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

RESEARCH NOTE

First Report of Fruit Rot Caused by *Fusarium decemcellulare* in Apples in Korea

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Abstract

In 2014, abnormal brown spots were observed on Hongro apples in fields in Gyeongsangbuk-do Province and during low-temperature storage. The spots were round, blight brown, and different from the symptoms of previously reported apple diseases. A fungal pathogen was isolated and cultured on potato dextrose agar, and it was morphologically similar to *Fusarium decemcellulare*. A pathogenicity test showed the same brown spots on both wounded and unwounded Hongro and Fuji apple cultivars. RPB1 and RPB2 sequences of *F. decemcellulare* KNU-GC01 matched with those of *F. decemcellulare* NRRL 13412 (98.3% and 97.6% similarities, respectively); both strains clustered together in the phylogenetic tree, indicating their close relationship at the species level. Therefore, *F. decemcellulare* is a newly reported pathogen that causes brown spots on apples in Korea.

Keywords: Apple fruit, Brown spot, Cultivar Fuji, Cultivar Hongro, *Fusarium decemcellulare*

OPEN ACCESS

Kor. J. Mycol. 2017 March, 45(1): 54-62
https://doi.org/10.4489/KJM.20170006

pISSN : 0253-651X
eISSN : 2383-5249

Received: 31 January, 2017
Revised: 22 February, 2017
Accepted: 22 February, 2017

Apple (*Malus pumila* Mill.) is cultivated worldwide, and its production was approximately 84 million tons in 2014 (FAOSTAT, Food and Agriculture Organization of the United Nations, http://faostat.fao.org/). In Korea, the cultivation area for apple is approximately 31,620 ha, of which cultivars Fuji and Hongro occupy about 70% and 15%, respectively; the total cultivation area of the two cultivars has gradually increased (KOSTAT, Statistics Korea, http://kostat.go.kr/portal/eng/). To date, about 40 pathogens have been reported for apple[1], among which, the causal agents of apple blotch, anthracnose, white rot, *Alternaria* leaf spot, and bacterial shoot blight are of economic concern for apple cultivation[2].

Because of the economic losses caused by the major apple diseases, rapid and accurate identification of the causal agent of the disease is an essential prerequisite for the fungicide spray program[3]. Apple anthracnose and white rot caused by *Colletotrichum* spp. and *Botryosphaeria dothidea*, respectively, are clearly distinct from each other on the basis of typical signs and symptoms[3], rendering their identification easier. Apple blotch caused by *Marssonina coronaria* can be observed on the fruit, but it is distinguished using its unique symptoms and signs[4]. Recently, abnormal brown spots were observed on Hongro fruits

not only in the field but also in low-temperature storage in Gyeongsangbuk-do Province. In this study, an unreported fungal pathogen was isolated from the affected fruits, and its morphological characteristics and phylogenetic relationships were analyzed. We have reported the fungal pathogen as a new causal agent of fruit rot in apples in Korea.

In 2014, abnormal brown spots were observed in a Hongro orchard located in Gimcheon in Gyeongsangbuk-do Province. The brown spots appeared on the lenticel and were brown and circular (Fig. 1A, 1B), and they were clearly differentiated from the typical symptoms of apple anthracnose and white rot[3]. In addition, similar symptoms were observed in apples in low-temperature storage (Fig. 1C, 1D). To isolate the causal agent from the abnormal brown spots, the surface of an apple was wiped with 70% EtOH and the diseased peel was removed using a sterilized blade. Then, the surface of the collected diseased tissue was sterilized in 70% ethanol and 1% sodium hypochlorite and washed 3 times with double-distilled water. The surface-sterilized tissues were transferred onto potato dextrose agar (PDA) plates and maintained in an incubator at 25°C. After 3 days, small white colonies were observed, and they were transferred onto a new PDA plate. The colonies formed abundant white to pink mycelia (Fig. 2A), and dark carmine-red pigment was produced under the PDA after 7 days (Fig. 2B). In addition, creamy yellow sporodochia were observed on the 20-day-old mycelia cultured on PDA (Fig. 2C). Two morphologically

