Effect of nitrogen resources and pH on growth and fruit body formation of *Coprinopsis phlyctidospora*

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Following 3 weeks of cultivation of *Coprinopsis phlyctidospora* the final pH of NO_3-N, asparagine-N (Asp-N) and Urea-N media increased, the final pH of NH_4-N media decreased, and high nitrogen concentrations induced a high final pH. The changes in reduction oxidation potential were opposite to that of pH. Most mycelial biomass was generated in Asp-N media, while least biomass was generated in NH_4-N media; high nitrogen levels promoted increased growth. During growth, NO_3-N was produced by the utilization of Asp-N, Urea-N and NH_4-N. The maximum yield of NO_3-N was found in the NH_4-N media. Under light, fruit bodies were formed in Urea-N, Asp-N and NO_3-N media. In darkness, fruit bodies were formed in Urea-N media only. Addition of urea and NH_4Cl to unsupplemented growth medium promoted the formation of fruit bodies.

Key words: *Coprinopsis phlyctidospora*, nitrogen, pH.

Introduction

Ammonia, urea, L-asparagine and nitrate can be utilized as sole nitrogen sources by many fungi (Morton and MacMillan, 1954; Pateman and Cove, 1967; Lewis and Fincham, 1970; Arima *et al*., 1972). The failure of some fungi to use ammonia is due to the toxicity of ammonia in its alkaline state or a change of pH in solution (Morton and MacMillan, 1954). The form of the supplied nitrogen and the pH of the media are therefore important criteria for fungal growth.

Both vegetative and reproductive growth of *Coprinopsis* spp. can be induced by ammonia or ammonium salts under alkaline conditions (Morimoto *et al*., 1981). The biochemical induction of fruiting of basidiomycetes had been widely studied (Eger, 1968; Rusmin and Leonard, 1978). Uno and Ishikawa (1973) reported that 3', 5'-cyclic AMP and a protein bound with 3', 5'-cyclic

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AMP were active compounds capable of inducing fruit body formation in *Coprinus macrorhizus* (*Coprinopsis cinerea*).

Light is required for both initiation and development of fruit bodies of *Coprinus cinereus* (*Coprinopsis cinerea*) on semi-synthetic agar medium (Morimoto and Oda, 1973). Morimoto *et al*. (1982) also reported that alkaline and some nitrogenous substances promoted fruiting in darkness. These results indicate that the induction of fruit body formation is affected by growth medium composition. In this experiment, urea, NH_4Cl, KNO_3 and L-asparagine were used as nitrogen sources for the cultivation of *Coprinopsis phlyctidospora*, in order to study the effects of nitrogen and pH on growth and reproduction of this fungus.

Materials and methods

Coprinopsis phlyctidospora [NBRC 030478 (IFO 030478)] was used as the study organism in this experiment. Growth medium comprised: KH_2PO_4, 0.3 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.3 $g \cdot L^{-1}$; $CaCl_2 \cdot 2H_2O$, 0.1 $g \cdot L^{-1}$; $ZnSO_4 \cdot 7H_2O$, 0.30 $mg \cdot L^{-1}$; $FeSO_4 \cdot 7H_2O$, 0.15 $mg \cdot L^{-1}$; $MnSO_4 \cdot 5H_2O$, 0.10 $mg \cdot L^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.10 $mg \cdot L^{-1}$; $(NH_4)_6Mo_7 \cdot H_2O$, 0.02 $mg \cdot L^{-1}$; thiamine hydrochloride, 0.50 $mg \cdot L^{-1}$; nicotinamide, 0.10 $mg \cdot L^{-1}$ and glucose, 20 $g \cdot L^{-1}$. Urea, L-asparagine, NH_4Cl, and KNO_3 were used as nitrogen resources. Nitrogen concentrations of 100 ppm, 300 ppm, 500 ppm and 800 ppm were used. The pH of each concentration was 5, 6, 7 and 8, respectively. These media were named as Urea-N media, NO_3-N media, Asp-N media, and NH_4-N media. Four solid media were made by adding 15 $g \cdot L^{-1}$ agar to 500 ppm nitrogen solution of Urea-N media, NO_3-N media, Asp-N media, and NH_4-N media, respectively. The initial pH was 6.5. A further solid medium (N-free media) was made of basic ingredients without a nitrogen source with an initial pH of 8.

