The Korean Journal of Mycology www.kjmycology.or.kr

RESEARCH ARTICLE

In Vitro Mycorrhization and Morphological Characterization of *Xanthoconium affine* with *Pinus densiflora*

Eun-Jin Wang, Yeongseon Jang, Kang-Hyeon Ka*

Division of Wood Chemistry and Microbiology, National Institute of Forest Science, Seoul 02455, Korea

*Corresponding author: kasymbio@korea.kr

Abstract

In this study, we investigated the culture conditions of four ectomycorrhizal fungi, namely, *Amanita spissacea* NIFoS 2719, *Pisolithus arhizus* NIFoS 2784, *Suillus spraguei* NIFoS 2848, and *Xanthoconium affine* NIFoS 2716, in solid and liquid culture media. In addition, we attempted to induce in vitro mycorrhization of the fungi with *Pinus densiflora*. Prior to liquid culture, we determined the optimal culture conditions for each species in solid media. The results revealed that all species examined are capable of growth in potato dextrose agar (PDA), malt extract agar (MEA), and modified Melin-Norkran's medium (MMN), although their preferred growth media were different. Liquid culture experiments showed that inorganic nitrogen did not enhance the mycelial growth of all four species. Therefore, we used MMN-based liquid inocula to promote the growth of ectomycorrhizal fungi in our symbiosis culture system. Mycorrhization was observed in *Xanthoconium affine* NIFoS 2716. Morphological analysis revealed that fungi-inoculated roots of *P. densiflora* form simple and dichotomous lateral roots with dense mycelia. In addition, inoculation with *X. affine* NIFoS 2716 promoted root and shoot developments.

Keywords: Morphological characterization, Mycorrhization, *Pinus densiflora*, *Xanthoconium affine*

Kor. J. Mycol. 2017 December, 45(4): 319-327
https://doi.org/10.4489/KJM.20170038

pISSN : 0253-651X
eISSN : 2383-5249

Received: 10 August, 2017
Revised: 23 October, 2017
Accepted: 31 October, 2017

Introduction

Ectomycorrhizal fungi are widespread in forest ecosystems. Major genera include *Amanita*, *Boletus*, *Pisolithus*, *Russula*, *Suillus*, and *Xanthoconium*. These ectomycorrhizal fungi have a symbiotic relationship with tree roots and thus contain root metabolites, such as enzymes, hormones, and bioflavonoids [1]. Kikuchi et al. [2] demonstrated that the flavonoids secreted by *Pinus densiflora* play roles in basidiospore germination of *Suillus bovinus*. Ectomycorrhizal fungi cover the root surface and penetrate through epidermal cells into the cortex. During ectomycorrhizal development, the inner tissue structures of ectomycorrhizal fungi, called hartig nets, are formed and exchange major nutrients in cortical cell zone [3] to

improve plant growth and development in ecosystems [4, 5].

In this study, we attempted to induce in vitro mycorrhization of four species ectomycorrhizal fungi, namely, *Amanita spissacea* NIFoS 2719, *Pisolithus arhizus* NIFoS 2784, *Suillus spraguei* NIFoS 2848, and *Xanthoconium affine* NIFoS 2716 in *P. densiflora*. Optimal culture media compositions for fungal growth were determined using solid and liquid media. These four species produce epigeous fruit bodies in the wild and are widespread in coniferous or mixed forests. However, artificial mycorrhizal symbiosis of these four species with *P. densiflora* has not been explored.

Materials and Methods

Fungal and plant materials

All fungal species used in this study were obtained from the National Institute of Forest Science (NIFoS). *A. spissacea* NIFoS 2719, *P. arhizus* NIFoS 2784, *S. spraguei* NIFoS 2848, and *X. affine* NIFoS 2716 were isolated from fruiting bodies in 2014 (Table 1). Samples were grown on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 25°C for 60 days and used as inocula. *P. densiflora* seeds were collected from trees in the National Institute of Forest Science (NIFoS) and stored at 4°C. Seed surfaces were sterilized in 30% H_2O_2 for 30 min. Seeds were germinated on PDA media at 25°C for one week.

Table 1. Information on four fungal species used in this study

Species	NIFoS species no.	Accession no.	Location	Origin of species	Mycorrhization[a] (%)
Amanita spissacea	2719	MF405926	Yesan, Chungnam	Basidiocarp tissue	0
Pisolithus arhizus	2784	MF405927	Taean, Chungnam	Basidiocarp tissue	0
Suillus spraguei	2848	MF405928	Gwanak, Seoul	Basidiocarp tissue	0
Xanthoconium affine	2716	MF405929	Yesan, Chungnam	Basidiocarp tissue	40

NIFoS, National Institute of Forest Science.
[a]Mycorrhization was expressed as the rate of fungal colonization of total inoculated groups in percentage (n = 5).

