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Investigation of microfungi from urea plots at pine forest in Bidoup – Nui Ba National Park, Lam Dong Province, Vietnam

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Abstract The 24 strains of anamorphic fungi were isolated from plots which applied urea 11 months ago, in the forest of *Pinus dalatensis* at Bidoup - Nui Ba National Park, Lam Dong Province, Vietnam. Based on morphology, isolated strains were identified as *Aspergillus*, *Penicillium*, *Trichoderma* and *Acremonium*. All of them had ability of cellulose decomposition when cultured on Czapek-Dox agar added carboxymethyl cellulose (CMC). Some strains showed a high cellulose decomposition. The effect of nitrate-nitrogen concentration on their mycelia growth were also demonstrated. Most of the highest dry biomass of mycelia were obtained when cultivated with 13g/L nitrate-nitrogen concentration. Some strains tolerated to nitrate-nitrogen concentration of 13g/L in medium.

Keywords: Ammonia fungi, mold, anamorphic fungi, cellulose, nitrate, *Pinus*

Introduction

Ammonia fungi (Sagara, 1975) are a fungal group that forms communities sequentially at the restricted sites of animal materials (e.g., decomposing carcasses and animal waste) or artificial disturbance of nitrogen compounds. The community of ammonia fungi is related to the host of vegetation, and considered a basic component of the normal fungal community in forest and grassland ecosystems. Till now, about 60 species, belong to Anamorphic fungi – Ascomycota – Basidiomycota, have been recorded as ammonia fungi (Suzuki, 2009a), but the species composition is belonged to each site.

Researches about ammonia fungi are fragmentary in global scale, especially sparse in tropical areas as Southeast Asia. The survey of ammonia

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fungi by the urea application in the field and/or laboratory started from Japan (Sagara and Hamada, 1965) and sequential extended to New Zealand, Europe, North America, Taiwan, Australia, Vietnam, China and Thailand. Till now, most of studies about ammonia were conduct in five countries: Japan (Sagara, 1975, 1992, 1995; Fukiharu and Hongo, 1995; Yamanaka, 1995a, b; Fukiharu and Horigome, 1996; Fukiharu *et al.*, 2000a, b; Sagara *et al.*, 2000; Imamura, 2001; Suzuki *et al.*, 2002), Taiwan (Fukiharu *et al.*, 1997), New Zealand (Suzuki *et al.*, 2003), Australia (Suzuki *et al.*, 2002) and Canada (Raut, 2011). Urea application were also conducted in few treated sites at Europe (Suzuki *et al.*, 2003), Vietnam (Ho, 2013), China (Fukiharu *et al.*, 2011) and Thailand (unpub. data).

In Vietnam, ammonia fungal study was firstly conducted by urea application in pure forest of *Pinus kesiya*, DaLat, Lam Dong Province from 2010 (Ho, 2013). As the results of this research, the record of *Hebeloma vinosophyllum* was new for Southeast Asia. Nguyen (2012) continued studied ecological succession of ammonia fungi in forests of *Quercus* spp. and *Pinus dalatensis* at Bidoup - Nui Ba National Park, Lam Dong Province, Vietnam with the record of 7 species (*Amblyosporium botrytis* Fres, *Ascobolus denudatus* Fries, *Lyophyllum tylicolor* (Fr.Fr.) M. Lange et Sivertsen , Coprinopsis spp., *Alnicola lactariolens* Clémonçon et Hongo, *Hebeloma* sp., *Laccaria* sp.).

Nonetheless, there is a few studies about ammonia anamorphic fungi communities (Sagara, 1975; Suzuki, 2009b), especially in Southeast Asia. Thus, this research was conducted to investigate the composition anamorphic fungi from plots treated urea after 11 months in forest of *Pinus dalatensis* at Bidoup - Nui Ba National Park, Lam Dong Province, Vietnam.

Materials and methods

Sample collection

10g mixture of soil and litter (3cm-depth) were collected from each of 5 urea plots in forest of *Pinus dalatensis* at Bidoup - Nui Ba National Park, Lam Dong Province, Vietnam. The urea was applied on the forest floor 11 month ago with different concentration of 100g/m^2 (urea plot T1), 200g/m^2 (urea plot T2), 400g/m^2 (urea plot T4), 800g/m^2 (urea plot T8) and 1500g/m^2 (urea plot T15).

