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SHORT COMMUNICATION

Growth promotion of *Suillus luteus* by adenosine in vitro

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Abstract In this work, adenosine has been shown to stimulate *Suillus luteus* mycelial growth, branching, and aggregates on solid substrate. Adenosine promoted *S. luteus* mycelial growth and branching, and the fungus responded significantly at a concentration as low as 0.01 mg/ml. The highest mycelial biomass and density of three strains were observed at 0.16 mg/ml in SNP-20, 0.08 mg/ml in SAF-501, and 0.16 mg/ml in PT-501. Adenosine enhanced mycelial aggregate at concentration of 0.02 mg/ml in SNP-20. The study suggests that adenosine promotes *S. luteus* mycelial growth, branching, and aggregation. Thus, it may be a good candidate as a biological elicitor of mycelial growth for *S. luteus*.

Keywords Aggregate · Branching

Suillus luteus (L.: Fr.) Gray is one of the well-known edible ectomycorrhizal (ECM) fungi with a worldwide distribution. Over the course of past decades, assessment of ECM fungal sporocarp production has been carried out, and some pot culture trials have been conducted to determine the golden edible ECM fungal growth, primordium, and basidiocarp formation (Yamada et al. 2001, 2007), which are greatly influenced by various factors such as fatty acids (Unestam and Sun 1995), wood ash (Hagerberg and Wallander 2002), surfactants or vegetable oil (Guerin-Laguette et al. 2003), and microbial interaction (Gamalero et al. 2003). Among these factors, much attention has been devoted to the bioactive compounds that improve the process of mycelial growth (Unestam and Sun 1995). So far, several bioactive compounds have been well assessed for their ability to promote ECM fungal growth, such as amino acids for *Hebeloma vinosophyllum* (Yamanaka 1999), rutin for *Pisolithus tinctorius* (Lagrange et al. 2001), olive oil for *Tricholoma matsutake* (Guerin-Laguette et al. 2003), and fulvic acid for *Leccinum aurantiacum* (Soukupová et al. 2008). Recently, Kikuchi et al. (2007) suggested that flavonoids induce germination of basidiospores of the ECM fungus *Suillus bovinus* on solid medium. However, to date, the development of mushrooms in ECM basidiomycetes has not been well described (reviewed in Kües and Liu 2000), which means the life cycle of ECM fungi is poorly understood. The lack of effective growth-activating elicitors (GAEs) for the mycelial growth process may contribute to the difficulty.

Adenosine is a precursor of cyclic adenosine 3′, 5′-monophosphate (cAMP) (Griffin 1993) and acts as an essential functional substance in edible mushrooms. cAMP also has broad biological activities to regulate fungal mycelial cellular synthesis (Laychock 1989), affecting mycelial branching (Kubo and Mihara 2007). Alexander and Lippert (1989) indicated that cAMP enhanced the mycelial growth of *Calvatia gigantea* by 24%. Interestingly, the level of cAMP increased only during fruiting development, as reported by Griffin (1993). A follow-up study by Ohta (1994) reported that *Lyophyllum shimeji* formed fruiting bodies on an artificial medium when cAMP was added in pure culture without a host plant. Thus, it was surmised that adenosine would play an essential role as a mycelial growth-signaling molecule component. A related study by Ohga (1989) had demonstrated that adenosine and

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adenosine triphosphate were the most important GAEs for the growth of *Lentinus edodes* in hot water extracts of onion. In addition, the results from the pure culture study on adenine and its derivatives as mushroom growth enhancers by Yoshikawa and Ooga (1989) suggested that addition of adenine or its derivatives significantly increased the carpophore weight of *Agrocybe aegerita*. Moreover, Lou and Montag (1994) found that the dominant fraction of nucleosides in mushrooms is adenosine. Later on, Domondon et al. (2004) revealed that adenosine acts as a biological elicitor to enhance mycelial growth in vitro and stimulates production of the edible mushrooms *Pleurotus pulmonarius* and *Stropharia rugosoannulata*. In view of these findings, we hypothesize that adenosine is essential and sufficient to stimulate the mycelial growth of ECM fungi. Consequently, the present study reported on adenosine, which promotes mycelial growth and branching and induces the mycelial aggregate of *S. luteus* in pure culture.

