Res. Bull. Pl. Prot. Japan No. 61：1 ～ 6（2025）

Short communication

First Report of *Acidovorax avenae* subsp. *citrulli* Intercepted from Seeds of *Cucurbita maxima* × *C. moschata* from China at Import Plant Quarantine Inspection in Japan

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Abstract: *Acidovorax avenae* subsp. *citrulli* (Aac; syn. *Acidovorax citrulli*), the causal agent of bacterial fruit blotch was intercepted from seeds of *Cucurbita maxima* × *C. moschata* imported from China at an import plant quarantine inspection in Japan. The bacterial isolates were pathogenic to the original host and 6 cucurbit plants, and were identified as Aac based on the bacteriological characteristics and the molecular biological analysis of 16S rRNA and *pilT* gene. In addition, seed transmission was demonstrated by grow-out assay. This is the first report of Aac on *Cucurbita maxima* × *C. moschata*.

Key Words: *Acidovorax avenae* subsp. *citrulli*, Aac, *Cucurbita maxima* × *C. moschata*, bacterial fruit blotch, seed transmission

Introduction

Acidovorax avenae subsp. *citrulli* (Aac; syn. *Acidovorax citrulli*), the causal agent of bacterial fruit blotch, is a seed-borne bacterial pathogen (Rane and Latin, 1992; Hopkins and Thompson, 2002) and a serious threat to cucurbit industry worldwide (Burdman and Walcott, 2012). Host plants imported from regions/countries where Aac occurs are required to undertake specific quarantine measures based on the Plant Protection Act in order to prevent the invasion of Aac into Japan. Emergency measures are implemented when there is new information on new host plants or distributions.

Seeds of Cucurbita hybrid (*Cucurbita maxima* × *C. moschata*) imported into the port of Yokohama from China were confirmed Aac positive by Loop-mediated Isothermal Amplification (LAMP) (Oya *et al*., 2008) at a plant quarantine inspection in December 2022.

The purpose of this study is to isolate and identify the isolates as Aac, and to demonstrate seed transmission on Cucurbita hybrid, the probable host plant, which has not been known in the world until now.

Material and methods

1. Isolation

LAMP-positive seed lot was used in this study. The Sweat-bag Seedling (SBS) method (Sato, 2010; Shirakawa, 2021) was carried out to propagate the target bacteria in the seeds. Using the SBS method, 1,000 seeds of Cucurbita hybrid were sowed onto paper towels and incubated at 28°C in plastic bags while maintaining saturated humidity. After 8-12 days, approximately 500ml of sterile water was added to the plastic bags to wash all sprouted seedlings. The washing solutions were collected and used as the isolation source. The target bacteria were propagated more using the host plant inoculation method (Ikegami *et al*., 2018), and the bacteria were isolated using a dilution plate method with Incomplete-AacSM (Kubota *et al*., 2011).

2. Pathogenicity

The isolates used in this study were grown for 2-3 days on yeast peptone agar (YPA) plates at 27°C, then suspended in sterile water at approximately 10^8 cfu/ml. Aac strain YPPS319 (Oya *et al*., 2008) and sterile water were used as positive and negative controls, respectively. Bundled needles were dipped in the suspensions and wound-inoculated to true leaves of the original host and 6 cucurbit plants (Table 1) at the stage of 2-4 true leaves. The inoculated plants were grown under moist conditions in a growth chamber at 30°C/27°C with 12h fluorescent light/dark for 7 days.

3. Bacteriological characteristics

The bacteriological characteristics were tested by API20NE (BioMerieux, France) according to the manufacturer's instructions. The profile index on API20NE was scored after 3 days incubation,

and searched using the API-96 database (Nishiyama, 1997).

4. Molecular biological analysis

Partial 16S rRNA and *pilT* gene were amplified with primers 16SF3/16SR3 (Sawada *et al*., 2011) and pilT-F/pilT-R (Feng *et al*., 2009), and the PCR fragments were directly sequenced. Homologies of the partial sequences were analyzed using a BLAST search of the National Center for Biotechnology Information (NCBI) database. Phylogenetic analysis was performed using each partial sequence of the isolates and several other *Acidovorax* species (*A. avenae*, *A. oryzae* and *A. cattleyae*) obtained from the NCBI database using the neighbor-joining method with MEGA11 (Tamura *et al*., 2021).

