

## Co-Occurrence of Two Phylogenetic Clades of *Pseudoperonospora cubensis*, the Causal Agent of Downy Mildew Disease, on Oriental Pickling Melon

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### ABSTRACT

The genus *Pseudoperonospora*, an obligate biotrophic group of Oomycota, causes the most destructive foliar downy mildew disease on many economically important crops and wild plants. A previously unreported disease by *Pseudoperonospora* was found on oriental pickling melon (*Cucumis melo* var. *conomon*) in Korea, which is a minor crop cultivated in the temperate climate zone of East Asia, including China, Korea, and Japan. Based on molecular phylogenetic and morphological analyses, the causal agent was identified as *Pseudoperonospora cubensis*, and its pathogenicity has been proven. Importantly, two phylogenetic clades of *P. cubensis*, harboring probably two distinct species, were detected within the same plots, suggesting simultaneous coexistence of the two clades. This is the first report of *P. cubensis* causing downy mildew on oriental pickling melon in Korea, and the confirmation of presence of two phylogenetic clades of this pathogen in Korea. Given the high incidence of *P. cubensis* and high susceptibility of oriental pickling melon to this disease, phytosanitary measures, including rapid diagnosis and effective control management, are urgently required.

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Downy mildew (Peronosporaceae; Oomycota) is an obligate biotrophic group that infects a wide range of monocotyledonous and dicotyledonous plants, including many economically relevant crops [1]. The genus *Pseudoperonospora* is a small group comprising only six species but includes two notoriously pathogenic species, *Pseudoperonospora cubensis* and *Pseudoperonospora humulii*. *P. cubensis* infects many cucurbitaceous crops, such as cucumber, gourd, melon, pumpkin, and watermelon [2,3], and more than 60 host plants have been listed [4,5]. *P. humuli* is one of the most critical threats to the cultivation of hops (Cannabaceae) [6,7]. Given their association with high economic losses, many recent studies have focused on the biology, host specificity, population structure, detection, and control of *Pseudoperonospora* species [3,8–13] as well as their taxonomy and phylogeny [14,15].

To date, five *Pseudoperonospora* species have been reported in Korea, namely *P. cannabina*, *P. cubensis*, *P. humuli* [15–18], *P. celtidis* [16], and *P. urticae* [19]. Among them, *P. cubensis* is the most destructive pathogen on both wild and cultivated Cucurbitaceae in Korea, including *Citrullus vulgaris*, *Cucumis melo*, *Cucumis sativus*, *Cucurbita moschata* [17], *Lagenaria siceraria* [20], and *Trichosanthes*

*kirilowii* [18]. Particularly, *P. cubensis* raises major economic concerns in the cultivation of *C. melo*, *C. sativus*, and *C. moschata*.

Recently, two phylogenetic clades within *P. cubensis*, Clades 1 and 2, were described by molecular sequence analysis [14]. Although the clustering showed no clear relevance with the pathotype or geographic distribution [13], Clade 1 seems to be associated with the recent severe epidemics of cucurbit downy mildew in Europe and the United States, while Clade 2 includes pre-epidemic samples originating from East Asia. Interestingly, both clades were often detected in the Czech Republic, Germany, and the USA [13,14]. To date, however, there has been no research to ascertain whether the cucurbit downy mildews in East Asia are caused by two clades, similar to the cases reported in the above countries, or by a particular group, and the identity of the predominant clade remains unclear.

Oriental pickling melon (*C. melo* var. *conomon*) is a minor crop cultivated in the temperate climate zone of East Asia, including China, Korea, and Japan. Young fruits are harvested and processed to make salty pickles by putting them in sake lees. From April to June 2020, typical symptoms of downy mildew were observed on the leaves of