Three strains of *S. luteus* were used in this study. SNP-20 and PT-501 were each isolated from fruiting bodies collected beneath Chinese pine (*Pinus tabulaeformis* Carr.) forests in Shangnan County and at Qinling Mountain (experimental forest at Huoditang, Northwest A & F University), respectively. SAF-501 was isolated from a fruiting body collected beneath *Populus euramericana* and *P. tabulaeformis* mixed forests in the microbiology experimental station in northwestern China (Tang et al. 2009). Identification was confirmed by rDNA internal transcribed spacer (ITS) sequences (Peintner et al. 2003). Sequences of the fungi are deposited at GenBank under accession numbers GQ330568, GQ330567, and GQ330566, respectively. Pure cultures were preserved in the Laboratory of Forestry Microbiology, College of Forestry, Northwest A & F University, China. Stock cultures were maintained routinely at 25 ± 2°C in darkness on potato dextrose agar (PDA) medium and subcultured weekly. PDA medium contained 1000 ml distilled water, 200 g potato, 20.0 g glucose, and 10.0 g agar. Adenosine (≥99.9%) was purchased from KAYON Biological Technology, China. Mycelia of *S. luteus* were first grown on modified Melin-Norkrans (MMN) medium (Marx 1969) containing 10 g/l glucose and 1.0% agar at 25 ± 2°C for 7 days. Mycelial disks (5 mm diameter) cut from the margin of actively growing stock colonies were grown on MMN medium (20 ml/9-cm-diameter Petri dish) supplemented with adenosine at different concentrations (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, or 0.64 mg/ml), and a no-adenosine medium was set as control. All inoculated plates were incubated at 25 ± 2°C in darkness. Mycelial diameter was measured every third day by a caliper in two directions until the entire surface of the agar plate medium was covered with mycelia. Once the agar plates were covered with mycelia, then incubation conditions were shifted to a growth chamber condition with a constant temperature of 15 ± 2°C, 80% humidity, and a 12:12 h photoperiod. Growth of the mycelium was regularly monitored until the surface of the growth media was homogeneously overgrown. The mycelial branching and aggregate were observed under a microscope (Olympus BX51, Japan). To determine mycelial biomass, the mycelium was either collected on a cellophane sheet 100 × 100 (Shanghai, China) or recovered from agar melted in a water bath (Guerin-Laguette et al. 2003), oven dried at 80°C for 48 h, and weighed after cooling in a vacuum desiccator. All the assays were replicated five times independently with each five plates. Data calculations were performed with the statistical software SAS System for Windows V8.1 (SAS Institute, USA).

In the present investigation, the growth rates of *S. luteus* increased in the presence of adenosine concentrations at 0.01–0.32 mg/ml. The highest growth rates, 7.3 mm/day in SNP-20, 7.8 mm/d in SAF-501, and 6.6 mm/day in PT-501, were obtained when the adenosine concentration was at 0.16, 0.08, and 0.16 mg/ml, respectively. However, no stimulatory effect of PT-501 was found at 0.64 mg/ml, and mycelial growth of the SNP-20 and SAF-501 strains was inhibited at this concentration (Table 1). Interestingly, branching clearly increased in the mycelium treated with adenosine (Fig. 1). We therefore concluded that mycelial growth rates and branching of *S. luteus* could be promoted by adenosine at micromolar levels.

When the three strains were cultured in 0.16 mg/ml adenosine medium, the mycelial growth increased by 2.05 (SNP-20), 2.45 (SAF-501), and 1.68 (PT-501) times that of the control, respectively. However, 0.64 mg/ml adenosine inhibited mycelial growth. Dry mycelial biomass and density of *S. luteus* grown on a range of concentrations of adenosine are summarized in Table 2. The dry weight of mycelia of the three strains of *S. luteus* increased linearly with increasing adenosine concentrations (0.01–0.08 mg/

Table 1 Effects of adenosine on growth rate of diameter (mm/day) of three strains of *Suillus luteus*: SNP-20, SAF-501, and PT-501

Adenosine concentration (mg/ml)	SNP-20 (mm/day)	SAF-501 (mm/day)	PT-501 (mm/day)
0	5.2 ± 0.5	4.9 ± 0.8	4.7 ± 0.9
0.01	6.6 ± 0.5	5.8 ± 0.4	5.2 ± 0.9
0.02	6.8 ± 0.5	5.8 ± 0.0	5.5 ± 0.5
0.04	6.8 ± 0.5	5.9 ± 0.9	5.8 ± 0.5
0.08	7.0 ± 0.9	7.8 ± 0.5	6.6 ± 0.5
0.16	7.3 ± 0.4	7.3 ± 0.5	6.6 ± 0.5
0.32	6.3 ± 0.4	6.1 ± 0.5	5.5 ± 0.5
0.64	5.1 ± 0.4	4.8 ± 0.5	4.7 ± 0.6

Each value is expressed as mean ± standard deviation ($n = 5$)