MY medium [malt extract (Difco) 20 g, yeast extract (Difco) 5 g and pure water 1000 ml] were also used, set up with initial pH values of 5, 6, 7 and 8, respectively. Agar 15 $g \cdot L^{-1}$ was added to MY medium to make MYA media. Liquid media (80 ml) were placed in 200 ml-Erlenmeyer flasks, while solid media were placed in Petri dishes (20 ml/dish).

Urea, L-asparagine, NH_4Cl, and KNO_3 were sterilized by filtering through cellulose acetate with a 0.45 μm pore size. Media pH was adjusted by adding 1 N NaOH and 1 N HCl.

The mycelia of *Coprinopsis phlyctidospora* were pre-cultured in MYA media in darkness for 7 days. Fresh discs of mycelia (4 mm diam.) were cut. One disc was placed at the centre of a Petri dish (90 mm diam.). Five discs were added to each 200 ml-Erlenmeyer flask. Treatments were repeated in triplicate with incubation at 25 ± 0.5°C in darkness. A further three replicates

were cultivated at 25 ± 0.5°C under light. Radial growth of mycelia in Petri dishes was measured (the mycelial diam. of Urea-N medium included the infringe of the Petri dish), while dry weight biomass was used to express biomass production in liquid culture. The numbers of fruit bodies were counted, the media pH, Eh (reduction oxidation potential), and NO_3-N content of liquid media were measured by pH electrode (Horiba, 6367-10D), Eh electrode (Horiba, 6861-10C) and nitrate electrode (Orion, 93-07), respectively.

Following 7 days of cultivation in MY media in darkness, 100, 300, 500 and 800 ppm of Urea-N, NO_3-N, asparagine-N, and NH_4-N was added to the flasks, respectively. The number of fruit bodies was measured after a further 10 days of cultivation in darkness.

Results

Effect of nitrogen on pH, NO_3-N, reduction oxidation potential and biomass production

Following three weeks of cultivation, the final pH of NO_3-N media was highest, ranging from 7.7 to 8.8. The final pH of NH_4-N media decreased to 3.7-4.1 (Table 1). When the nitrogen concentration was 100 ppm, the final pH of Asp-N media and Urea-N media was lower than the initial pH. A high initial pH resulted in a high final pH value. When the nitrogen concentration was over 100 ppm, the final pH of Asp-N media and Urea-N was over 8 (Table 1). Among the 4 nitrogen concentrations tested, high concentration resulted in high final media pH, i.e. the final pH of solution was in the following order: 800 ppm > 500 ppm > 300 ppm > 100 ppm (Table 1).

The mycelia production of *Coprinopsis phlyctidospora* growing in NH_4-N media was too low to harvest. In the other three media, mycelia production in Asp-N media was the greatest. Mycelia production in NO_3-N media was similar even under different pH and different nitrogen concentrations (Table 1). This indicates that a high concentration of nitrogen promotes mycelial growth. When the nitrogen concentration was 100-500 ppm, high pH promoted higher biomass production, while a nitrogen level of 800 ppm impeded biomass production under high pH conditions.

Mycelia produced NO_3-N by utilizing NH_4-N, Asp-N and Urea-N. The highest concentration of NO_3-N was produced in NH_4-N media, while the lowest was produced in Asp-N media. High concentrations of nitrogen resulted in high concentrations of NO_3-N (Table 1). The reduction oxidation potential of NH_4-N media was the greatest, followed by Urea-N media and the reduction oxidation potential of NO_3-N media was the lowest. The changes in reduction