Samples were dried at room temperature, blended and sieved with pore size 2mm x 2mm.

Fungal isolation

5g dry sample was diluted up 10^{-5} and 0.1mL of each dilution was spread on Czapek Dox Agar ($NaNO_3$ 3.0g, KCl 0.5g, K_2HPO_4 1.0g, $MgSO_4$. $7H_2O$ 0.5g, $FeSO_4$. $7H_2O$ 0.01g, Saccharose 20g, Agar 15g) and Potato Dextrose Agar (Peeled potato 200g, Dextrose 20g, Agar 15g) media. For suppression of bacteria growth, chloramphenicol (1%) was added to the medium. Two plates of each dilution were incubated for 24 – 96 hours at room temperature. Each morphologically unique fungal colony was sub-cultured on Czapek-Dox Agar and Potato Dextrose Agar media.

Identification of isolated strains

Isolated strains were identified to genus based on morphological characteristics and taxonomy guides (Clements *et al.*, 1964; Ridgway, 1912; Watanabe, 2002).

Cellulose decomposition of isolated strains

Isolated strains were sub-cultured on Czapek Dox Agar with carboxymethylcellulose (CMC) 1% as carbon source. After incubated for 3 – 5 days at room temperature, all plates were stained with Lugol solution (I_2 1g, KI 2g, H_2O 300mL) for observing the clear zone. The radius of decomposed zone was the difference of clear zone radius and colony radius.

Effect of nitrogen-nitrate on the mycelial growth of isolated strains

Isolated strains were pre-cultivated on Potato Dextrose agar medium in petri dishes. Inoculum disks of fungi were cut with a 4 mm-diameter cork border from sub-peripheral regions of actively growing colonies and placed into 20 mL of liquid Basal synthetic medium (Maltose 40g, KH_2PO_4 0.3g, $MgSO_4$. $7H_2O$ 0.3g, $CaCl_2$. $2H_2O$ 0.1g, $ZnSO_4$. $7H_2O$ 0.3mg, $CuSO_4$. $5H_2O$ 0.1mg, $MnSO_4$. $5H_2O$ 0.1mg, Na_2MoO_4. $2H_2O$ 0.02mg, $FeSO_4$. $7H_2O$ 0.15mg, Thiamine HCl 0.02mg, Niacine 0.1mg, Agar 15g) in a 100mL glass bottle, incubated at room temperature. KNO_3 was added to the medium as the nitrogen source with different concentrations (0g/L, 0.13g/L, 1.3g/L and 13g/L N-NO_3). Three bottles for each treatment were prepared. After 10 days, mycelia were collected and dried at 60^{o}C for 48 hours and put in a desiccator for 2 hours. The dry weight of mycelia was determined.

Statistical analysis

Data were analysed by ANOVA two factors without replication, using MS Excel 2013 software.

Results

Morphological identification

From 5 urea plots, 24 strains of anamorphic fungi were isolated. Their taxonomy results and morphological characteristics were shown in Table 1 and Figures 1 – 4.

Table 1. Taxonomy of 24 fungal strains from 5 urea plots

Urea plot No.	Urea amount (g/m^2)	Strain No.	Genus
T1	100	T1-2	***Trichoderma***
		T1-5	***Trichoderma***
		T1-10	***Penicillium***
		T1-12	***Trichoderma***
		T1-16	***Aspergillus***
		T1-19	***Trichoderma***
T2	200	T2-1	***Trichoderma***
		T2-5	***Trichoderma***
		T2-13	***Trichoderma***
		T2-17	***Trichoderma***
T4	400	T4-1	***Aspergillus***
		T4-6	***Acremonium***
		T4-10	***Trichoderma***
T8	800	T8-2	***Trichoderma***
		T8-3	***Acremonium***
		T8-5	***Penicillium***
		T8-6	***Aspergillus***
T15	1500	T15-1	***Aspergillus***
		T15-3	***Trichoderma***
		T15-5	***Trichoderma***
		T15-7	***Trichoderma***
		T15-8	***Trichoderma***
		T15-11	***Trichoderma***
		T15-14	***Aspergillus***

