

New Records of Endophytic *Paecilomyces inflatus* and *Bionectria ochroleuca* from Chili Pepper Plants in Korea

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Abstract Two new species of endophytic fungi were encountered during a diversity study of healthy tissues of chili pepper plants in Korea. The species were identified as *Paecilomyces inflatus* and *Bionectria ochroleuca* based on molecular and morphological analyses. Morphological descriptions of these endophytic isolates matched well with their molecular analysis. In the present study, detailed descriptions of internal transcribed spacer regions and morphological observations of these two fungi are presented.

Keywords *Bionectria ochroleuca*, Chili pepper, Endophytic fungi, Morphology, *Paecilomyces inflatus*

Endophytes are microorganisms that reside within internal tissues of living plants without visibly harming the host plant [1]. Endophytic microorganisms have been found in all plant families [2], and represent many species in different climate regions of the world [3-7]. Endophytic attention has increased in recent years because of their taxonomic diversity [8], multiple functions, including the potential for use as genetic vectors [9], and their host plant growth promotion and fitness [10, 11]. Furthermore, they are the source of secondary metabolites [7, 12] and biological control agents [13]. Chili peppers that belong to the genus *Capsicum* are probably the most widely consumed spice in the world [14] and cultivated crop plants may live in association with a variety of mycoflora.

The genus *Paecilomyces* was first introduced by Bainier [15], who described it as being closely related to *Penicillium*, but differing in the absence of green colored colonies and in having short cylindrical phialides [16]. The *Paecilomyces*

inflatus described by Onions and Barron [17] is the only monophialidic species of *Paecilomyces* that is commonly isolated from forest soil. However, most species of nectrioid fungi have been assigned to the genus *Nectria*, which includes about 1,000 names. Based on morphological and molecular studies, Rossman *et al.* [18] revised the concept of Nectriaceae and established the family Bionectriaceae typified by *Bionectria* Speg. Bionectriaceous fungi are decomposers of plant debris, pathogens of plants and insects and biological control agents [19]. *Bionectria ochroleuca* is characterized by pale yellow or white ascomata and two-celled, hyaline ascospores. An anamorph of the *B. ochroleuca* is *Gliocladium rosea*, which is normally isolated from forest areas [20]. Interestingly, these two species were isolated from chili pepper as endophytes. In this study, we characterized *P. inflatus* and *B. ochroleuca* isolated from healthy symptomless root tissues of chili pepper in Korea by molecular and morphological analysis.

MATERIALS AND METHODS

Isolation of endophytic fungi. Chili pepper plant (*Capsicum annuum* L.) tissues were collected from a field in Daejeon, which is in Chungnam Province in the central portion of the Republic of Korea, in 2009. Leaf, stem and root samples of plants were randomly excised and brought to the laboratory in separate sterile polyethylene bags, where they were processed for isolation within 5 hr of collection. Briefly, samples were washed in running tap water to remove dust and debris, dried in the air and then cut into 1 cm segments. For surface sterilization, the segments were soaked in 95% ethanol for 1 min, then in sodium hypochlorite (4% available chlorine) for 3 min, and 95%

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ethanol for 30 sec. The samples were subsequently washed in sterile distilled water three times and dried in a laminar air flow chamber. Next, ten segments per sample were placed horizontally on dichloran rose bengal chloramphenicol agar (DRBC; Difco, Detroit, MI, USA) and potato dextrose agar (PDA; Difco) supplemented with streptomycin sulfate to inhibit bacterial growth. Developing hyphal tips of emerged colonies were collected after incubation at 25°C for 5, 10, and 25 days and sub-cultured on PDA for 8~10 days. Pure cultures of isolates were maintained in PDA slant tubes and 20% glycerol stock solution and deposited in the culture collection of the Chungnam National University Fungal Herbarium. In this study, molecular and morphological characteristics of two isolates, CNU081043 and CNU081055, were examined.

Genomic DNA extraction and PCR amplification.

Genomic DNA was extracted from mycelium using the method described by Deng *et al.* [21]. Amplification of the internal transcribed spacer (ITS) region was performed using the ITS5 and ITS4 primers, after which the PCR products were purified using a Wizard PCR prep kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were then directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Gel electrophoresis and data collection were performed using an ABI prism 310 Genetic Analyzer (Applied Biosystems).