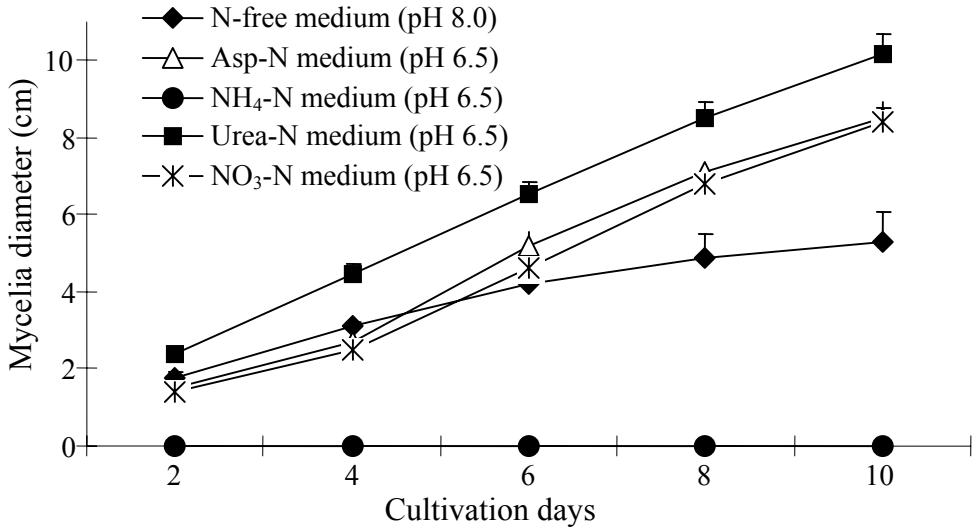

Fig. 1. Growth rates of *Coprinopsis phlyctidospora* in five nitrogen media (values are the means with standard errors of three replicates).

oxidation potential were opposite to that of pH. Liquid media with a high nitrogen concentration had a low reduction oxidation potential. Media with a low pH had a high reduction oxidation potential (Table 1). The mycelia grew fastest on Urea-N media at 1.2 cm diam. per day. Mycelia in the N-free medium (pH 8.0) grew the second fastest during the first 4 days (0.8 cm day^{-1}), but 4 days later the mycelia showed almost no growth. The mycelia showed no growth in NH_4-N medium (Fig. 1).

Formation of fruit bodies

In darkness, the mycelia formed fruit bodies only in Urea-N media (Table 1). Under light, the mycelia formed fruit bodies in Urea-N media, NO_3-N media and Asp-N media, with most fruit bodies being formed in Urea-N media (Table 2). Large numbers of fruit bodies was produced in the high pH/high nitrogen growth medium. After 7 days of cultivation in MY media in darkness, the addition of urea promoted the formation of fruit bodies. Fruit bodies also developed upon the addition of NH_4Cl, but only at a high pH. The addition of L-asparagine and KNO_3 had no effect on fruit body formation (Table 3).

Discussion

Effect of nitrogen on pH, NO_3-N, reduction oxidation potential and biomass production

Coprinopsis phlyctidospora generated the highest biomass in Asp-N media (Table 1). The availability of readily utilized organic nitrogen (L-asparagine) is considered to responsible. Keller (1996) also reported that fungi including some mycorrhizal fungi grew well on asparagine, arginine, glutamine and protein. Mycelia production was found to be lowest in inorganic nitrogen NH_4-N media, and its pH decreased to about 3-4. Utilization of ammonia salts [NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$] has been reported to induce a rapid drop in pH, consequently mycelia growth was inhibited (Morton and MacMillan, 1954; Jongbloed *et al*., 1990; Yamanaka, 1999). Besides drastic changes in pH, the accumulation of secondary metabolites was also considered to be responsible for the inhibition of fungal growth (Cooke and Whipps, 1993).

Darlington and Scazzocchio (1967) reported that if one nitrogen source was utilized by a fungus for a long time, the pH of the media would rise. In this experiment, the pH of NO_3-N, Urea-N and Asp-N media increased following three weeks of cultivation. This association between pH and nitrogen metabolism has also been reported by Sprent (1987).

In this experiment, *Coprinopsis phlyctidospora* not only grew in NO_3-N, but also produced NO_3-N via NH_4^+, urea, and L-asparagine (Table 1). Fries (1955) showed that *Coprinus ephemerus* (*Coprinellus ephemereus*), *Coprinus fimetarius* (*Coprinopsis cinerea* or *Coprinopsis radiata*), *Coprinus micaceus* (*Coprinellus micaceus*), and *Coprinus narcoticus* (*Coprinopsis narcotica*) could use nitrate as a sole N source. Since nitrate can be produced by nitrification easily when environmental conditions, e.g. pH, temperature and aeration are optimum. Many fruit bodies of *Coprinopsis phlyctidospora* were formed in forest soils, following urea treatment and the soil had a high concentration of NO_3-N (Suzuki et al., 2002). In this experiment, high NO_3-N production occurred in NH_4-N media, suggesting that the fungus is oxidizing NH_4-N to NO_3-N.

Growth rate of mycelia in N-free media (pH = 8) was great at first, although the growth stopped after four days (Fig. 1) because of nutrition limitation. This indicates that alkaline conditions promote the growth of the mycelia of *Coprinopsis phlyctidospora*.