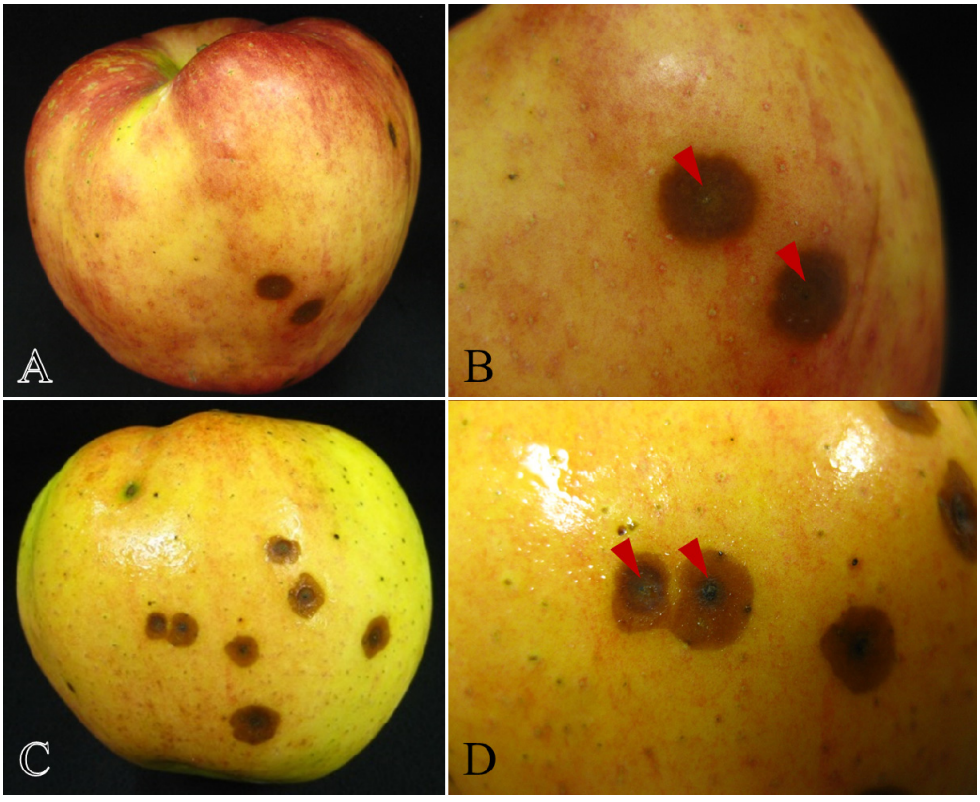

Fig. 1. Photographs of showing abnormal brown spot on cultivar Hongro diseased apple fruits collected from Gimcheon in Gyeongsangbuk-do Province in 2014. A, Collected from field; B, Close-up view of an infected apple of A; C, Collected from low-temperature storage; D, Close-up of an infected apple of C. Red arrowhead indicate lenticels on the surface.

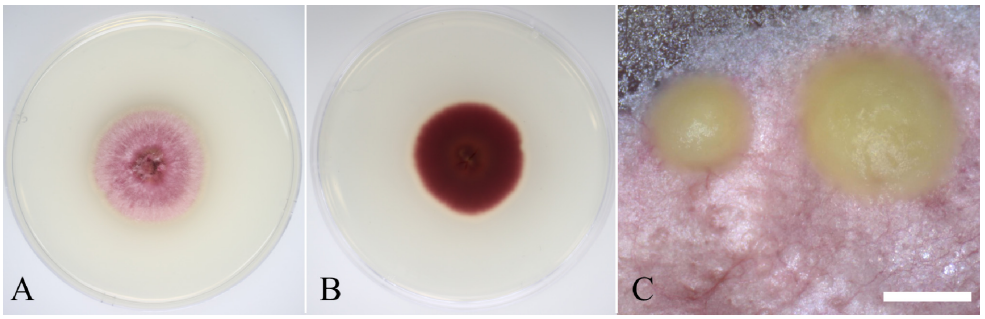

Fig. 2. Morphological characteristics of the isolated *Fusarium decemcellulare* KNU-GC01 on potato dextrose agar (PDA). A, Feature of the colony after 7 days of growth on PDA; B, Reverse side of the 7 days growth colony showed dark carmine-red pigment; C, Creamy yellow sporodochia on the after 20 days cultured mycelium (scale bar = 1 mm).