5. Grow-out assay

About 350 seeds of LAMP-positive seed lot were planted into a plastic tray (35×50×7 cm) filled with commercial soil, and it was enclosed in transparent plastic box to maintain high humidity. It was then incubated in a growth chamber at 30°C/25°C with 12h fluorescent light/dark for about 2 weeks. The bacteria were isolated from the lesions and confirmed Aac by LAMP.

Results

1. Isolation

Direct isolation from the SBS solutions did not go well. Using the host plant inoculation method, Aac-like colonies were isolated from inoculated cotyledon (Fig. 1A). Two representative isolates (22A64-1, 22A64-2) were subjected to the following tests.

2. Pathogenicity

The isolates and positive control produced symptoms on all tested plants (Table1 and Fig. 1B-E), and negative control showed no symptoms. The isolates were re-isolated from the symptoms. The pathogenicity was strong for pumpkin (*Cucurbita maxima*), watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus*), but mild for Cucurbita hybrid, Japanese pumpkin (*Cucurbita moschata*), zucchini (*Cucurbita pepo*) and melon (*Cucumis melo*).

3. Bacteriological characteristics

In API20NE tests, the same results were obtained from the two isolates. After 3 days incubation, the isolates were positive for nitrate

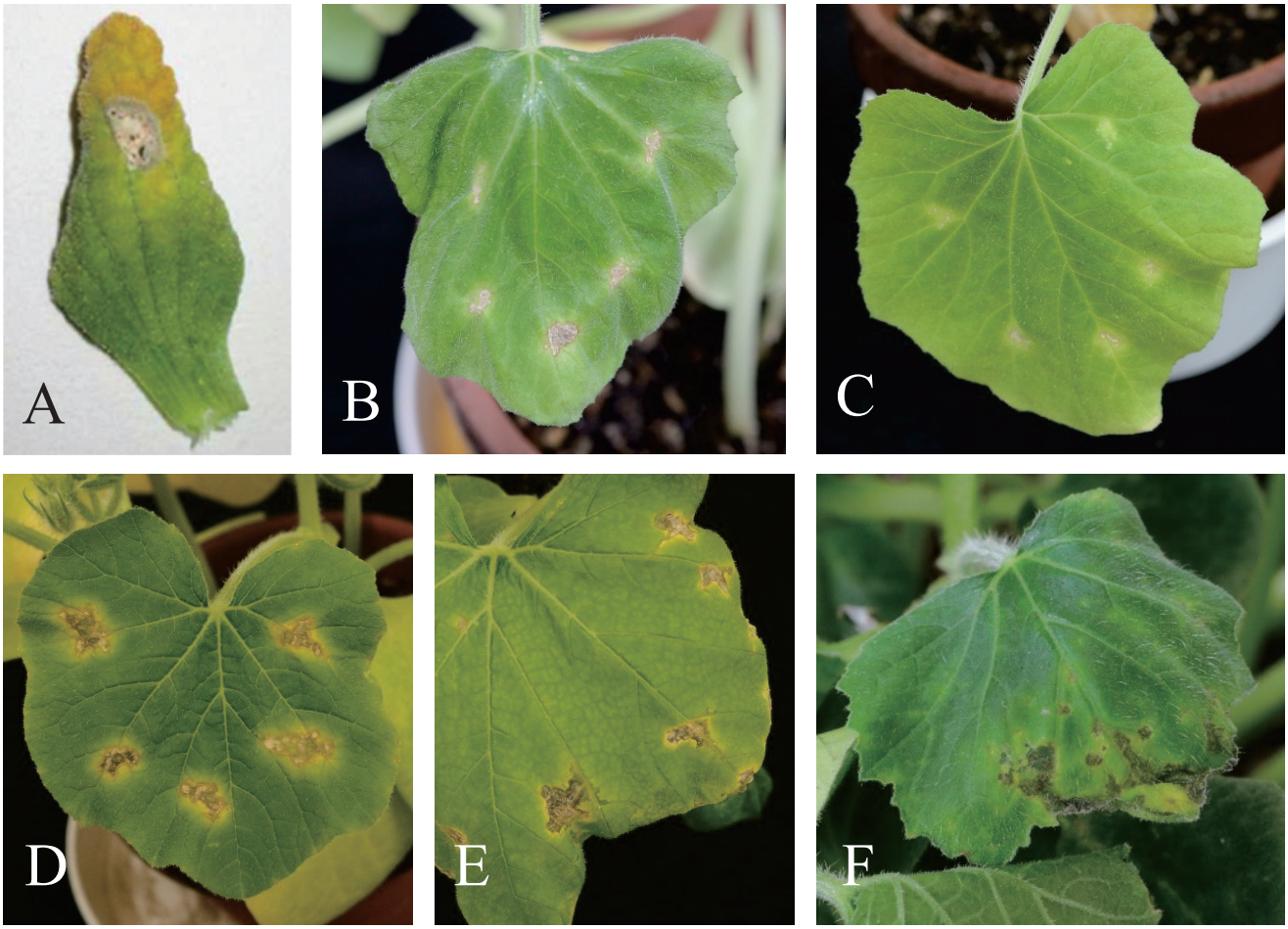

Fig. 1. Symptoms in host plant inoculation method, pathogenicity test, and Grow-out assay.
A: Pumpkin cotyledon 2 weeks after inoculation using host plant inoculation method. B-E: Cucurbita hybrid (B, C) and Pumpkin (D, E) leaves 7 days after wound-inoculation with the isolate 22A64-2 (B, D) and Aac strain YPPS319 (C, E). The pathogenicity of these two strains was strong for Pumpkin (D, E), but mild for Cucurbita hybrid (B, C). F: Cucurbita hybrid (LAMP-positive seed lot) leaf 2 weeks after sowing in Grow-out assay.

Table 1. Pathogenicity of isolates to cucurbit plants.