Fruit body formation

In darkness, fruit bodies were only formed in Urea-N media, while *Coprinopsis phlyctidospora* formed fruit bodies in Urea-N, Asp-N and NO_3-N

Table 1. Biomass production and culture fluid properties of *Coprinopsis phlyctidospora*.

Media	Nitrogen (ppm)	Initial pH	Final pH	Fruit body number	NO_3-N content (μg)	Final Eh (mV)	Mycelia weight (dry/mg)
Asp-N	100	5	4.9 ± 0.1	0	3.8 ± 0.3	153.5 ± 14.0	112.4 ± 10.4
		6	5.3 ± 0.1	0	2.0 ± 0.2	189.0 ± 14.0	154.1 ± 16.4
		7	6.6 ± 0.2	0	1.4 ± 0.1	226.8 ± 21.0	189.3 ± 17.1
		8	6.8 ± 0.2	0	4.0 ± 0.3	213.6 ± 10.1	199.7 ± 18.2
	300	5	8.3 ± 0.2	0	7.8 ± 0.1	108.8 ± 7.3	241.5 ± 17.2
		6	8.2 ± 0.2	0	5.6 ± 0.5	137.3 ± 13.7	253.2 ± 24.1
		7	8.2 ± 0.2	0	6.3 ± 0.4	138.9 ± 9.4	278.9 ± 18.7
		8	8.2 ± 0.2	0	2.3 ± 0.2	151.5 ± 10.1	271.0 ± 17.2
	500	5	8.4 ± 0.2	0	38.3 ± 1.5	106.3 ± 7.6	298.2 ± 19.8
		6	8.3 ± 0.2	0	29.4 ± 1.8	126.0 ± 14.0	301.5 ± 24.4
		7	8.2 ± 0.2	0	19.8 ± 0.6	130.8 ± 7.3	290.4 ± 15.9
		8	8.5 ± 0.2	0	17.6 ± 0.9	141.8 ± 15.8	279.5 ± 29.6
	800	5	8.5 ± 0.2	0	47.6 ± 1.3	98.2 ± 5.8	317.2 ± 18.4
		6	8.3 ± 0.2	0	42.2 ± 2.0	107.4 ± 10.7	321.4 ± 13.8
		7	8.3 ± 0.2	0	26.5 ± 1.0	127.2 ± 7.5	310.2 ± 24.1
		8	8.7 ± 0.2	0	25.4 ± 0.9	137.2 ± 8.1	312.8 ± 18.4
NO_3-N	100	5	7.7 ± 0.2	0	19.7 ± 0.7	166.2 ± 15.1	191.4 ± 17.3
		6	7.8 ± 0.2	0	17.3 ± 0.8	150.1 ± 16.7	220.5 ± 13.5
		7	8.1 ± 0.2	0	15.3 ± 0.7	134.9 ± 12.3	214.2 ± 19.4
		8	8.2 ± 0.2	0	30.7 ± 1.0	162.6 ± 14.8	210.5 ± 11.4
	300	5	8.5 ± 0.2	0	62.8 ± 1.9	135.5 ± 9.0	199.6 ± 13.1
		6	8.7 ± 0.2	0	162.1 ± 3.7	119.7 ± 12.0	225.6 ± 22.1
		7	8.4 ± 0.2	0	181.8 ± 2.1	113.0 ± 7.5	218.6 ± 14.5
		8	8.6 ± 0.2	0	48.4 ± 1.3	110.6 ± 7.4	240.1 ± 15.8
	500	5	8.6 ± 0.2	0	372.2 ± 6.3	132.8 ± 9.5	212.7 ± 15.2
		6	8.6 ± 0.2	0	255.8 ± 7.2	124.5 ± 13.8	230.4 ± 17.5
		7	8.6 ± 0.2	0	298.3 ± 4.3	111.9 ± 6.2	224.9 ± 12.4
		8	8.8 ± 0.2	0	274.3 ± 8.2	104.0 ± 11.6	243.9 ± 14.8
	800	5	8.7 ± 0.2	0	670.1 ± 14.2	129.4 ± 7.6	221.8 ± 12.9
		6	8.6 ± 0.2	0	342.2 ± 10.0	123.2 ± 12.3	236.1 ± 15.9
		7	8.6 ± 0.2	0	530.7 ± 9.1	96.9 ± 5.7	249.3 ± 15.7
		8	8.8 ± 0.2	0	335.5 ± 5.7	87.4 ± 5.1	251.3 ± 11.2
Urea-N	100	5	3.9 ± 0.1	5 ± 0.8	1.7 ± 0.2	352.6 ± 32.1	120.5 ± 7.1
		6	5.8 ± 0.1	4 ± 0.7	3.2 ± 0.1	275.3 ± 30.6	150.8 ± 8.4
		7	6.3 ± 0.2	11 ± 2.1	3.7 ± 0.1	288.3 ± 26.2	179.6 ± 10.1
		8	6.5 ± 0.2	17 ± 1.7	2.4 ± 0.0	289.2 ± 26.3	183.7 ± 10.9
	300	5	8.5 ± 0.2	8 ± 1.2	10.6 ± 0.0	83.2 ± 5.6	160.2 ± 10.4
		6	8.3 ± 0.2	9 ± 0.4	12.1 ± 0.1	134.5 ± 13.5	200.1 ± 16.9
		7	8.3 ± 0.2	16 ± 1.1	11.7 ± 0.0	127.6 ± 8.5	210.3 ± 13.5
		8	8.2 ± 0.2	15 ± 0.9	10.2 ± 0.1	152.5 ± 10.2	248.9 ± 16.4