Mycelial growth on media

Mycelial growth on solid media. All species were inoculated on 85 mm petri dishes. For culture on solid media, we examined four culture media, namely, PDA, malt extract agar (MEA; Difco, Detroit, MI, USA), Sabouraud dextrose agar (SDA; Difco, Detroit, MI, USA), and modified Melin-Norkran's medium [MMN; 10 g of glucose, 0.5 g of KH_2PO_4, 0.15 g of $MgSO_4 \cdot 7H_2O$, 1.2 mL of $FeCl_3$ (1% sol.), 0.05 g of $CaCl_2$, 0.025 g of NaCl, 3 g of

malt extract, 0.25 g of $(NH_4)_2HPO_4$, and 100 μg of thiamin×HCl in 1 L of water]. Cultures were incubated at 25°C for 60 days.

Mycelial growth on liquid media. The four liquid media, namely, MMN, M0, M1, and M2, were used for this study; MMN medium with 10 g/L glucose as a carbon source instead of sucrose, M0 with no nitrogen source, M1 with ammonium nitrogen [$(NH_4)Cl$] as nitrogen source, and M2 with nitrate nitrogen (KNO_3) as nitrogen source.

Mycorrhizal induction

Liquid inocula of fungal species. For each species, a small sample (6 mm × 6 mm) was cut and inoculated in a 500 mL Erlenmeyer flask containing 200 mL of sterile MMN broth. Fungal cultures were incubated at 25°C for 60 days.

Mycorrhizal induction. For mycorrhizal induction, 1 L culture bottles were added with 600 mL of granite soil and 100 mL of PDMP (6 g of potato dextrose broth, 0.75 g of malt extract, and 0.25 g of peptone per liter). Culture bottles were then autoclaved at 100°C and 121°C consecutively for 20 min. Germinated seeds were transferred into sterilized culture bottles and grown for one week. Afterwards, each seedling was injected with liquid inoculum. Inoculated seedlings were grown on 3,500 lx at 23°C for seven months.

Mycorrhizal observation and seedling growth assessment. *X. affine* NIFoS 2716 samples were washed and observed under a microscope (Leica DES8 APO; Leica Microsystems, Wetzlar, Germany). Mycorrhizae were placed in water and observed at 10× magnification. Hartig net samples of *X. affine* NIFoS 2716 were cut into 10 μm sections using a freezing microtome (Leica CM 1900; Leica Microsystems) and observed under a microscope (Leica DEDM 2500; Leica Microsystems). Growth characteristics of *P. densiflora* seedlings, including shoot height, and dry biomass of shoots and roots, were measured.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Duncan's test ($p < 0.05$) was performed to analyze the data. Statistical analysis was performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Mycelial growth of fungal species using different media and inorganic nitrogen sources

Mycelial growth on solid media. For in vitro mycorrhization, mycelia in liquid media were needed. Prior to liquid culture, we determined the optimal culture conditions for each species using solid media. Mycelial growth was evaluated on PDA, MEA, SDA, and MMN.

A. spissacea NIFoS 2719 showed better mycelial growth on SDA than in other media

(Fig. 1). On the other hand, *P. arhizus* NIFoS 2784 showed optimum growth on PDA in 85 mm PDA petri dishes. However, *P. arhizus* NIFoS 2784 showed the least growth in MEA. *S. spraguei* NIFoS 2848 mycelia grew to less than 50 mm after 60 days of cultivation on all culture media tested and showed the least growth among all species examined. In addition, *S. spraguei* NIFoS 2848 showed no growth on SDA. *X. affine* NIFoS 2716 showed the fastest growth on MMN compared to the other species. *X. affine* NIFoS 2716 showed the highest growth after 30 days of inoculation on 85 mm petri dishes containing MMN. Both *P. arhizus* NIFoS 2784 and *X. affine* NIFoS 2716 showed the least growth on MEA. All four species were capable of growth on PDA, MEA, and MMN, although their preferred growth media were different. These results indicated that the four species can be cultivated on PDA and MMN.