From urea plot T1 (100g urea/m^2), 1 *Aspergillus* strain, 1 *Penicillium* and 3 *Trichoderma* strains were isolated. From urea plot T2 (200g urea/m^2), all isolated strains belonged to *Trichoderma*. 3 isolated strains of urea plot T4 (200g urea/m^2) were identified as *Aspergillus*, *Acremonium* and *Trichoderma*. In urea plot T8 (800g urea/m^2), *Aspergillus*, *Acremonium*, *Penicillium* and

Trichoderma were also collected. Finally, 2 strains of *Aspergillus* and 5 strains of *Trichoderma* were isolated from urea plot T15 (1500g urea/m^2).

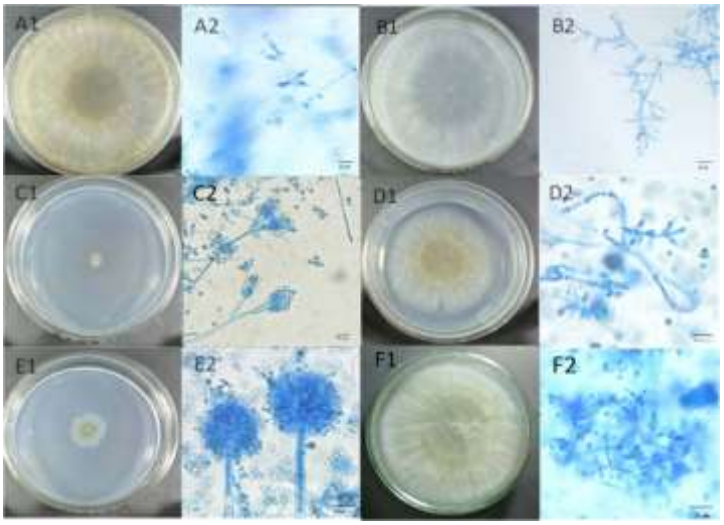

Figure 1. Colony and reproductive structure of anamorphic fungi from urea plot T1. A-F: T1-2, T1-5, T1-10, T1-12, T1-16, T1-19. T1-2, T1-5, T1-10, T1-12, T1-19 on Potato Dextrose Agar in 3 days. T1-10, T1-16 on Czapek Dox Agar in 7 days. Bar: 10 μm.

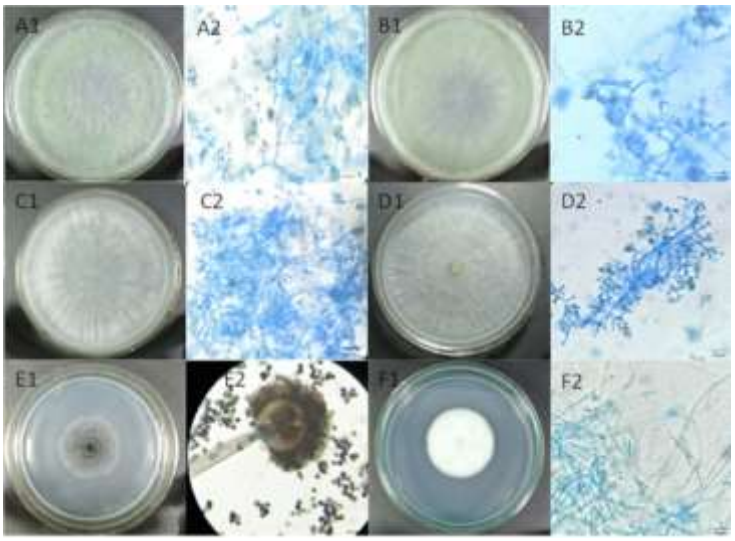

Figure 2. Colony and reproductive structure of anamorphic fungi from urea plots T2 and T4. A-F: T2-1, T2-5, T2-13, T2-17, T4-1, T4-6. T2-1, T2-5, T2-13, T2-17 on Potato Dextrose Agar in 3 days. T4-1, T4-6 on Czapek Dox Agar in 7 days and 5 days, respectively. Bar: 10 μm.