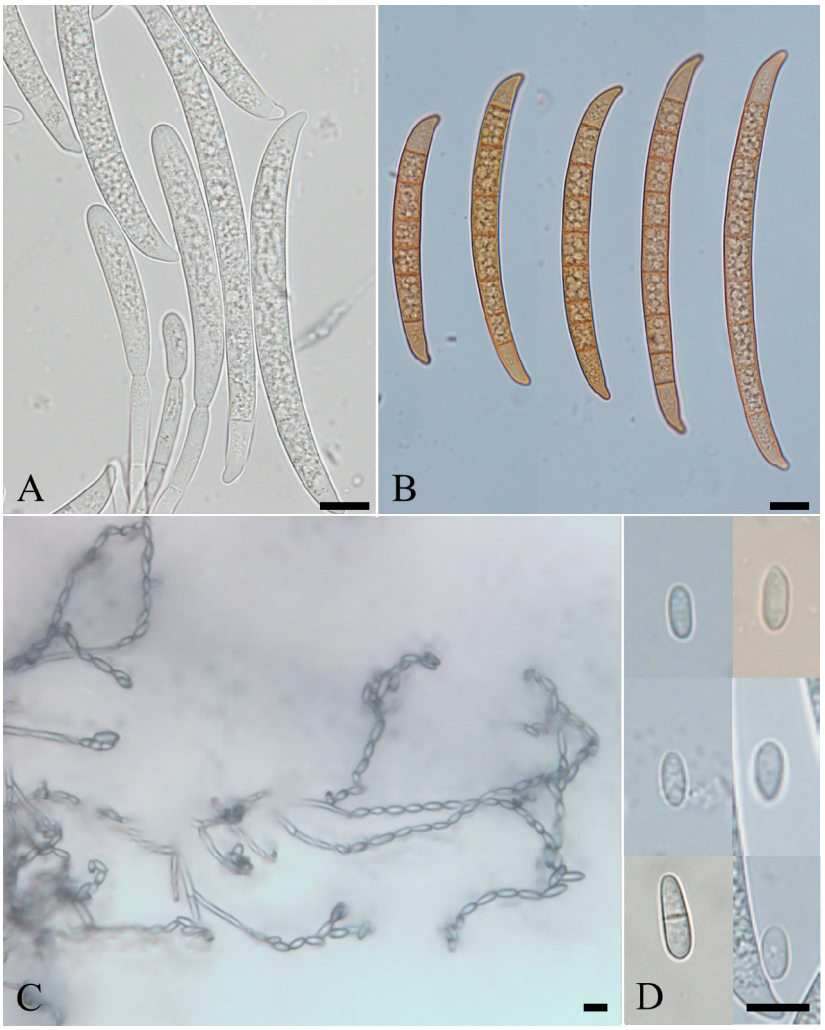

Fig. 3. The observed macroconidia and microconidia of *Fusarium decemcellulare* KNU-GC01. A, Monophialides and immature and mature macroconidia; B, Macroconidia, predominantly 5~9 septate depends on the length; C, Microconidia produced in long chains on potato dextrose agar; D, Microconidia, 0~1 septate and oval (scale bars: A~D = 10 μm).

identical fungal strains, KNU-GC01 and KNU-GC02, were isolated. The morphological characteristics were observed using a light microscope (BX-50; Olympus, Tokyo, Japan). Monophialides were found in the sporodochia, and macroconidia were formed at the end of the monophialides (Fig. 3A). The macroconidia were stained with 1% safranin O solution to observe them more clearly. The macroconidia were slightly falcate, curved, thick-walled, 6 to 9 septate, with a tapering apical cell, and 71.3~84.6 × 5.8~7.5 μm (n = 50; Fig. 3B). The microconidia were produced in a long chain and were branched (Fig. 3C), oval, aseptate or septate, and 5.3~16.0 × 3.0~4.7 μm (n = 50; Fig. 3D). When compared with the cultural and morphological characteristics of previously reported *Fusarium* spp. in fruit [5-7], the isolated fungal strains were found to be the closest to *Fusarium decemcellulare* (Table 1). In addition, KNU-GC01 and KNU-GC02 showed identical morphological and cultural characteristics (data not shown).