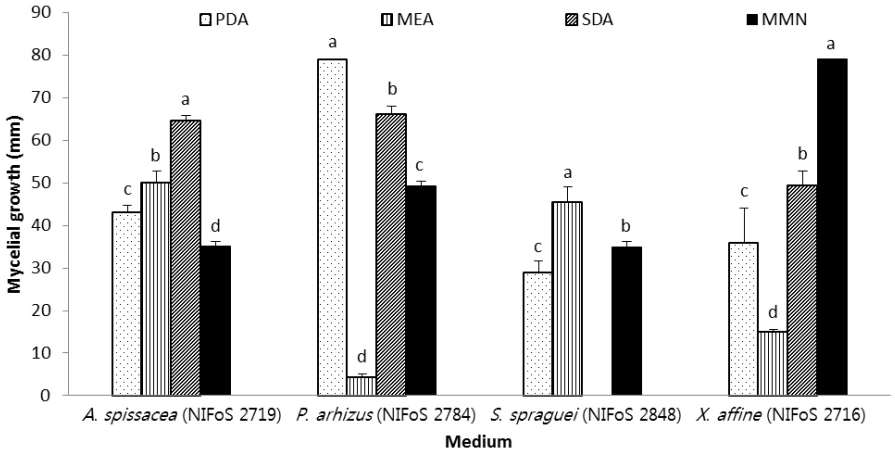

Fig. 1. Mycelial growth of four fungal species in four different solid media. Mycelia were grown on potato dextrose agar (PDA), malt extract agar (MEA), Sabouraud dextrose agar (SDA), and modified Melin-Norkran's medium (MMN). Values are presented as mean ± SD (n = 3). Mean differences are considered significant at $p < 0.05$ after Duncan's multiple range test.

Morphological analysis showed that the mycelial density of *Amanita spissacea* NIFoS 2719 was higher on PDA than on MEA and MMN (Fig. 2A~2D). *P. arhizus* NIFoS 2784 showed lower mycelial density on MMN than on PDA (Fig. 2E~2H). *S. spraguei* NIFoS 2848 on MMN exhibited low mycelial density with little aggregation (Fig. 2L). *X. affine* NIFoS 2716 showed lower mycelial density when cultivated on MMN than on PDA (Fig. 2M, 2P). Morphological analysis of mycelia in the different culture media revealed that all four species generally produce low mycelial density on MMN.