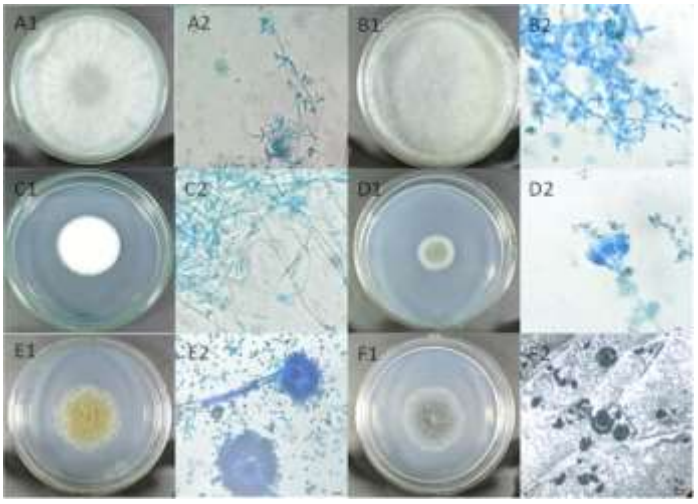

Figure 3. Colony and reproductive structure of anamorphic fungi from urea plots T4, T8 and T15. A-F: T4-10, T8-2, T8-3, T8-5, T8-6, T15-1. T4-10, T8-2 on Potato Dextrose Agar in 3 days. T8-3 on Czapek Dox Agar in 5 days. T8-5, T8-6, T15-1 on Czapek Dox Agar in 7 days. Bar: 10 µm.

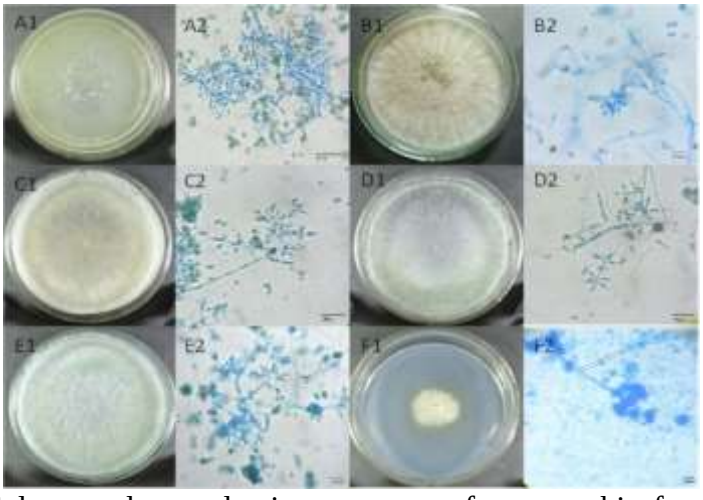

Figure 4. Colony and reproductive structure of anamorphic fungi from urea plot T15. A-F: T15-3, T15-5, T15-7, T15-8, T15-11, T15-14. T15-3, T15-5, T15-7, T15-8, T15-11 on Potato Dextrose Agar in 3 days. T15-14 on Czapek Dox Agar in 7 days. Bar: 10 µm.

The taxonomy result showed that *Aspergillus* spp., *Acremonium* spp., *Penicillium* spp. and *Trichoderma* spp. occurred in urea plots. *Trichoderma* spp. were occurred in all 5 urea plots while strains of *Aspergillus* were occurred in 4 urea plots, except for urea plot T8. *Penicillium* spp. were collected from 2 urea

plots T1 and T8. *Acremonium* spp. were collected from 2 urea plots T4 and T8. The absent of *Aspergillus* spp., *Acremonium* spp., *Penicillium* spp. in some urea plots might cause by the small amount of sample collection. In the future study, more amount of sample should be collected to increase the frequency of fungal isolation.

Cellulose decomposition of isolated strains

After 3 days (*Trichoderma*) or 5 days (*Aspergillus, Acremonium* and *Penicillium*) of cultivation, all 24 strains grew and formed colonies on Czapek Dox Agar added CMC 1%. Colonies of *Trichoderma* strains were larger than 20mm and some of them were larger than 50mm. Colonies of *Aspergillus* strains, *Acremonium* strains and 1 *Penicillium* strain were larger than 15mm. Only 1 *Penicillium* strain T1-10 grew slowly and formed 5.5mm colony. The data revealed that 24 isolated strains utilized cellulose as a carbon source (Table 2).