Plant (Scientific Name)		Isolates 22A64-1	Isolates 22A64-2	Positive control YPPS319	Negative control sterile water
Cucurbita Hybrid (*Cucurbita maxima* × *C. moschata*)	Cultivar A	10/10[*1]	5/5	5/5	0/5
		(+)[*2]	(+)	(+)	(-)
	Cultivar B	10/10	10/10	5/5	0/5
		(+)	(+)	(+)	(-)
Pumpkin (*Cucurbita maxima*)		10/10	10/10	5/5	0/5
		(++)	(++)	(++)	(-)
Japanese Pumpkin (*Cucurbita moschata*)		10/10	10/10	5/5	0/5
		(+)	(+)	(+)	(-)
Zucchini (*Cucurbita pepo*)		10/10	10/10	5/5	0/5
		(+)	(+)	(+)	(-)
Watermelon (*Citrullus lanatus*)		2/2	2/2	1/1	0/1
		(++)	(++)	(++)	(-)
Melon (*Cucumis melo*)		4/4	3/4	2/2	0/2
		(+)	(+)	(+)	(-)
Cucumber (*Cucumis sativus*)		10/10	10/10	5/5	0/5
		(++)	(++)	(++)	(-)

[*1] Number of diseased leaves / total number of inoculated ones

[*2] ++ : Severe symptoms, + : Mild symptoms, - : No symptoms

reduction, oxidase activity, assimilation of L-arabinose, potassium gluconate, adipic acid and malic acid, and they were negative for indole production, glucose fermentation, arginine dihydrolase, urease activity, esculin hydrolysis, gelatin hydrolysis, β-galactosidase activity, assimilation of D-glucose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, capric acid, trisodium citrate and phenylacetic acid. Therefore, the profile index of the isolates scored 1001464, which corresponds to Aac.

4. Molecular biological analysis

The obtained sequences of the respective genes were completely identical among the two isolates (16S rRNA: 1427bp, *pilT*: 421bp). From a blast and phylogenetic analysis of 16S rRNA sequences, the isolates suggest that they belong to the genus *Acidovorax* although these were not distinguishable between *A. avenae*, *A. oryzae*, *A. cattleyae* and Aac (Data not shown). The *pilT* sequences of the isolates shared high identities (over 99%) with the available sequences of Aac, and phylogenetic analysis of *pilT* gene on these 4 species showed that the isolates form a monophyletic group with Aac (96% bootstrap value) and were clearly separated from the others (Fig. 2).