Sequence analysis. The sequences were compared with those available in the GenBank database by BLAST search analysis. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using CLUSTAL X [22], after which the alignment was refined manually using PHYDIT ver. 3.2 [23]. Neighbor-joining trees were reconstructed for ITS gene sequences with Kimura's 2-parameter distance model [24] using the MEGA 4 program [25]. Bootstrap analysis using 1,000 replications was performed to assess the relative stability of the branches.

Sequence data were deposited in GenBank and assigned accession numbers KC285890 for isolate CNU081043 and KC285891 for CNU081055.

Morphological characterization. Morphological characteristics of isolates CNU081043 and CNU081055 were examined on corn meal agar (CMA), malt extract agar (MEA), oat meal agar (OMA), and PDA. Small discs (0.5 cm diameter) were cut from the margin of developing cultures, inoculated on three points of the Petri dish for CNU081043 and in the center of plates for CNU081055 and incubated at 20~35°C in the dark to determine the favorable growth conditions. The mycelia, phialides, penicillus and conidiophores were observed using a BX50 microscope (Olympus, Tokyo, Japan). The conidia, phialides and

conidiophores were measured using an Artcam 300MI digital camera (Artray, Tokyo, Japan). Colors were named using a mycological color chart [26]. Morphological characteristics of the isolate were then compared with previous descriptions.

RESULTS AND DISCUSSION

Taxonomy of the isolate CNU081043.

Molecular analysis: To determine the phylogenetic relationship among the endophytic isolate CNU081043 from chili pepper and its related species, the ITS region was compared. BLAST searches revealed 99% sequence similarity between the endophytic fungal isolate (CNU081043) and its relevant sequences in GenBank. Isolate CNU081043 and GenBank isolates *P. inflatus* (isolate H34, accession no. GU466291) and *Acremonium atrogriseum* (AB540569) clustered together in a group that matched the reference *P. inflatus* well with a high bootstrap value (100%). There was only one nucleotide difference between CNU081043 and *P. inflatus* isolate H34, but two or more nucleotide differences were observed among other related species from the GenBank database (Fig. 1).

Morphological characterization: Taxonomic descriptions and microphotographs of morphological structures of the species are shown in Table 1 and Fig. 2.

Paecilomyces inflatus (Burnside) J. W. Carmich. 1962 (Table 1, Fig. 2).

Colony on MEA: Slow growing, attaining a diameter 33 (34.02)~35 mm in 14 days at 25°C. Appearing powdery, velvety and cottony when freshly isolated, becoming more floccose to funiculose and tougher from an increase in vegetative hyphae after several transfers. Vegetative hyphae hyaline, smooth-walled (Fig. 2A and 2B).

Colony on PDA: Growing slowly on PDA media at 25°C. The colony length ranged from 33 (34)~35 mm after 14

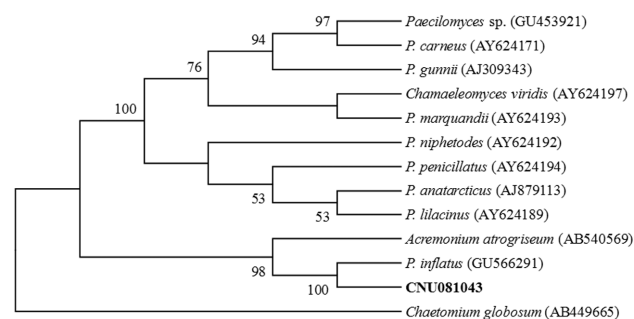


Fig. 1. Neighbor-joining phylogenetic tree of the endophytic isolate CNU081043 and its relevant species from GenBank based on internal transcribed spacer gene sequences. Numbers at the nodes indicate bootstrap values from a test of 1,000 replications. The scale bar indicates the number of nucleotide substitutions. The present isolate is shown in bold. Evolutionary analyses were conducted using the MEGA5 program [25].

Table 1. Comparison of morphological characteristics between the endophytic fungal isolate CNU081043 and *Paecilomyces inflatus*

Characteristics		CNU081043	<i>Paecilomyces inflatus</i> [16]
Colony	Color	White to pale yellow, reverse pale yellow in PDA	Pale yellow
	Size	3.3~3.5 cm diam. on PDA 3.3~3.5 cm diam. on MEA	3.5 cm diam. on MEA
Conidiophores		Conidiogenous structures irregular, distinct conidiophores usually lacking	Conidiophores short, cylindrical, up to 14 μm long, 1.5~3.2 μm wide, smooth-walled, hyaline, unbranched
Phialide	No.	Mostly single, occasionally 2~3	1~3 phialides
	Size	(9.0~) 12.8 (~18.0) \times (1.5~) 2.3 (~3.5) μm	Phialides 5~15 \times 2.4~3.7 μm
Conidia	Shape	Conidia single celled, lemon-shaped or citriform, smooth walled with distinct connectives in both sides/ends	Conidia hyaline, smooth-walled or finely roughened, lemon-shaped to fusiform
	Size	(2.0~) 3.0 (~4.5) \times (1.0~) 2.2 (~3.0) μm in diameter	2.8~5.2 \times 1.8~3.1 μm (avg. 3.6 \times 2.3 μm)

PDA, potato dextrose agar; MEA, malt extract agar.