Table 2. Growth and cellulose decomposition of 24 fungal strains from 5 urea plots

Strain No.	Genus	Colony radius (mm)	Clear zone radius (mm)	Decompostion zone radius (mm)
T1-2	*Trichoderma*	51.5	0.0	**0.0**
T1-5	*Trichoderma*	22.0	0.0	**0.0**
T1-10	*Penicillium*	5.5	**7.5**	1.0
T1-12	*Trichoderma*	35.0	0.0	**0.0**
T1-16	*Aspergillus*	16.5	0.0	**0.0**
T1-19	*Trichoderma*	54.5	0.0	**0.0**
T2-1	*Trichoderma*	52.0	0.0	**0.0**
T2-5	*Trichoderma*	60.0	0.0	**0.0**
T2-13	*Trichoderma*	54.0	0.0	**0.0**
T2-17	*Trichoderma*	28.5	0.0	**0.0**
T4-1	*Aspergillus*	33.5	0.0	**0.0**
T4-6	*Acremonium*	18.0	0.0	**0.0**
T4-10	*Trichoderma*	37.0	0.0	**0.0**
T8-2	*Trichoderma*	46.0	0.0	**0.0**
T8-3	*Acremonium*	28.0	0.0	**0.0**
T8-5	*Pennicillium*	15.5	**28.5**	6.5
T8-6	*Aspergillus*	40.5	**49.5**	4.5
T15-1	*Aspergillus*	35.5	0.0	**0.0**
T15-3	*Trichoderma*	54.0	0.0	**0.0**
T15-5	*Trichoderma*	49.5	0.0	**0.0**
T15-7	*Trichoderma*	31.0	0.0	**0.0**
T15-8	*Trichoderma*	49.5	0.0	**0.0**
T15-11	*Trichoderma*	37.5	0.0	**0.0**
T15-14	***Aspergillus***	**18.5**	29.0	5.3

However, clear zones were observed around colonies of 2 strains *Penicillium* T1-10, T8-5 and 2 strains *Aspergillus* T8-6, T15-14 after the Lugol staining. It means that these strains have a high activity of cellulose decomposition. These results also showed that all isolated strains might have a role in decomposition of cellulose material in the forest.

Effect of nitrogen-nitrate on the mycelial growth of isolated strains

Most of isolated strains could grow in the medium with the high concentration of N-NO_3 (13g/L), except the strain *Penicillium* T1-10.
The strain *Aspergillus* T1-16 formed the highest dry weight of mycelia in 13g N-NO_3/L while other strains from urea plot T1 formed the highest dry weight of mycelia in 1.3g N-NO_3/L. Nonetheless, most strains isolated from urea plots T2, T4, T8 and T15 formed the highest dry weight of mycelia in 13g N-NO_3/L, except that *Acremonium* spp. T4-6 and T8-3 had the highest dry weight of mycelia in 1.3g N-NO_3/L.

Table 3. Dry weight (mg) of mycelia of 24 fungal strains cultured in basal synthetic media with different concentrations of N-NO_3 (g/L)