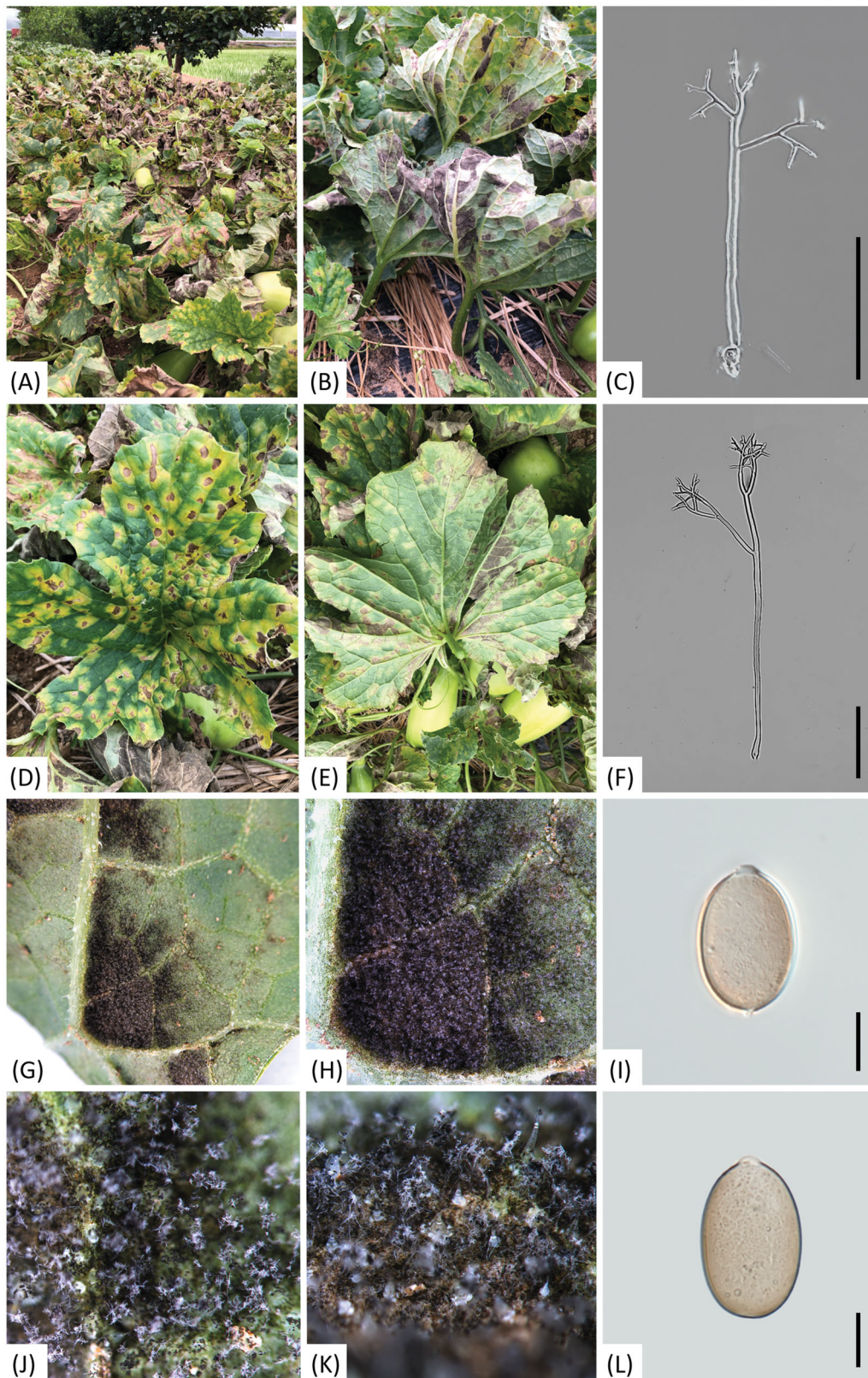
**Figure 1.** Geographic distribution of *Pseudoperonospora cubensis* specimens collected on oriental pickling melon (*Cucumis melo* var. *conomon*) in Korea. The four plots where *P. cubensis* specimens were collected are marked on Google Earth map with different colored circles; red for plot 1, blue for plot 2, purple for plot 3, and yellow for plot 4.

oriental pickling melon (*C. melo* var. *conomon*) growing in Gunsan, Korea (Figure 1). The disease incidence was high, reaching 30–50% from April to May, but was approximately 90% in June. This study aimed to identify the causal agent of downy mildew on oriental pickling melon in Korea and to determine the causal clade among two phylogenetic clades of *P. cubensis*, using detailed morphological and multi-locus phylogenetic investigations.

On oriental pickling melon, downy mildew infection resulted in discoloration of the leaf tissues, with yellowish to brownish spots on the upper leaf surfaces (Figure 2(A,B,D,E)). The lesions were water-soaked, polyangular, and clearly delimited by the leaf veins (Figure 2(G,H)). A distinctive gray to dark brown, dense oomycete growth was observed on the corresponding abaxial leaf surface (Figure 2(J,K)). As the disease progressed, the spots turned blackish and often merged to cover larger areas. Several leaves showing downy mildew symptoms were collected within each of four melon plots (116, 164, 780, and 800 m<sup>2</sup>). Figure 1 displays the site map and a close view of sample locations, which were programmatically marked on Google Earth. All samples were deposited in the Kunsan National University Herbarium (KSNUH), and information

on the dried herbarium samples has been provided in Table 1.