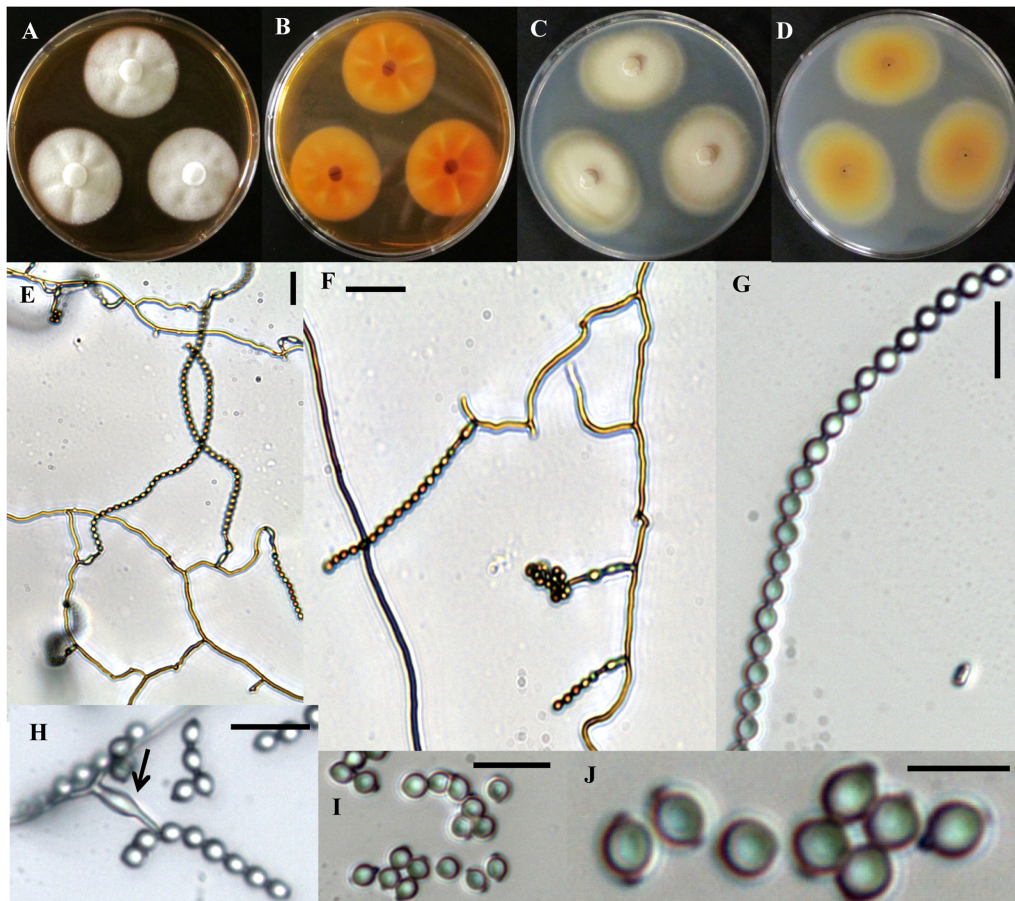


Fig. 2. Morphology of the isolate CNU081043 (*Paecilomyces inflatus*). Obverse and reverse colony on malt extract agar (A, B) and potato dextrose agar (C, D), respectively, after 2 wk at 25°C. Production of conidial chain from vegetative hyphae: E, smaller view; F, larger view with single phialide (rarely 2 phialides). G, Conidial chain; H, Conidia bearing phialides; I, J, Lemon shaped conidia (scale bars: E, F = 20 μm , G~I = 10 μm , J = 5 μm).

days, was white to pale yellow in color, and reverse pale yellow. The optimum temperature for the growth of this fungus on PDA media was 25°C (Fig. 2C and 2D).