Note: All cultivation carried out at 25°C in darkness.
Values are the means with standard errors of three replicates.

Table 1. (continued).

Media	Nitrogen (ppm)	Initial pH	Final pH	Fruit body number	NO_3-N content (µg)	Final Eh (mV)	Mycelia weight (dry/mg)
	500	5	8.6 ± 0.2	12 ± 0.7	13.1 ± 0.1	79.6 ± 5.7	170.1 ± 12.5
		6	8.5 ± 0.2	13 ± 1.1	16.7 ± 0.5	112.5 ± 12.5	209.8 ± 13.4
		7	8.5 ± 0.2	16 ± 1.9	23.3 ± 0.4	115.6 ± 6.4	220.4 ± 12.1
		8	8.5 ± 0.2	22 ± 2.4	19.7 ± 0.5	135.7 ± 15.1	255.7 ± 15.4
	800	5	8.6 ± 0.2	7 ± 0.6	12.6 ± 0.0	71.9 ± 4.2	171.4 ± 9.1
		6	8.6 ± 0.2	11 ± 1.2	18.0 ± 0.5	85.4 ± 8.5	279.6 ± 18.7
		7	8.6 ± 0.2	16 ± 2.1	41.3 ± 0.6	45.0 ± 2.7	291.1 ± 17.1
		8	8.7 ± 0.2	19 ± 1.4	40.0 ± 0.7	86.2 ± 5.1	248.6 ± 14.7
NH_4-N	100	5	3.8 ± 0.1	0	31.6 ± 0.2	288.9 ± 26.3	0
		6	3.7 ± 0.1	0	30.3 ± 1.0	294.2 ± 32.7	0
		7	3.8 ± 0.1	0	27.5 ± 0.5	262.0 ± 23.8	0
		8	3.9 ± 0.1	0	31.0 ± 1.2	278.7 ± 25.3	0
	300	5	3.8 ± 0.1	0	34.8 ± 0.2	242.1 ± 16.0	0
		6	3.9 ± 0.1	0	30.9 ± 1.4	240.6 ± 24.1	0
		7	3.8 ± 0.1	0	36.3 ± 1.0	259.8 ± 17.3	0
		8	3.9 ± 0.1	0	31.4 ± 0.9	275.4 ± 18.4	0
	500	5	3.9 ± 0.1	0	55.9 ± 1.6	239.1 ± 17.1	0
		6	4.0 ± 0.1	0	47.8 ± 2.1	244.4 ± 27.2	0
		7	3.9 ± 0.1	0	53.3 ± 0.7	251.2 ± 14.0	0
		8	4.0 ± 0.1	0	42.8 ± 2.4	266.0 ± 29.6	0
	800	5	3.9 ± 0.1	0	68.2 ± 1.0	241.5 ± 14.3	0
		6	4.0 ± 0.1	0	75.9 ± 3.3	232.5 ± 23.3	0
		7	4.0 ± 0.1	0	67.7 ± 2.2	224.3 ± 13.2	0
		8	4.1 ± 0.1	0	62.0 ± 1.9	225.6 ± 13.3	0