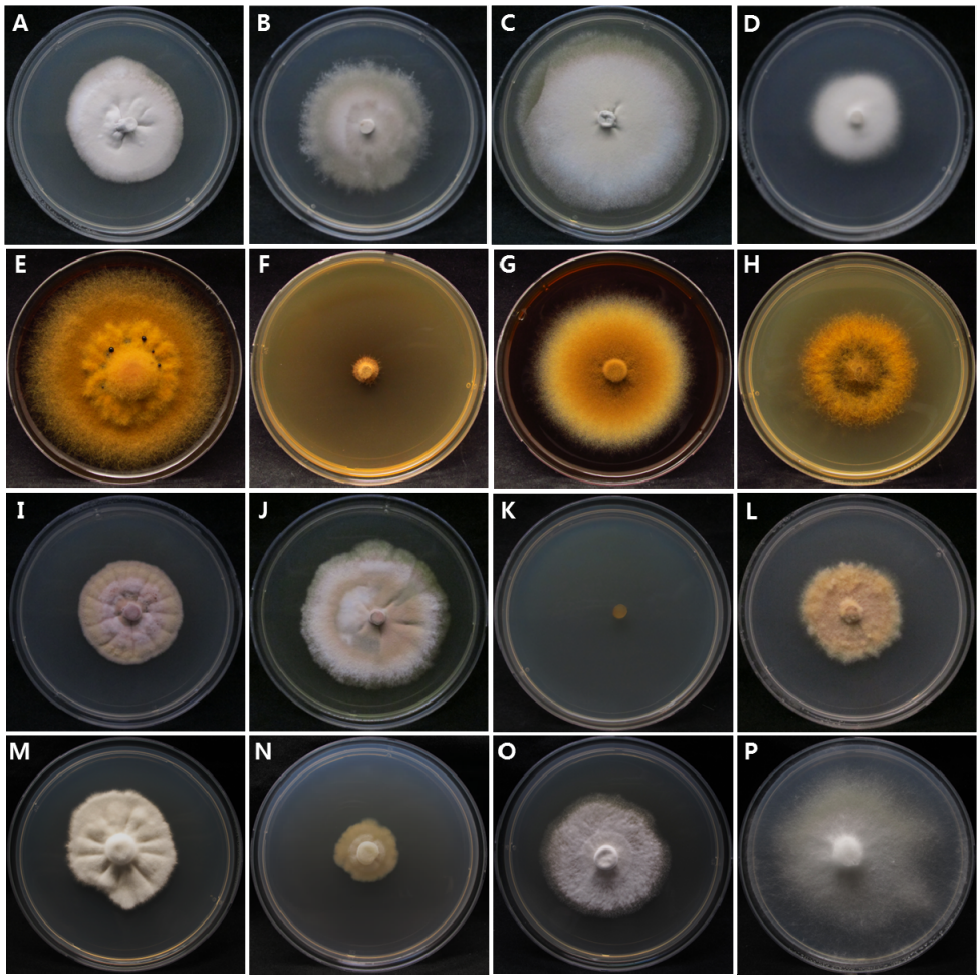

Fig. 2. Morphological characteristics of four fungal species. A~D, *Amanita spissacea* NIFoS 2719; E~H, *Pisolithus arhizus* NIFoS 2784; I~L, *Suillus spraguei* NIFoS 2848; M~P, *Xanthoconium affine* NIFoS 2716; A, E, I, M, Mycelia on potato dextrose agar; B, F, J, N, Mycelia on malt extract agar; C, G, K, O, Mycelia on Sabouraud dextrose agar; D, H, L, P, Mycelia on modified Melin-Norkran's medium.

Mycelial growth on liquid media. All liquid media were based on MMN because ectomycorrhizal fungi are typically grown on MMN at pH conditions ranging from 5.8 to 6.2. Moreover, all four species showed low mycelial densities, which are ideal for liquid inoculation [6, 7]. We then measured the dry weights of the four species after cultivation in MMN media using different inorganic nitrogen sources. Inorganic nitrogen is abundant in forest ecosystems and is a major nutrient of ectomycorrhizal fungi [8]. Ammonium nitrogen is especially known to play an important role in promoting ectomycorrhizal growth [9].

A. spissacea NIFoS 2719 showed no differences in mycelial growth under different nitrogen sources (Fig. 3). *P. arhizus* NIFoS 2784 showed better mycelial growth in MMN and M2 than in M1 and M0. *S. spraguei* NIFoS 2848 produced abundant mycelia in MMN

and showed the least growth in M0. Thus, our results were similar to those reported in a previous study [10]. Dry weights were approximately 2.9-fold higher in mycelia grown in MMN than those grown in M0. Mycelial growth in M2 was lower than in M1 media. As shown in Fig. 3, *X. affine* NIFoS 2716 showed the highest dry weight after cultivation in MMN. Dry weights of mycelia grown in MMN were approximately 2.8-fold higher than those grown in M0. Results showed small differences in mycelial growth after cultivation in different inorganic nitrogen sources.

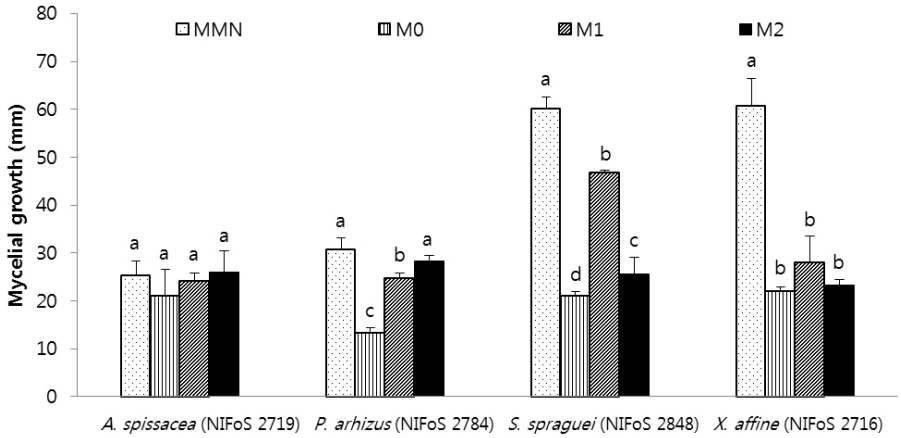

Fig. 3. Mycelial growth of four fungal species on four different liquid media containing organic and inorganic nitrogen sources. The modified Melin-Norkran's medium (MMN) contains 10 g/L glucose instead of sucrose as a carbon source. M0 is MMN without added nitrogen sources. M1 is MMN containing ammonium nitrogen. M2 is MMN with nitrate nitrogen [13]. Values are presented as mean ± SD (n = 3). The mean difference is considered significant at $p < 0.05$ after Duncan's multiple range test.