Colony on OMA: Slow growing at 25°C, with the diameter ranging from 33 (34)~38 mm after 14 days. Velvety to granular, greenish white to pale yellow. The fungus did not

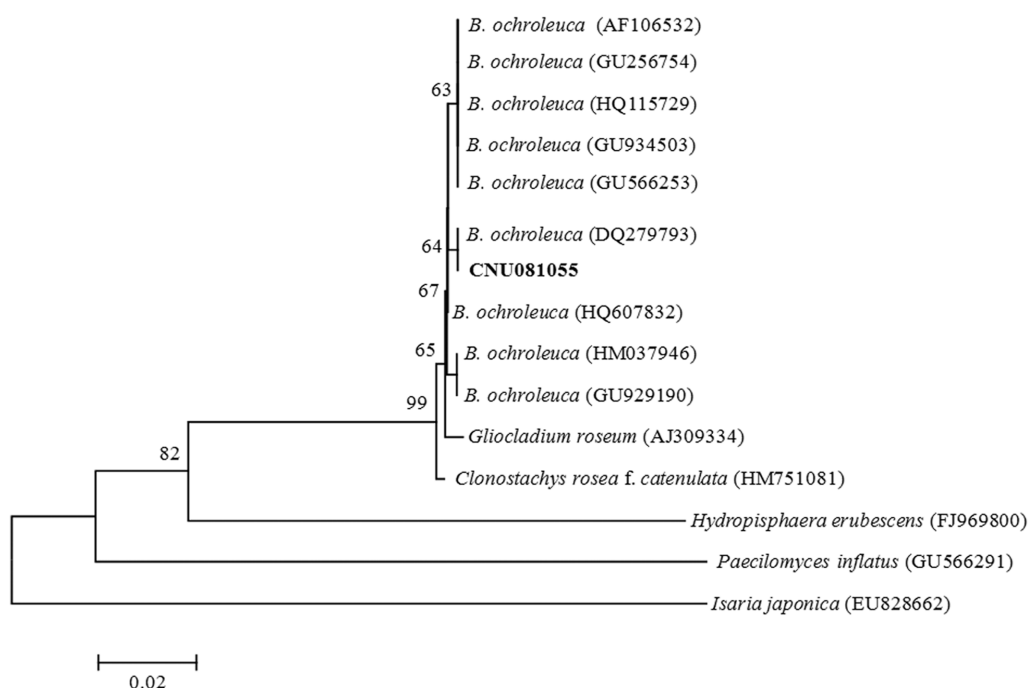


Fig. 3. Neighbor-joining phylogenetic tree of the endophytic fungi CNU081055 and its relevant species from GenBank based on internal transcribed spacer gene sequences. Numbers at the nodes indicate bootstrap values from a test of 1,000 replications. The scale bar indicates the number of nucleotide substitutions. The present isolate is shown in bold. Evolutionary analyses were conducted using the MEGA5 program [25].

grow at 35°C and the optimal colony growth occurred at 25°C.

Colony on CMA: Growing slowly on CMA. The diameter ranged from 31 (33.51)~35 mm when grown for 14 days at 25°C. Colony transparent white. Vegetative hyphae hyaline, smooth-walled.

Conidiophores, phialides, and conidia: Conidiogenous structures irregular, usually consisting of one to three phialides on short branches of trailing hyphae, distinct conidiophores usually lacking. Phialides produced on short branches on hyphae, mostly consisting of single phialides borne irregularly on aerial hyphae or occasionally 2~3 phialides borne on top of a short branch. Phialides lateral or terminal, flask-shaped, with a swollen basal part and tapering abruptly into a long neck. Phialide lengths are (9.0~) 12.8 (~18.0) × (1.5~) 2.3 (~3.5) μm, hyaline and smooth walled. Conidia single celled, lemon-shaped or citriform, smooth walled with distinct connectives in both sides/ends, arranged in very long chains that differentiate them from *Penicillium*, (2.0~) 3.0 (~4.5) × (1.0~) 2.2 (~3.0) μm in diameter. Ascospores or chlamydo-spores were not observed (Table 1, Fig. 2).

Isolate examined: On roots of chili pepper; CNU081043. Only one *Paecilomyces* was isolated from this plant. This is a rarely isolated endophytic fungus, and its isolation frequency was 0.21.

Distribution: Common species, found especially in forest soil. *Paecilomyces* are rarely isolated endophytic fungi and *P. inflatus* is the first report in Korea.

Taxonomy of the isolate CNU081055.