To confirm pathogenicity, *F. decemcellulare* KNU-GC01 isolated in this study was inoculated into 5 healthy Hongro and Fuji fruits. For the inoculum, the spore suspension was prepared using *F. decemcellulare* cultured for 20 days, and the conidial concentration was determined with a hemocytometer and adjusted to approximately 1.75×10^5 conidia/mL. The surface of a healthy apple was wiped with 70% EtOH and then air-dried. Two points of the lenticel were wounded using a sterilized needle, and paper disks containing 20 μL of the spore suspension were attached and sealed using foil. Identical paper disks were also attached to unwounded lenticels and sealed using foil. Fruits inoculated with sterilized water were used as the control. All the inoculated fruits were incubated at 25°C, and after 3 days, the disks were removed. After 15 days, brown spots could be observed on both wounded and unwounded Hongro fruits (Fig. 4A~4D), although the size and shape of the spots were somewhat smaller on the Hongro fruits. However, pathogenicity was confirmed for Fuji (Fig. 4E~4H). From each of the inoculated fruits, *F.*

Table 1. Comparison of morphological characteristics of isolated fungi from abnormal brown spot with the previous descriptions of *Fusarium* spp. on fruit disease

Characteristics		Present isolate	*Fusarium decemcellulare* [a]	*Fusarium avenaceum* [b]
Macroconidia	Shape	slightly falcate, curved, robust and thick walled	very long, curved, thick walled, fusiform and hyaline	long and slender, slightly falcate and thin walled
	Septa	6~9	5~9 (usually 7~9)	usually 5
	Length (μm)	71.3~84.6	60~76	40~80
	Width (μm)	5.8~7.5	5~7	3.5~5
Microconidia	Shape	oval	oval	fusoid, rarely observed
	Septa	0~1	0~1	1~2
	Length (μm)	5.3~15.2	7.5~12.9	ND
	Width (μm)	3.1~4.7	2~4.7	ND
Chlamydospores		not observed	absent	absent
Color of sporodochia		yellow on PDA	yellow on PDA	pale orange color on CLA

PDA, potato dextrose agar; ND, not described; CLA, carnation leaf agar.
[a] Description by Leslie and Summerell[6]; Serrato-Diaz et al.[5].
[b] Description by Leslie and Summerell[6]; Wenneker et al.[7].