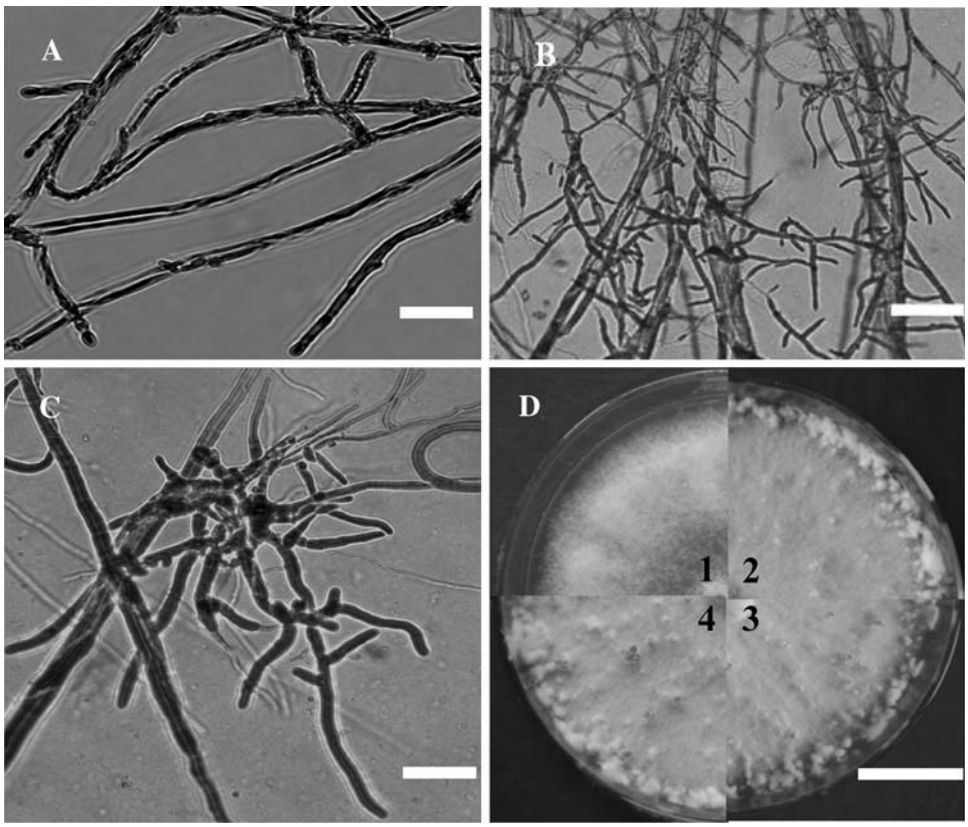

Fig. 1 Colonies of *Suillus luteus* SNP-20 after 9 days culturing. Appearance of mycelial branching grown on no-adenosine medium (**A**) and on adenosine-containing medium (**B**: 0.04 mg/ml; **C**: 0.16 mg/ml). **D** Mycelial aggregate on the medium, where the numbers (*1–4*) represent adenosine concentrations at 0, 0.02, 0.08, and 0.32 mg/ml, respectively. *Bars* **A**, **C** 40 μm; **B** 80 μm; **D** 2 cm

Table 2 Effects of adenosine on dry mycelial biomass and density of three strains of *S. luteus*: SNP-20, SAF-501, and PT-501

Adenosine concentration (mg/ml)	SNP-20		SAF-501		PT-501	
	DW (g)	MD (mg DW/cm^2)	DW (g)	MD (mg DW/cm^2)	DW (g)	MD (mg DW/cm^2)
0	0.21 ± 0.01c	3.4 ± 0.2	0.09 ± 0.04c	1.4 ± 0.1	0.19 ± 0.02d	3.1 ± 0.3
0.01	0.27 ± 0.06cb	4.2 ± 0.9	0.15 ± 0.08bc	2.3 ± 0.6	0.21 ± 0.02cd	3.2 ± 0.3
0.02	0.27 ± 0.07bc	4.3 ± 1.1	0.15 ± 0.04bc	2.4 ± 0.6	0.22 ± 0.01bcd	3.5 ± 0.2
0.04	0.28 ± 0.05bc	4.4 ± 0.8	0.20 ± 0.01b	3.1 ± 0.7	0.22 ± 0.04bcd	3.5 ± 0.5
0.08	0.32 ± 0.04b	5.1 ± 0.7	0.31 ± 0.06a	4.9 ± 0.7	0.26 ± 0.01b	4.1 ± 0.2
0.16	0.43 ± 0.06a	6.8 ± 1.0	0.22 ± 0.03b	3.4 ± 0.4	0.32 ± 0.07a	5.0 ± 0.1
0.32	0.31 ± 0.04b	5.0 ± 0.7	0.19 ± 0.06b	2.9 ± 0.8	0.25 ± 0.02bc	3.9 ± 0.2
0.64	0.23 ± 0.03c	3.6 ± 0.4	0.12 ± 0.03c	1.8 ± 0.4	0.18 ± 0.05d	2.8 ± 0.7