media in light (Tables 1, 2). This indicates that light plays an important role for the normal fruiting of this fungus. The maximum number of fruit bodies was formed in Urea-N media (Table 2), which indicates that urea is important in inducing formation of fruit bodies. Morimoto *et al*. (1982) reported that urea promoted fruit body formation in *Coprinus stercorarius* (*Coprinopsis stercorea*). Rogers (1973) reported that on cornmeal agar containing malt and yeast extracts, and a hot water horse dung extract, *Coprinopsis stercorarius* formed fruit bodies both in light and in darkness. Only the fruiting bodies which developed in light, however, were fertile. MacDougal (1903) described similar effects of light in the same species, suggesting that urea and light is important in the formation of fruit bodies, with light being more important.

The addition of urea and NH_4-N promoted the formation of fruit bodies, while Asp-N and NO_3-N showed no effect (Table 3). The reduction oxidation potential decrease of fungi grown in NH_4-N and Urea-N media was great

Table 2. Fruit bodies production* by *Coprinopsis phlyctidospora* during growth in light.

Media**	Nitrogen (ppm)	Initial pH 5	6	7	8
NH_4-N	100	0	0	0	0
	300	0	0	0	0
	500	0	0	0	0
	800	0	0	0	0
NO_3-N	100	12 ± 2.1	17 ± 4.1	21 ± 1.9	27 ± 2.4
	300	18 ± 1.7	19 ± 1.9	27 ± 3.2	35 ± 2.9
	500	25 ± 3.1	25 ± 2.1	33 ± 1.9	39 ± 1.8
	800	23 ± 4.1	26 ± 3.1	29 ± 2.1	34 ± 2.9
Urea-N	100	14 ± 1.2	16 ± 0.8	28 ± 2.5	34 ± 1.7
	300	19 ± 1.7	22 ± 2.7	34 ± 3.1	41 ± 3.4
	500	23 ± 2.4	27 ± 1.4	36 ± 2.3	49 ± 4.4
	800	24 ± 3.1	25 ± 5.1	38 ± 3.4	39 ± 3.7
Asp-N	100	10 ± 3.2	15 ± 1.8	21 ± 3.5	24 ± 2.2
	300	16 ± 2.5	19 ± 2.7	26 ± 3.1	32 ± 2.4
	500	19 ± 2.4	25 ± 1.4	36 ± 2.3	35 ± 2.4
	800	21 ± 3.1	26 ± 5.1	27 ± 3.4	34 ± 3.7

*Number of fruit bodies; **nitrogen added to basal growth medium.
Values are the means with standard errors of three replicates.

Table 3. Fruit bodies production* by *Coprinopsis phlyctidospora* during growth in darkness.

Nitrogen**	Nitrogen (ppm)	Initial pH 5	6	7	8
NH_4Cl	100	0	0	0	4 ± 0.7
	300	0	0	3 ± 0.4	8 ± 1.5
	500	0	0	4 ± 0.7	9 ± 0.8
	800	0	0	8 ± 2.1	11 ± 2.5
KNO_3	100	0	0	0	0
	300	0	0	0	0
	500	0	0	0	0
	800	0	0	0	0
Urea	100	7 ± 1.2	10 ± 0.8	16 ± 2.5	24 ± 1.7
	300	11 ± 1.7	15 ± 2.7	24 ± 3.1	29 ± 3.4
	500	13 ± 2.4	15 ± 1.4	25 ± 2.3	33 ± 2.4
	800	14 ± 3.1	17 ± 5.1	27 ± 3.4	30 ± 3.7
Asparagine	100	0	0	0	0
	300	0	0	0	0
	500	0	0	0	0
	800	0	0	0	0

*Number of fruit bodies; **nitrogen added to MY growth medium.
Values are the means with standard errors of three replicates.

(Table 1). This indicates that reduced substances from Urea-N and NH_4-N media may play a role in inducing fruit body formation. Morimoto *et al*. (1981) and Morimoto and Oda (1973) reported that free NH_4^+ was necessary for the induction of fruit body formation, and in the field, urea, aqueous ammonia, and ammonia salts were reported to affect the succession of ammonia fungi (Sagara, 1975).

Mycelia production of *Coprinopsis phlyctidospora* was low in Urea-N and poor in NH_4-N media, but the addition of urea and NH_4Cl (pH 7-8) promoted fruit body formation (Tables 1, 3). Alkaline substances and urea may have a stimulating effect on fruit body formation in some fungi.

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