Generally, ectomycorrhizal growth in MMN was higher than growth on inorganic nitrogen media [11], thereby indicating that inorganic nitrogen did not significantly enhance mycelial growth of the four fungal species. In addition, Jeon et al. [12] demonstrated that the highest dry weight of *X. affine* KFRI 1412 was obtained after cultivation on MMN medium.

Mycorrhizal formation

In this study, the four fungal species and *Pinus densiflora* were synthesized cultivated under sterile conditions. After seven months of inoculation, the roots of *P. densiflora* were observed. Only *X. affine* NIFoS 2716 showed mycorrhizal formation with *P. densiflora* (Table 1). The mycorrhization of *Amanita spissacea* has not been investigated. However, *P. arhizus* and *S. spraguei* have been previously shown to form mycorrhizae with pine hosts [13, 14]. *S. spraguei* formed ectomycorrhization with the *Strobus* (white pine), but showed

little or no association with the *Pinus*. The *P. densiflora* species used in this is classified under the *Pinus*. Therefore, further studies must be conducted using other pine hosts.

Morphological analysis showed that the uninfected roots of *P. densiflora* had brownish lateral roots and abundant root hairs (Fig. 4A). Inoculated roots of *P. densiflora* showed simple and dichotomous lateral roots and rhizomorphs. Brown lateral roots were covered by dense mycelia, which were short and highly branched with numerous root tips and appeared white and light brown. The ends of the lateral roots were thicker than those of the uninfected roots. The hairs of inoculated roots were less developed than those of uninfected roots. The fungal mantles covered the root surface and formed the external mantle. Ectomycorrhizal symbiosis was identified based on the presence of the thick mantles of *P. densiflora* (Fig. 4D~4F).

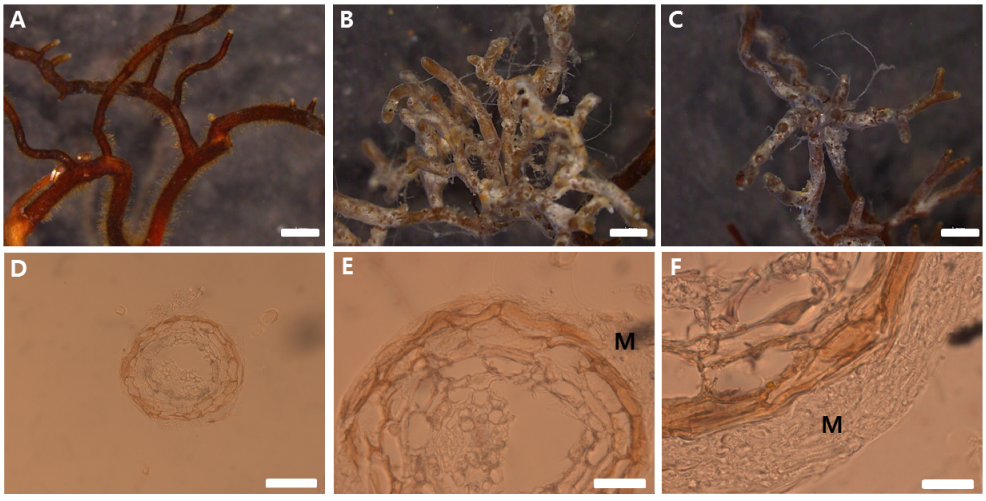

Fig. 4. Morphology of *Xanthoconium affine* NIFoS 2716. A, uninfected *Pinus densiflora* roots; B, C, *X. affine* NIFoS 2716 ectomycorrhizal formation on *P. densiflora*; D~F, fungal mantle of *X. affine* NIFoS 2716 ectomycorrhiza on *P. densiflora*; M, fungal mantle (scale bars: A~C = 1 mm, D = 100 μm, E = 50 μm, F = 20 μm).