Strain No.	Genus	N0			N1			N2			N3		
		0			0.13			1.3			**13**		
T1-2	*Trichoderma*	14.4	±	1.0	82.4	±	11.2	94.6	±	37.7	93.2	±	**45.9**
T1-5	*Trichoderma*	14.8	±	1.4	147.2	±	16.0	211.1	±	12.1	35.7	±	**7.1**
T1-10	*Penicillium*	21.1	±	8.2	50.9	±	9.7	22.8	±	0.7	15.1	±	**2.3**
T1-12	*Trichoderma*	20.1	±	4.0	58.4	±	6.6	78.6	±	12.3	38.3	±	**24.4**
T1-16	*Aspergillus*	19.4	±	5.4	101.1	±	6.4	113.4	±	15.2	161.8	±	**34.1**
T1-19	*Trichoderma*	18.7	±	3.4	87.6	±	26.1	111.2	±	8.5	97.9	±	**50.4**
T2-1	*Trichoderma*	22.1	±	3.9	49.6	±	12.7	119.0	±	16.4	274.8	±	**24.7**
T2-5	*Trichoderma*	17.2	±	1.4	79.5	±	11.2	112.4	±	38.6	265.0	±	**27.7**
T2-13	*Trichoderma*	25.9	±	5.5	155.1	±	54.1	134.0	±	8.1	185.1	±	**40.7**
T2-17	*Trichoderma*	24.8	±	4.8	140.8	±	20.1	175.0	±	65.1	242.9	±	**4.0**
T4-1	*Aspergillus*	40.3	±	38.0	147.5	±	8.2	261.8	±	2.8	443.1	±	**11.9**
T4-6	*Acremonium*	31.6	±	5.9	164.7	±	14.3	176.7	±	2.5	113.4	±	**31.8**
T4-10	*Trichoderma*	21.2	±	2.4	102.3	±	4.8	155.6	±	3.1	392.3	±	**52.3**
T8-2	*Trichoderma*	23.2	±	3.5	85.7	±	19.0	123.9	±	4.9	149.6	±	**30.8**
T8-3	*Acremonium*	33.7	±	3.0	140.1	±	23.4	142.5	±	48.8	54.2	±	**4.6**
T8-5	*Pennicillium*	7.7	±	2.4	122.4	±	1.3	178.4	±	2.2	315.1	±	**4.7**
T8-6	*Aspergillus*	10.4	±	1.0	121.5	±	12.8	218.7	±	18.1	404.1	±	**37.9**
T15-1	*Aspergillus*	26.6	±	9.0	133.6	±	7.1	355.3	±	19.0	424.3	±	**16.9**
T15-3	*Trichoderma*	22.7	±	1.4	101.8	±	30.2	145.0	±	24.4	158.9	±	**45.0**
T15-5	*Trichoderma*	13.6	±	4.4	111.3	±	9.5	126.3	±	18.5	446.3	±	**27.0**
T15-7	*Trichoderma*	26.8	±	1.2	100.4	±	60.1	147.3	±	49.5	365.5	±	**54.8**
T15-8	*Trichoderma*	25.0	±	4.6	120.0	±	10.5	159.7	±	17.4	261.0	±	**14.3**
T15-11	*Trichoderma*	26.6	±	9.0	98.0	±	26.4	132.5	±	37.7	310.8	±	**20.4**
T15-14	***Aspergillus***	**8.3**	**±**	**2.0**	**218.9**	**±**	**70.9**	**191.9**	**±**	**5.5**	**318.3**	**±**	**18.8**

Mean ± Standard Deviation (n=3)