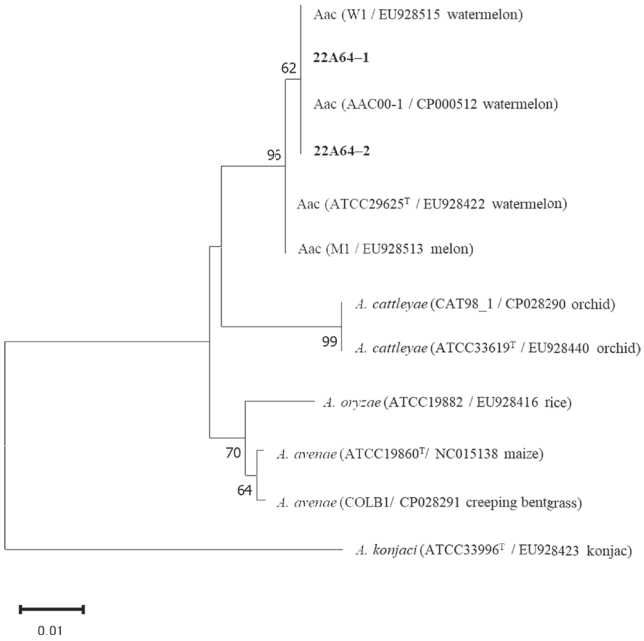

Fig. 2. Neighbor joining tree using the maximum composite likelihood based on *pilT* sequences of the isolates, *A. avenae, A. oryzae, A. cattleyae* and Aac. Numbers at nodes indicate bootstrap values (more than 60%) for 1,000 replicates, scale bar corresponds 0.01 substitutions per site. Strain numbers, accession numbers and isolation sources for sequences are in parentheses.

5. Grow-out assay

On some of germinated seedlings, brownish lesions surrounded by a yellow halo on true leaves were observed after 2 weeks inoculation (Fig. 1F). The lesions were observed on 28% of the total germinated seedlings, however primary and secondary infection could not be distinguished. Seven isolates obtained from the lesions of each seedling were confirmed Aac positive by LAMP.

Discussion

Based on these results, we identified the isolates from seeds of Cucurbita hybrid as Aac. Cucurbita hybrid has not been reported as a host plant for Aac before, and we thus propose that Cucurbita hybrid is added as a new host plant.

Moreover, we demonstrated seed transmission on Cucurbita hybrid by Grow-out assay. The results here provide an evidentiary base for the introduction and maintenance of quarantine measures to prevent domestic invasions of Aac.

Acknowledgments

We conducted this study using Japanese Patent No. 4633645 (Sato, 2011). We would like to thank the National Agriculture and Food Research Organization for permitting us the use of this patent. We would also like to thank Hiroshi Uematsu (Haneda airport Sub-station Yokohama Plant Protection Station) for his invaluable advice.

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和　文　摘　要

我が国の輸入植物検疫で中国産セイヨウカボチャ及びニホンカボチャの交雑種の種子から初めて分離されたスイカ果実汚斑細菌病菌

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2022 年 12 月、横浜港に輸入された中国産セイヨウカボチャ及びニホンカボチャの交雑種（*Cucurbita maxima* × *C. moschata*: カボチャ交雑種）種子の輸入検査において、LAMP 法によりスイカ果実汚斑細菌病菌（*Acidovorax avenae* subsp. *citrulli*: Aac）が検出された。種子からの分離菌株は、カボチャ交雑種等のウリ科植物に病原性を示し、細菌学的性状並びに 16S rRNA 及び *pilT* の分子系統解析に基づいた同定の結果、Aac と同定した。さらに、当該種子の栽培検定により種子伝染を実証した。カボチャ交雑種における Aac の発生は世界的に未報告であるため、カボチャ交雑種を Aac の宿主植物に追加することを提案する。