Effect of *X. affine* inoculation on seedling growth

Conditions for cultivation of seedlings inoculated with *X. affine* NIFoS 2716 are important because inoculated seedlings in sterile conditions must adapt to the natural, non-sterile environment. In this study, we investigated shoot heights, dry weights of shoots and roots, and SR ratio (dry weight of shoot / dry weight of root) to evaluate seedling growth. The shoot heights of inoculated *P. densiflora* seedlings were significantly lower than those of the controls (Fig. 5A). On the other hand, the shoot weights of inoculated seedlings were higher, indicating that inoculation with promoted volume growth than elongation (Fig. 5B). The root dry weight of control and inoculated seedlings were 6.5 mg and 8.67 mg, respectively, which were lower than the obtained shoot dry weights (Fig. 5C). The dry

weights of shoots and roots of the inoculated seedlings were approximately 1.8- and 1.3-fold higher than those of the controls, respectively. Seedlings inoculated with *X. affine* NIFoS 2716 showed significantly higher SR ratios than those of the controls (Fig. 5D). Root biomass was influenced by soil conditions; in particular, reduced availability of water or nutrients leads to lower SR ratios [15]. Accordingly, *X. affine* NIFoS 2716 inoculation exerted a positive effect on dry weights of shoots and roots.

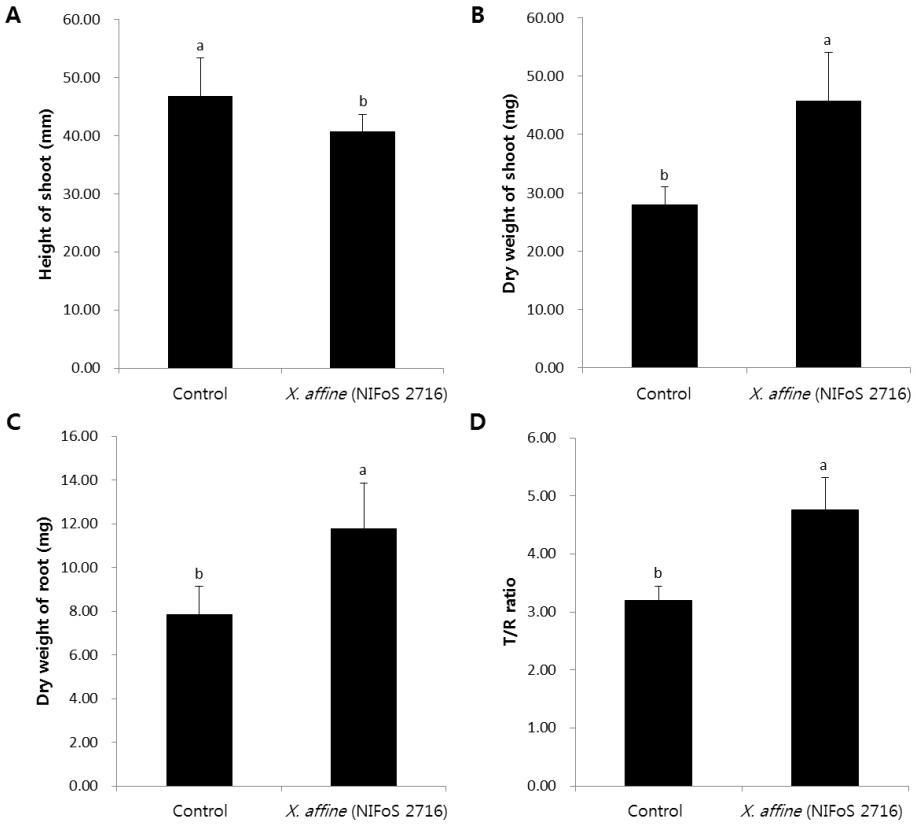

Fig. 5. Growth of *Pinus densiflora* seedlings after seven months of *Xanthoconium affine* NIFoS 2716 inoculation A, Shoot height; B, Shoot dry weight; C, Root dry weight; D, SR ratio (Shoot dry weight / Root dry weight). Values are presented as mean ± SD (n = 5). The mean difference is significant at the $p < 0.05$ after t-test.

In conclusion, our findings demonstrate the first evidence of ectomycorrhizal symbiosis of *X. affine* NIFoS 2716 and *P. densiflora* in vitro. In our symbiosis culture system, the liquid inocula were based on MMN medium to promote the growth of mycelia and maintain low mycelial density. Results of the morphological study showed that the inoculated roots of *P. densiflora* produced simple and dichotomous lateral roots and dense mycelia with white to light brown color. In addition, inoculation with *X. affine* NIFoS 2716 enhanced root and shoot developments of *P. densiflora*. The above results suggested that *X. affine* NIFoS 2716 can promote plant growth.

Acknowledgements

This study was supported by grants (FP 0801-2010-01) from the National Institute of Forest Science, Republic of Korea.

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