials

3.3.1. Boiled extracts

Wheat straw and grass chaff, 200 g each, were boiled separately for 15 min in 2 l each of water. The following combinations were prepared to determine the effect of boiled grass chaff extract as a supplement: (a) 100 ml of boiled wheat straw extract, (b) 90 ml of boiled wheat straw extract + 10 ml of boiled grass chaff extract, (c) 70 ml of boiled wheat straw extract + 30 ml of boiled grass chaff extract, (d) 100 ml of boiled grass chaff extract and (e) 100 ml of distilled water. Each of the treatments was placed in flasks and 1.5 g of agar was added.

3.3.2. Crude grass chaff extracts

The different crude grass chaff extracts were dissolved in acetone at a concentration of 160 mg/ml and added to hand-warm water agar at a final concentration of 1.6 mg of crude extract per ml of water agar.

3.3.3. Fractions

The different fractions obtained during isolation were dissolved in methanol at a concentration of 50 mg/ml and added to hand-warm fruiting medium (Chiu and To, 1993) at a final concentration of 0.05 mg and 0.1 mg per ml of medium.

3.3.4. Pure compounds

The isolated compound **1** was compared with α-adenosine (Sigma-Aldrich) and β-adenosine (Sigma-Aldrich) for primordial stimulation. These compounds were dissolved in water-methanol (70:30) at a concentration of 0.05 mg/ml and added to hand-warm fruiting

medium at a final concentration of 0.05, 0.025 and 0.012 mg per ml of medium.

3.3.5. Inoculation and incubation

All media were dispensed in polystyrene Petri plates at a volume of 20 ml/plate. After solidification, agar plates were inoculated centrally with 9 mm mycelial discs of *P. pulmonarius* or *S. rugosoannulata*. The inoculated plates were incubated in dark conditions at 24 °C. After 7 days, mycelial diameter was measured with a vernier calliper. Growth density was photographed, rated and recorded with a plus sign. Plates were subsequently maintained in fruiting conditions (±20 °C with a photoperiod of 12/12 h (day/night) and a light intensity of 1000 lux) for possible primordial formation. The primordia were recorded regularly for a period of 30 days.

3.4. Fruiting trial

The basal substrates for the growing tests were chopped and hammermilled wheat straw supplemented with chaff of perennial ryegrass, *Lolium perenne*. Wheat straw and grass chaff were pasteurized separately by immersion in water at 65 °C for 1 min. The pasteurized substrates were cooled at room temperature and drained in a sieve up to 70% moisture (Oei, 1991). When the substrates were cooled, the following treatments were prepared: (a) 100% wheat straw, (b) 100% wheat straw + 12 mg β-adenosine kg^{-1} wet straw, (c) 100% wheat straw + 25 mg β-adenosine kg^{-1} wet straw and (d) 70% wheat straw + 30% grass chaff. The combined wet weight of wheat straw and grass chaff supplement per replicate was 1 kg. Each treatment was carried out in three repetitions. Each replicate was inoculated with 50 grams of mushroom spawn. Prepared oat grain spawn was used for inoculation. Fifty grams of oat grain spawn were inoculated alternately while filling the polyethylene bags (25×40 cm) with substrates layer by layer. The topmost layer of the substrate was inoculated with more spawn grains to hasten faster colonization and prevent growth of competitor microorganisms. β-Adenosine dissolved in 50 ml of water was added in droplets in the bags of inoculated wheat straw. Bags were closed with adhesive tape and the basal corners of the bag were cut to provide aeration and for drainage of excess water. For *Stropharia* trials, the inoculated bags were incubated in dark condition for 3 weeks at a temperature of 25 °C. After full mycelial colonization, the bags with were opened and a casing layer composed of 50% deep garden soil and 50% black peat was applied at a depth of 3 cm. The fully colonized bags with casing layer were transferred in a fruiting room provided with artificial light of 1000 lux with a photoperiod of 12 h/12 h and at a temperature of ±20 °C. For *Pleurotus pulmonarius* trials, the inoculated bags were incubated in dark conditions for 2 weeks and then exposed to light conditions like the bags of *Stropharia*. Casing layer was not, however, necessary for *Pleurotus* cultivation. The mean yield of carpophores was determined for both species at the end of the fruiting trials.

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