For a detailed microscopic examination, sporangiophores and sporangia formed underneath the infected leaves were transferred to a drop of lactic acid on a glass slide, covered with a coverslip, and gently warmed using an alcohol lamp. The microscope measurements were examined under the BX53F DIC-light microscope (Olympus, Tokyo, Japan) with the DigiRetina 16M digital camera (Tucsen, Fuzhou, China) or under the Zeiss Imager M2 AX10 microscope (Carl Zeiss, Jena, Germany) with the AxioCam 512 camera (Carl Zeiss, Jena, Germany). Measurements were reported as follows; (minimum–)standard deviation toward the minimum – mean – standard deviation toward the maximum (–maximum). Sporangioophores emerging through the stomata were tree-like, hyaline, straight, or slightly curved, (202.6–)256.3–355.3(–421.9)(av. 305.8) × (4.7–)5.9–8.2(–10.2)(av. 7.1)(*n* = 100), and monopodially branched in 3–4 orders (Figure 2(C,F)). Ultimate branchlets were in pairs, straight to slightly curved (3.6–)6.3–11.6(–15.6)(av. 9.0) μm long, (1.0–)1.3–1.9(–2.5)(av. 1.69) μm wide at the base (*n* = 100), with a truncate or rarely swollen tip. Sporangia were brownish, ovoidal, or lemon-shaped,



**Figure 2.** Downy mildew disease caused by *Pseudoperonospora cubensis* on oriental pickling melon (*Cucumis melo* var. *conomon*) in Korea. (A and B) Downy mildew outbreak in a field of oriental pickling melon; (D and E) Vein-limited spots above (D) and below (E) an infected leaf; (G and H) Close-up view of vein-limited downy mildew growth developing on the lower surface; (J and K) Dense sporangiophores with grayish, numerous sporangia; (C and F) Sporangiophore; (I and L) Sporangium. Scale bars: 100  $\mu\text{m}$  for sporangiophore; 10  $\mu\text{m}$  for sporangium.

measured (19.9–)22.6–27.0(–29.8)(av. 24.8)  $\times$  (14.3–)15.4–17.9(–19.6)(av. 16.7)  $\mu\text{m}$ , with a length/width ratio of (1.30–)1.41–1.57(–1.66)(av.

1.49)( $n = 100$ ) (Figure 2(I,L)). Resting organs were not observed. Morphological observations revealed that this oomycete unequivocally belonged to the

**Table 1.** Information of *Pseudoperonospora* collections parasitic to oriental pickling melon (*Cucumis melo* var. *conomon*) used in this study.

Plot number (size m <sup>2</sup> )	Herbarium number	Geographic location	GPS	Date	DNA number	Phylogenetic clade	GenBank Acc. No. ( <i>cox2</i> )
1 (ca. 800)	KSNUH621	Chango-ri 88-2, Seongsan- myeon, Gunsan-si, Korea	35°59'7.67"N 126°49'33.39"E	June 23 2020	D822A	1	MW273007
					D822B	1	MW273021
					D822C	1	MW273027
					D822D	1	MW273025
					D822E	1	MW273026
					D822F	1	MW273020
					D822G	1	MW273017
2 (ca. 780)	KSNUH623	Chango-ri 106-1, Seongsan- myeon, Gunsan-si, Korea	35°59'14.0"N 126°49'41.4"E	June 23 2020	D824A	1	MW273008
					D824B	1	MW273019
					D824C	1	MW273016
3 (ca. 164)	KSNUH625	Chango-ri 103-1, Seongsan- myeon, Gunsan-si, Korea	35°59'10.6"N 126°49'39.0"E	June 23 2020	D826A	1	MW273015
					D826B	2	MW273010
					D826C	2	MW273012
					D826D	1	MW273014
					D826E	2	MW273013
					D826F	2	MW273009
4 (ca. 116)	KSNUH626	Chango-ri 55-5, Seongsan- myeon, Gunsan-si, Korea	35°59'18.0"N 126°49'36.7"E	June 23 2020	D827A	1	MW273018
					D827B	2	MW273022
					D827C	2	MW273006
					D827D	2	MW273023
					D827E	2	MW273011
					D827F	2	MW273024