Phylogenetic analysis: To determine the phylogenetic relationship among the endophytic isolate CNU081055 from chili pepper and its related species, the ITS region was analyzed. The results revealed 99~100% sequence similarity between the endophytic fungal isolate (CNU081055) and its relevant sequences in GenBank. Isolate CNU081055 and *B. ochroleuca* GCA-605-5 (DQ279793), which was isolated from *Gladiolus grandiflorus* in Mexico, showed 100% sequence similarity. The ITS sequence of the present isolate also showed 99% sequence similarity (1 nucleotide differences) with *B. ochroleuca* isolate ATT093 (HQ607832) and other *Bionectria* spp. isolated from different regions of the world. Furthermore, the isolate showed similarity with its anamorphs *Gliocladium roseum* isolate G97012 (AJ309334) and *Clonostachys rosea* f. *catenulata* isolate NRRL:22970 (HM751081). Finally, the phylogenetic tree revealed that sequences of CNU081055 and *B. ochroleuca* isolate GCA-605-5 clustered together in a group in which the reference *B. ochroleuca* matched with a high bootstrap value (64%) (Fig. 3).

Morphological characterization: Taxonomic descriptions and microphotographs of the morphological structures of the species are shown in Table 2 and Fig. 4.

***Bionectria ochroleuca* (Schweinitz) Schroers & Samuels 1997 (Table 2, Fig. 4).**

Colony on PDA: Growth fast at 25°C, with colonies reaching 35~40 mm in diameter in 7 days. Colonies

Table 2. Comparison of morphological characteristics between the endophytic fungal isolate CNU081055 and *Bionectria ochroleuca*

Characteristics		CNU081055	<i>Bionectria ochroleuca</i> [20]
Colony	Color	White to yellowish on PDA White, yellow ring formed on OA	Yellowish
	Size	3.5~4.0 cm diameter on PDA 3.5~3.8 cm diameter on MEA	3.5 cm diameter on MEA
Stripes		Wide at base; (20~) 75 (~250) μ m long	Wide at base; (25~) 70 (~200)
Phialide	No.	3~5	2~5
Perithecia		Formed	Formed
Stroma	Shape	Well developed stroma	Well developed stroma; rarely solitary from mycelium
Conidia	Shape	Slightly curved; broadly rounded	Slightly curved; distally broadly rounded; slightly flattened on one side
	Size (μ m)	(3.4~) 5.3 (~7.3) \times (2.0~) 2.8 (~4.7) μ m in diameter	(4.2~) 4.8 (~6.6) \times (2.0~) 2.4 (~3.4) μ m in diameter

PDA, potato dextrose agar; OA, oatmeal agar; MEA, malt extract agar.