Each value is expressed as mean ± standard deviation ($n = 5$); means with different small letters within a column are significantly different at the 0.05 level

DW dry weight mycelial biomass; *MD* mycelial density

ml). The highest mycelia biomasses and densities of the three strains were observed at 0.16 (SNP-20), 0.08 (SAF-501), and 0.16 mg/ml (PT-501), respectively (Table 2). The results showed that all adenosine concentrations tested induced mycelial aggregates of strain SNP-20. No mycelial aggregate development was detected on the medium without adenosine. Fewer mycelial aggregates were observed in PT-501 and SAF-501 than in SNP-20.

Studying the effects of bioactive products on ECM fungal mycelial growth in pure culture promotes the understanding of the role of the different factors in the life cycle of those basidiomycetes. To date, several bioactive compounds have been well described for their ability to promote mycelial growth of ECM fungi, such as 1-methoxy-3-indolymethyl glucosinalate and 4-methoxy-3-indolymethyl glucosinolate for *Paxillus involutus* and

P. tinctorius (Zeng et al. 2003), flavonoids for *S. bovinus* (Kikuchi et al. 2007), and rutin for *P. tinctorius* (Lagrange et al. 2001). In a related study, Domondon et al. (2004) extracted the adenosine from ryegrass (*Lolium multiflorum*) and showed that adenosine could act as an effective biological elicitor to stimulate the growth of *P. pulmonarius* and *S. rugosoannulata*. In this study, adenosine as investigated was effective in stimulating mycelial growth and branching of the *S. luteus* in pure culture without the host plant. To our knowledge, this is the first report of a positive effect of adenosine on stimulating mycelial growth and branching and inducing the mycelial aggregate process of ECM fungi.

Mycelial growth rates of *S. luteus* were stimulated by adenosine at 0.01–0.32 mg/ml, but 0.64 mg/ml adenosine in the medium inhibited mycelial growth. The highest mycelial growth rates were different among the three strains tested, which suggests considerable intraspecific variation among *S. luteus* strains. Higher concentrations of adenosine inhibited mycelial growth. There are two possibilities for this phenomenon. (1) The mycelial catabolism was suppressed by the higher concentrations of adenosine because of osmotic pressure as ectomycorrhizal fungi do not grow under high osmotic pressure (Hatakeyama and Ohmasa 2004a). (2) It is well known that adenosine is a precursor of cAMP, and the higher concentration improves the activity of cAMP-dependent protein kinase; however, the enzyme could suppress mycelial growth (Lubbehusen et al. 2004). This effect was also found in other carbon and nitrogen components (Hatakeyama and Ohmasa 2004b). Kusuda et al. (2007) reported catabolite suppression by higher concentrations of extra glucose in the ectomycorrhizal fungus *Tricholoma matsutake*.

In the present investigation, aggregation and branching of the mycelium was observed on plates treated with adenosine (see Fig. 1). Mycelial densities of the fungus treated with all concentrations of adenosine were much higher than that of control. These effects were also found in other basidiomycetes, such as *P. pulmonarius* and *S. rugosoannulata*, and the concentrations of 0.012, 0.025, and 0.05 mg/ml adenosine significantly increased the mycelial aggregation of *P. pulmonarius* (Domondon et al. 2004). Kubo and Mihara (2007) demonstrated that cAMP promotes mycelial branching in *Mucor globosus*. The reason suggested by Domondon et al. (2004) was that there is a ready source for the synthesis of cAMP from adenine for mycelial cellular synthesis and growth.

In this study, therefore, additional adenosine in the medium might induce the synthesis of cAMP and consequentially trigger mycelial aggregates of *S. luteus*. Interestingly, fewer mycelial aggregates were observed in PT-501 and SAF-501 than in SNP-20. The most probable explanation is that the ability of ECM fungus to form mycelial aggregates in pure culture depended on the strain (Ohta 1994). As discussed here, the present study includes only three strains of *S. luteus*. Although the results from limited microcosm research should be extrapolated, they are essential in assessing the positive effects of adenosine on the growth of *S. luteus* in pure culture. More research should be undertaken to determine the mechanisms of these positive effects in the future.

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