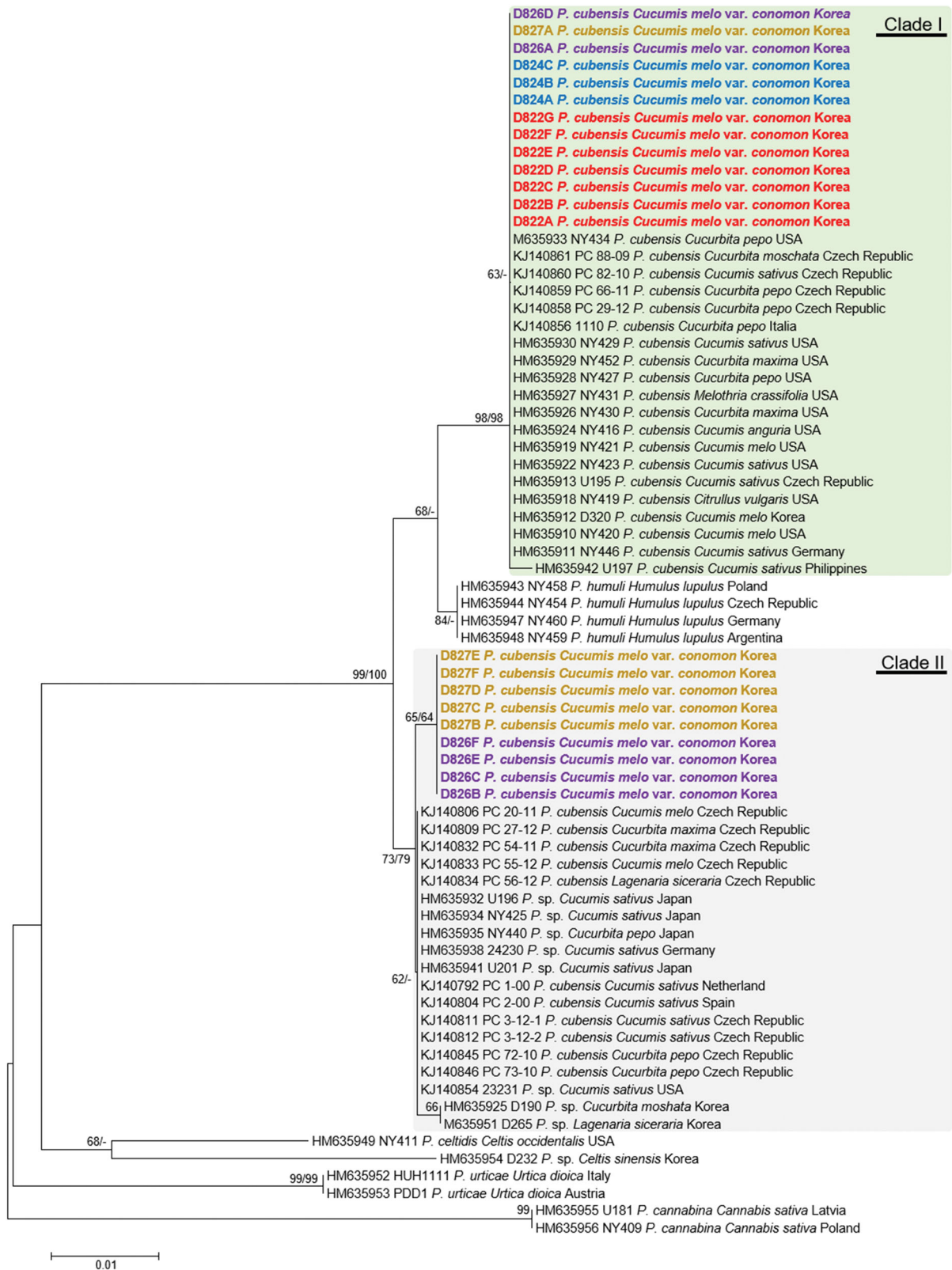
genus *Pseudoperonospora*, and the characteristics were consistent with those described from *P. cubensis* (Berk. & Curt.) Rostov. [21].

For molecular sequence analysis, genomic DNA was extracted from the infected plant tissue of the herbarium specimens using the MagListo 5M plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The mitochondrial cytochrome c oxidase II (*cox2*) gene was sequenced using primers *cox2*-F [22] and *cox2*-RC4 [23]. Amplicons were visualized on 1.2% agarose gel, purified using the AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea), and sequenced by a DNA sequencing service (Macrogen, Seoul, Korea), with the primers used for amplification. The resulting sequences were edited using the DNASTAR software package version 5.05 (DNASTAR, Madison, WI) and deposited in GenBank (see Table 1). In addition to the reference sequences of *Pseudoperonospora* species available in NCBI GenBank, they were aligned using MAFFT version 7 [24] with the Q-INS-I algorithm [25]. Minimum evolution (ME) and maximum likelihood trees were constructed with MEGA version 7.0 [26], using the Kimura 2-parameter model and performing 10,000 bootstrap replicates.