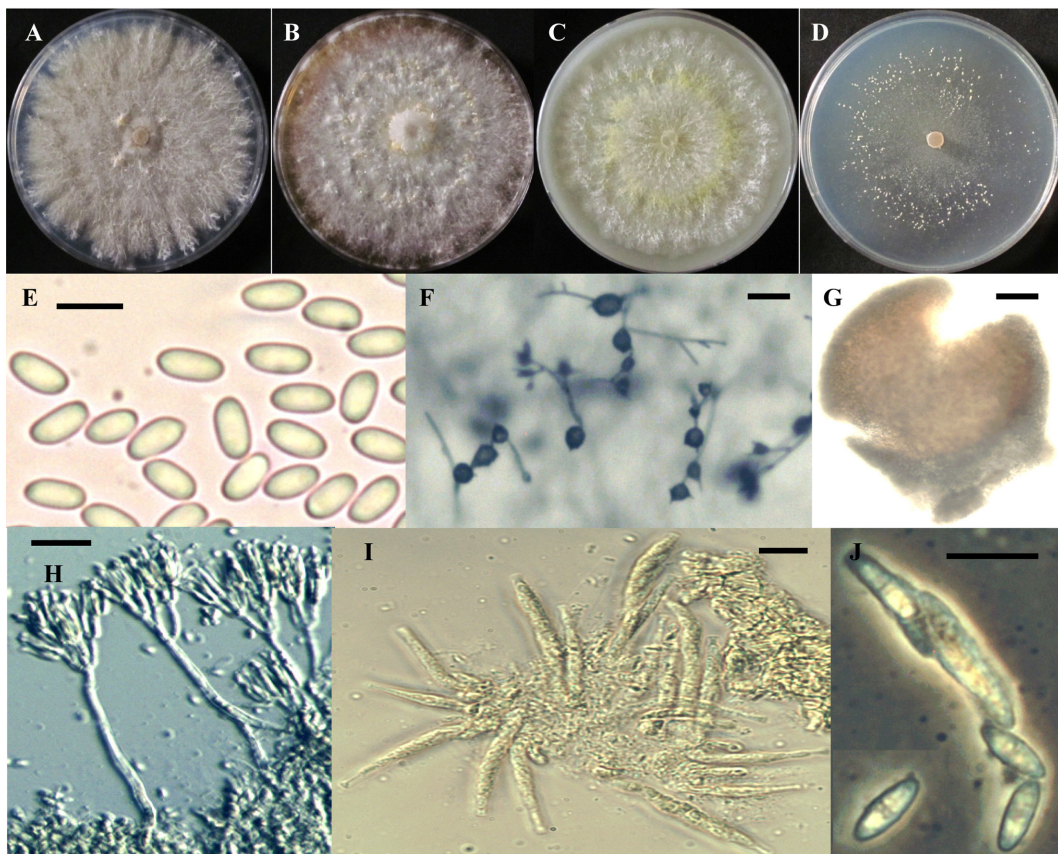


Fig. 4. Colonies of CNU081055 (*Bionectria ochroleuca*) on potato dextrose agar (A), malt extract agar (B), oatmeal agar (C), and cornmeal agar (D) after 2 wk at 25°C, conidia from secondary conidiophore (E), ascoma (F, G), secondary conidiophores (H), asci (I), double celled ascospores (J) (scale bars: E, H = 20 μ m, F = 200 μ m, G = 50 μ m, I, J = 10 μ m).

whitish to yellowish. Surface textures plane velutinous. Reverse white to light yellowish. Aerial mycelium strongly developed in thick, often erect hyphal strands. Surface unpigmented or with slight yellow pigmentation, appearing white because of aerial mycelium and white conidial masses, or in yellow or orange hues with yellowish white to orange-white granules because of conidial masses (Fig. 2A).

Colony on MEA: Fast growing fungi, with colony diameters of 25~29 mm after 7 days at 25°C. The optimum temperature for colony growth was 25°C. Colony unpigmented and white. Aerial mycelium very strongly developed and erect hyphal strands occurring often. Reverse whitish. Granules (cyst like structures) were produced on the colonies after 3 wk of mycelia growth, and the number of granules

increased after 4 wk (Fig. 2B).

Colony on OMA: Colony diameter 35~38 mm after 7 days at 25°C; optimum growth observed at 27°C, with growth possible at 33°C. Yellow pigment generally diffusing beyond the colony, pigment only visible in the agar inside the colony margins. Colony reverse on OMA yellowish white to light yellow, with orange or brownish hues occurring with time and becoming generally orange-white to light orange or carrot-red after incubation under UV. Surface mycelium optimally developed, felty to tomentose, arranged in strands, particularly toward the colony centre, or granulose because of conidial masses from solitary or aggregated conidiophores. Surface yellow or orange hues because of pigmentation of the agar or with yellowish white to orange-white granules because of conidial masses (Fig. 2C).

Colony on CMA: Colony diameter reaching 40 mm after 7 days at 25°C, fast growing fungi. The suitable temperature for the growth of this fungi ranged from 24~27°C. The colony color was transparent white to light brownish and produced granulose structures on CMA plates because of conidial masses from solitary or aggregated conidiophores. Surface mycelium developed on CMA (Fig. 2D).

Conidiophores, phialides, penicillius and conidia: Conidiophores dimorphic. Primary conidiophores verticillium-like, formed throughout the colony, arising from the agar surface. Stipes (20~) 75 (~250) µm long, 3.5~5.5 µm wide at the base, generally longer than the 30~120 µm high branching portion. Phialides divergent, in whorls of 3~5, or singly from lower levels, straight, each producing a small, hyaline drop of conidia. Secondary conidiophores solitary or aggregated, particularly around the colony center. Branches and phialides appressed, phialides slightly flask-shaped, with the widest point below the middle, slightly tapering in the upper part. Conidia from primary conidiophores larger, frequently less curved, (3.4~) 5.3 (~7.3) × (2.0~) 2.8 (~4.7) µm. Perithecia formed frequently in single ascospore isolates, crowded in large numbers on a well-developed stroma.

Isolate examined. On the roots of chili pepper; CNU081055. Only one *Bionectria* was isolated from this plant. This organism is a rarely isolated endophytic fungus and this is the first report of its occurrence in Korea.

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