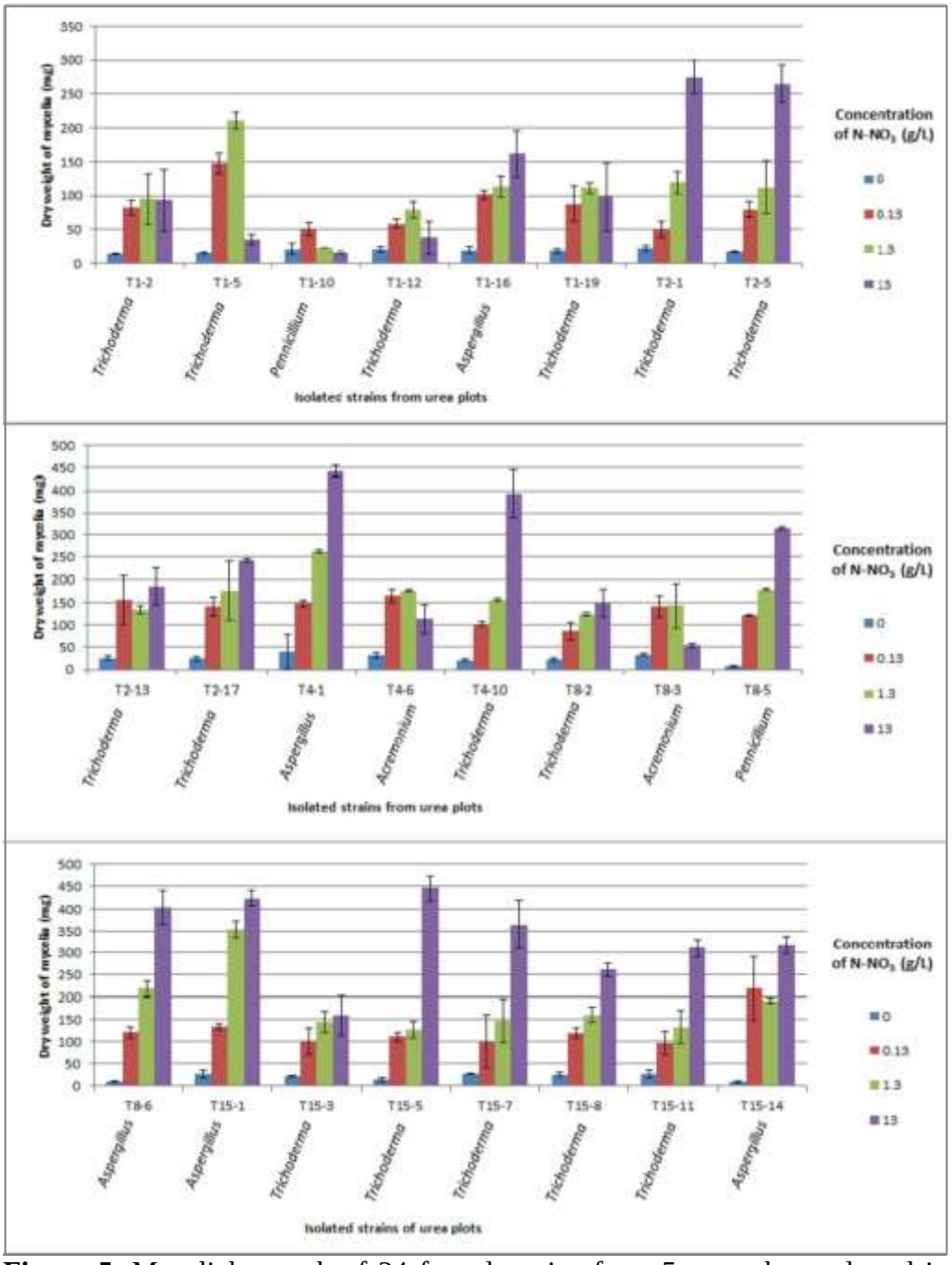

Figure 5. Mycelial growth of 24 fungal strains from 5 urea plots cultured in basal synthetic media with different concentrations of N-NO3 (g/L). Vertical bar indicate the Standard Deviation (n=3)

These above results that 24 isolated strains were able to utilize nitrate in the forest. Moreover, many strains such as *Trichoderma* spp. T2-1, T2-5, T4-10, T15-5, T15-7, T15-8, T15-11; *Aspergillus* spp. T4-1, T8-6, T15-1, T15-14; and *Penicillium* sp. T8-5 formed the biomass of mycelia over 250 mg of dry weight. These strains might survive in the environment of high nitrate concentration (Table 3 and Figure 5).

Discussion

Total 24 strains of anamorphic fungi were isolates from 5 urea plots which were applied urea 11 months ago in pine forest *Pinus dalatensis*. All strains have ability of cellulose decomposition and nitrate utilization.

Until recent, many researches on ammonia fungi have been conducted but no researches on soil micro-fungi. The research results should provide the first image of mycoflora of ammonia mould in soil.

According to Sagara (1975) and Suzuki (2009b), *Amblyosporium botrytis*, *Cladorrhinum foecundissimum* and *Doratomyces purpureofuscus* were records as ammonia anamorphic fungi. These species appeared on the field ca. 1 week to 3 months after urea application at forests in Japan. In our study, most of recorded species were belonged to *Trichoderma* and *Aspergillus*. The component of anamorphic fungi from urea plots were different, maybe because mixtures of soil and litter were collected 11 months after urea application at a forest in Vietnam. From the preliminary comparison between the anamorphic fungi collected from this study and those from other researches, it is necessary to do further researches the successive occurrence and species component of ammonia anamorphic fungi in forests of Vietnam from the start of urea application to 2 years after.

This study also confirmed the adaptation in high ammonia concentration of several *Trichoderma* and *Aspergillus* species which were isolated from urea plots. Following study of Babla (2012), *Trichoderma viride* strains shown the significant positive activities of extracellular urease. The taxonomy of *Trichoderma* and *Aspergillus* strains in this study should be confirmed in next studies for expanding the list of ammonia fungi.

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