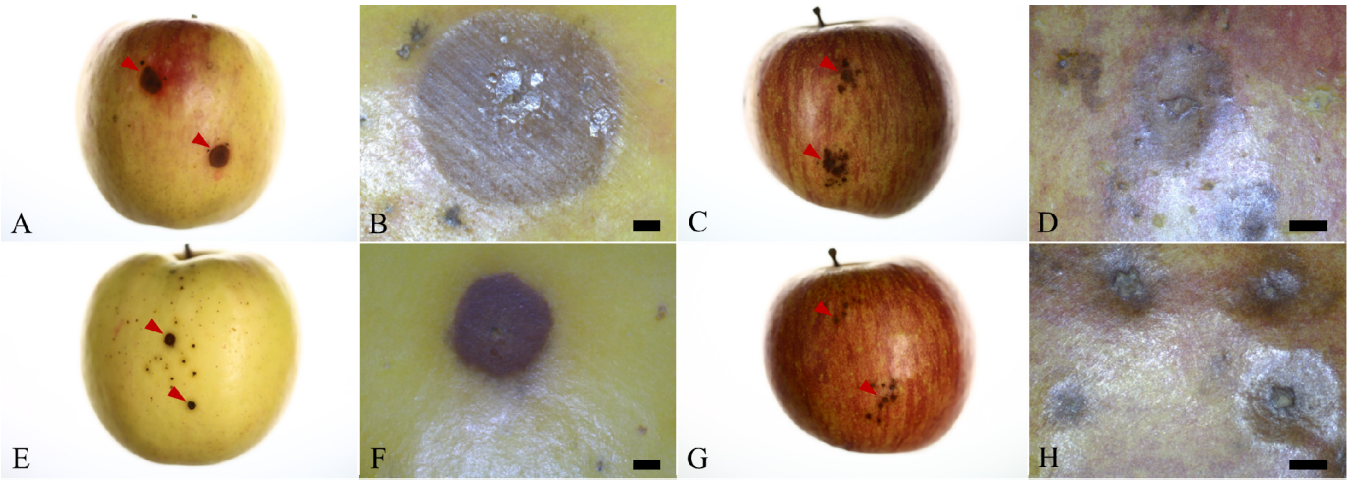

Fig. 4. Pathogenicity test on cultivar Hongro and Fuji with wounded and non-wounded inoculation using conidial suspension of *Fusarium decemcellulare* KNU-GC01. A, wounded Hongro 15 days post inoculation (dpi); B, enlarged picture of A; C, wounded Fuji 15 dpi; D, enlarged picture of C; E, non-wounded Hongro 15 dpi; F, enlarged picture of E; G, non-wounded Fuji 15 dpi; H, enlarged picture of G. Red arrowhead indicate the symptoms (scale bars: B, D, F, H = 1 mm).

decemcellulare was re-isolated (data not shown).

According to previous studies, the resistance and susceptibility of apple diseases have been reported to be dependent on the apple cultivar[8-10]. In addition, Hongro has been reported to be susceptible to apple anthracnose, while Fuji is a resistant cultivar[11]. Thus, it was assumed that Hongro is more susceptible to *F. decemcellulare* than Fuji. Meanwhile, no specific changes were observed in the control fruit.

For molecular identification of the isolate KNU-GC01, total genomic DNA was prepared using the HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea), according to the manufacturer's protocol, and the internal transcribed spacer (ITS) region was amplified and sequenced using the ITS1F/ITS4 primer pair[12]. Then, 519 bp was obtained from KNU-GC01, and BLAST revealed that the ITS sequences showed 99.6% and 100% similarity with that of *F. decemcellulare* CBS 315.73 (accession no. KM231809) and *Albonectria rigidiuscula* (anamorph: *F. decemcellulare*) strain 6940 (accession no. HM054147) and 99% similarity with that of *Fusarium avenaceum* (accession no. FJ478097) and *Fusarium proliferatum* (accession no. KP050559) (data not shown). Therefore, it was assumed that the ITS region was not appropriate for identifying the isolate KNU-GC01 among *Fusarium* spp.

Previous studies suggest that a combined molecular phylogeny based on the DNA-directed RNA polymerase II largest (RPB1) and second largest (RPB2) subunits can show accurate evolutionary relationships within *Fusarium* spp.[13]. Therefore, partial regions of RPB1 and RPB2 were amplified and sequenced in this study. To identify the species, partial RPB1 was amplified and sequenced using the Fa/G2R primer pair, and partial RPB2 was amplified and sequenced using the 5f2/7cr and 7cf/11ar primer pairs[14-16]. Each PCR product was purified using the Solgent PCR Purification Kit and sequenced (SolGent, Daejeon, Korea). Partial sequences of RPB1 (2,139 bp) and RPB2 (1,865 bp) were obtained and deposited in NCBI GenBank (LC212975 and LC214751, respectively). To analyze the

phylogenetic relationships, additional sequences of RPB1 and RPB2 from other *Fusarium* spp. were retrieved from NCBI GenBank. A phylogenetic tree was constructed using the maximum likelihood method with the MEGA 7 program. In addition, sequence identities of *F. decemcellulare* KNU-GC01 were analyzed using GENETYX-WIN, version 3.2. RPB1 and RPB2 sequences of the KNU-GC01 isolate showed 98.3% and 97.6% similarities to those of *F. decemcellulare* strain NRRL 13412 (JX171453 and JX171567, respectively) isolated from the coffee tree (*Coffea arabica*) (Fig. 5). Although the KNU-GC01 isolate showed low similarities to *F. decemcellulare* strain NRRL 13412, it was assumed that the genetic variation observed in RPB1 and RPB2 was due to the isolation source, host, and isolated region. The isolate KNU-GC01 clustered together with *F. decemcellulare* NRRL 13412, with a high bootstrap value of 100% in the phylogenetic tree constructed using the combined partial RPB1 and RPB2 sequences; this confirmed their close relationship at the species level (Fig. 5). Thus, *F. decemcellulare* KNU-GC01 was confirmed using morphological and molecular biological characteristics and was deposited in the Korea Agricultural Culture Collection (accession no. KACC48069).