Phylogenetic structures of *Pseudoperonospora* species, including 22 Korean specimens of oriental pickling, were inferred by the ME and ML methods using *cox2* mtDNA sequences, a barcoding locus of oomycetes. As the two trees were congruent, only one ME tree is shown in Figure 3. In both analyses, the Korean specimens were divided into two different clades, matching each of the two phylogenetic clades of *P. cubensis*. A sequence dissimilarity of 1.4% (7 out of 472 characters were different) was

observed between the two clades. Clade 1 includes *Pseudoperonospora* specimens of *Citrullus*, *Cucurbita*, *Cucumis*, and *Melothria* spp. originating from the Czech Republic, Germany, Italy, Korea, the Philippines, and the USA, with a high bootstrapping value of 98/98%. Clade 2 includes *Cucurbita*, *Cucumis*, and *Lagenaria* specimens originating from the Czech Republic, Germany, Japan, Korea, the Netherlands, Spain, and the USA, with a moderate value of 73/79%. Within Clade 2, the Korean specimens formed a subgroup with a weak value of 65/64%. Of the four melon plots of the downy mildew collections, only Clade 1 was detected in plots 1 ( $n=7$ ) and 2 ( $n=3$ ), Clades 1 and 2 coexisted in the same number in plot 3 ( $n=6$ ), and Clade 2 was predominant in plot 4 ( $n=6$ ).

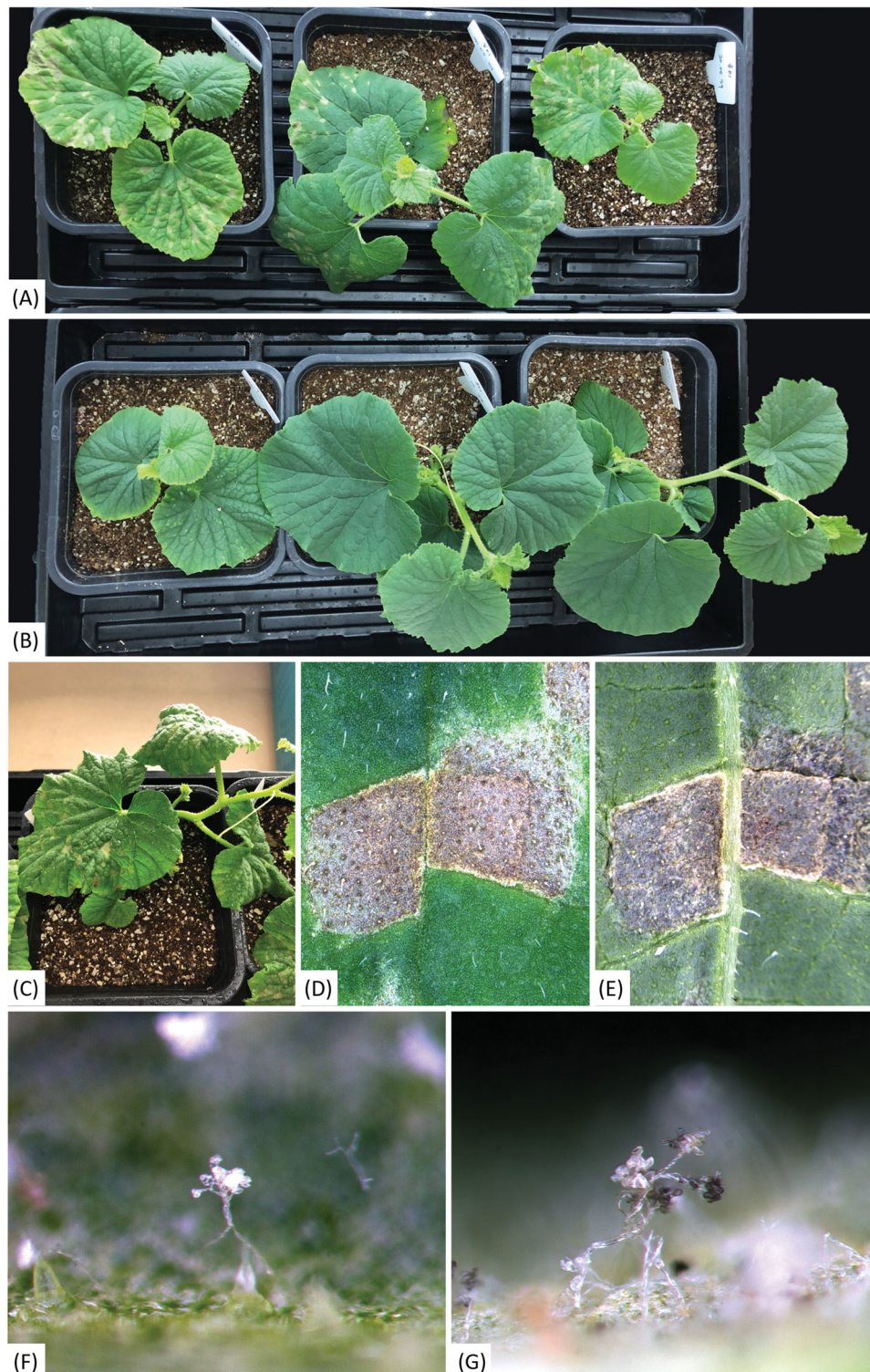
For the pathogenicity test, sporangia were harvested from each infected leaf of two clades (826A in Clade 1 and 826B in Clade 2) collected at site 3. They were suspended in sterile water ( $1.0 \times 10^6$  sporangia/mL) and sprayed onto the lower leaf surface of five healthy *C. melo* var. *conomon*. Five control plants were sprayed with distilled water. All plants were incubated in a growth chamber at 22 °C under a relative humidity of 95%. After 7 d, both isolates (826A and 826B) developed downy mildew symptoms on the inoculated leaves. Since there is no difference in disease incidence and characteristics between the two isolates, only the results of the isolate 826A are shown in Figure 4. It formed yellow to green polygonal lesions on the leaves (Figure 4(A)), whereas the control plants remained symptomless (Figure 4(B)). White sporangiophores and dark grayish sporangia were observed on the vein-limited spots of the inoculated leaves (Figure