According to a previous study, *F. decemcellulare* (teleomorph: *Albonectria rigidiuscula*) is distributed more frequently in tropical and subtropical areas[5]. *F. decemcellulare* has been reported to cause galls in mango (*Mangifera indica*)[17-19]; inflorescence wilt and flower necrosis in rambutan (*Nephelium lappaceum*), longan (*Dimocarpus longan*), and mango; small brown skin lesions on the ripening fruit of avocado (*Persea americana*)[20]; and dieback in atemoya (*Annona squamosa* L. × *Annona cherimola*) and mango[21, 22]. However, to the best of our knowledge, there have been no reports on fruit rot or brown spots in apples.

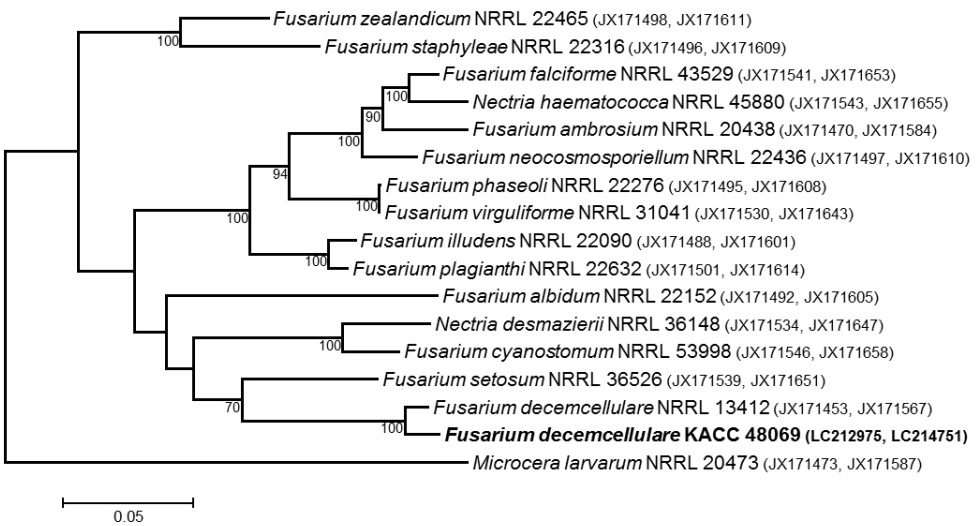

Fig. 5. Maximum likelihood phylogenetic tree, based on the combined partial RPB1 and RPB2 gene sequences, showing relationship between *Fusarium decemcellulare* KACC 48069 and closest *Fusarium* species. *Microcera larvarum* (NRRL 20473) was used as the out-group. The numbers above the branches represent the bootstrap values obtained for 1,000 replicates (values smaller than 70% were not shown). The scale bar represents a phylogenetic distance of 0.05%.

Several *Fusarium* spp. have been associated with fruit rot or core rot symptoms in apples in the field and under postharvest conditions[23-26]. In Greece, *F. avenaceum* and *F. proliferatum* have been reported as minor causal agents of postharvest fruit rot in various apple cultivars such as Red Delicious, Golden Delicious, Granny Smith, and Fuji[25]. In addition, *Fusarium equiseti*, *F. proliferatum*, *Fusarium solani*, and *Fusarium tricinctum* have been associated with browning, moldy core, and core rot symptoms in Fuji grown under field conditions in China[26]. Among them, *F. avenaceum* is the predominant fungal agent that causes wet core rot, which has been reported in Croatia, Netherland, USA, Italy, and Greece[7, 23, 25, 27, 28]. Wet core rot causes not only economic losses but also safety problems for consumers because of the production of mycotoxins such as aurofusarin and enniatin[24]. Similarly, *F. decemcellulare* produces naphthoquinone and trichothecene mycotoxins[29, 30]. The pathogenicity of *F. decemcellulare* confirmed in Hongro and Fuji, two major cultivars that are widely cultivated in Korea, suggests that the brown spots caused by *F. decemcellulare* can spread nationwide. In this study, abnormal brown spots were observed and studied in an orchard in Gimcheon, Gyeongsangbuk-do Province. Further studies are required to survey the nationwide incidence rate of brown spots caused by *F. decemcellulare*. In addition, it is important to find control methods for the brown spots to protect food and prevent economic losses.

Acknowledgements

This research was supported by Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ0104812016), Rural Development Administration, Republic of Korea.

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