**Figure 3.** Minimum evolution tree of *Pseudoperonospora* species using *cox2* mtDNA sequences. Bootstrapping values (minimum evolution BP/maximum likelihood BP) higher than 60% are shown above the branches (10,000 replicates). The scale bar equals the number of nucleotide substitutions per site. *Pseudoperonospora* specimens collected in four plots were marked as different colors; red for plot 1, blue for plot 2, purple for plot 3, and yellow for plot 4.

4(C-E)). Their morphological characteristics (Figure 4(F,G)) and *cox2* sequences were consistent with those initially investigated.

Based on morphological characteristics, molecular sequencing data, and pathogenicity test, the pathogen was identified as *P. cubensis*. Previously, *P.*



**Figure 4.** Pathogenicity assay of *Pseudoperonospora cubensis* isolate D826A on oriental pickling melon (*Cucumis melo* var. *conomon*). (A) Oriental pickling melon plants a week after inoculation; (B) Uninoculated plants a week after inoculation; (C–E) Vein-limited spots above (C and D) and below (E) an infected leaf; (F and G) Sporangioophores emerging from stomata, with immature sporangia without melanin pigment (F) and mature sporangia with melanin pigment (G).

*cubensis* has been recorded on *C. melo* var. *conomon* in Japan [27] and under laboratory conditions by Zitter et al. [28]. To our knowledge, this is the first report of downy mildew caused by *P. cubensis* on *C. melo* var. *conomon* in Korea. With the recent increase in the popularity of oriental pickling melons, the growing area of this crop in Korea is

gradually expanding. However, the high disease severity and low resistance in melons pose a potential risk for oriental pickling melon production.

Beyond the geographic origin and distribution of the two clades of *P. cubensis*, it should be noted that both clades coexist within a few countries [13,14], including Korea. Additionally, this study has

demonstrated that two clades of *P. cubensis* can coexist even within a small crop plot at the same time. However, it remains unclear whether such a simultaneous coexistence is ubiquitous in other global cucurbit farms, and there is no research on the extent of damage caused by each clade and a disease management method specific to the clade. As the overlapping host ranges and indistinguishable morphological characteristics of the clades pose challenges for their detection and diagnosis in the field and laboratory, there is an urgent need to develop a clade-specific molecular diagnostic method and to study whether they can be distinguished at the species level.

### Disclosure statement

No potential conflict of interest was reported by the author(s).

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