Prevalence of *Acidovorax citrulli* in Commercial Cucurbit Seedlots During 2010–2018 in China

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Abstract

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB), a serious threat to cucurbit fruit and seed production worldwide. In recent years, the BFB has spread to many areas of China, mainly via the inadvertent distribution of contaminated commercial seeds. To assess the prevalence of seedborne *A. citrulli* in commercial watermelon and other cucurbitaceous seedlots in China, a 9-year survey was conducted between 2010 and 2018. A total of 4,839 seedlots of watermelon and other cucurbitaceous species were collected from 13 major seed production areas of China and tested by a semiselective media-based colony PCR technique for *A. citrulli*. Overall, *A. citrulli* was detected in 18.00% (871/4,839) of all cucurbitaceous seedlots. The bacterium was detected in 21.59% (38/176), 19.19% (33/172), 23.44% (214/913), 40.76% (247/606), 13.28% (85/640), 15.40% (95/617), 13.25% (73/551), 8.03%

(48/598), and 6.71% (38/566) of all commercial seedlots tested from the 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, and 2018 growing seasons, respectively. Additionally, the prevalence of *A. citrulli* in cucurbit seedlots was determined for different seed production areas. The prevalence of *A. citrulli* in cucurbitaceous seedlots produced in Xinjiang, Gansu, Ningxia, Inner Mongolia, and 9 other provinces was 18.76% (582/3103), 26.34% (103/391), 21.47% (82/382), 11.11% (14/126), and 10.75% (90/837), respectively. This is the first survey for *A. citrulli* in commercial cucurbit seeds in China, and the relatively high prevalence suggests that commercial seeds represent a substantial source of primary inoculum that can threaten cucurbit seed and fruit production in China.

Keywords: prokaryotes, epidemiology, pathogen survival, small fruits

Acidovorax citrulli is the causal agent of bacterial fruit blotch (BFB) (Schaad et al. 2008) (formerly *Acidovorax avenae* subsp. *citrulli* (Willems et al. 1992) = *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (Schaad et al. 1978), a serious threat to cucurbitaceous crop (mainly watermelon [*Citrullus lanatus*] and muskmelon [*Cucumis melo*]) production worldwide. The bacterium is seedborne and seed transmitted, and infested/infected seeds represent the most important primary inoculum source for BFB epidemics (Hopkins and Thompson 2002). BFB has caused significant economic losses in cucurbit fruit and seed production worldwide. Yield losses have ranged from 60% (Latin and Hopkins 1995) in severe epidemics to 80 to 100% in some regions of China (Jin et al. 2014; Shi and Yu 2015). Unfortunately, little is known about the biology of cucurbit seed infestation by *A. citrulli* under seed production field conditions; hence there are currently no effective strategies to prevent seed infection. Additionally, since BFB management strategies that rely on pathogen exclusion and seed treatments are not 100% effective, the most effective

BFB control strategy is exclusion of *A. citrulli* by using pathogen-free seeds (Burdman and Walcott 2012).

Perennially, China is the world's leading producer of watermelon and muskmelon (Xu 2011). Large differences between day and night temperatures and low relative humidity make Xinjiang Province, China highly suitable for watermelon and muskmelon cultivation. As a result, Xinjiang Province has become the largest region for watermelon and muskmelon seed production. In particular, Xinjiang represents more than 45% cucurbitaceous seed production in China (Zhao 2012). In recent years, BFB has occurred in watermelon and muskmelon crops in many provinces in China, including Xinjiang (Zhang and Mo 1996), Gansu (Xue et al. 2009), Ningxia (Jin et al. 2014), Shandong (Zhao et al. 2009), Fujian (Cai et al. 2005), and Guangdong (Ren et al. 2010). The main cause of BFB outbreaks in these provinces has been the use of *A. citrulli*-infested seeds (An et al. 2013; Zhao 2012).

To enhance cucurbit crop tolerance to Fusarium wilt, grafting and transplanting of greenhouse-grown seedlings have been used for commercial watermelon production worldwide (Nisini et al. 2002). Cucurbit grafting and seedling transplanting became a common practice around 1990 in China (Bie and Present 2009), and currently, with the exception of a few regions, direct seeding in commercial fields has been completely replaced by seedling transplanting. Seed-to-seedling transmission of BFB is heavily dependent on high relative humidity (Burdman and Walcott 2012; Rosenberg et al. 2018); hence greenhouse conditions, including high temperatures, high relative humidity, and high plant populations, are conducive to seedling transmission and rapid spread of *A. citrulli*. Additionally, many domestic seed producers do not employ effective disease management strategies to reduce the likelihood of *A. citrulli* seed infestation. Based on local Chinese planting habits and economic pressures, the 3- to 5-year crop rotation with noncucurbit hosts that is widely recommended for BFB management is almost impossible to apply. Hence, the risk of BFB epidemics is higher in China. Since 2003, BFB outbreaks have occurred in greenhouse-grown seedlings in different areas of China (An et al. 2013; Bie and Present 2009; Jin et al.

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2014; Zhao et al. 2009), resulting in widespread disease in grafted cucurbit seedlings and significant economic losses. To reduce the risk of BFB epidemics, only *A. citrulli*-free seeds should be planted. Hence, it is essential to determine the prevalence of seedborne *A. citrulli*, especially in major watermelon-growing provinces of China. To assess this, we conducted a 9-year survey for *A. citrulli* in commercial cucurbit crop seedlots in China.

Materials and Methods

Sampling of commercial cucurbit seedlots. A 9-year seed survey was conducted in China for *A. citrulli* from 2010 to 2018. A total of 4,839 cucurbit species seedlots were collected from 13 provinces of China and tested for *A. citrulli* as described below.

About four-fifths of seedlots were sampled from the northwest of China (Xinjiang, Ningxia, Gansu, and Inner Mongolia) due to the high percentage of cucurbitaceous seed production in this region. For each sample, 1,000 seeds were collected.

Seed sample preparation and testing for *A. citrulli*. In order to test for *A. citrulli* cells that may be in or on seeds, samples ($n = 500$

seeds/lot) were individually “cracked” (not crushed) with a sterilized fingernail clipper (Supplementary Fig. S1) and incubated in 100 ml of PBST (phosphate buffered saline + Tween 20) with agitation on a shaker for 4 h at room temperature. Subsequently, the seed wash was pelleted by centrifugation at $6,000 \times g$ for 3 min and the pellet was resuspended in 50–100 μ l of PBST.

Colony PCR. Colony PCR (Woodman 2008) was used for rapid bacterial identification, which eliminated the need to purify genomic DNA (Pardesi and Tompkins 2015). We routinely used semiselective media-based colony PCR assay with BX-L1/BX-S-R2 primers (Bahar et al. 2008) to detect and recover *A. citrulli* in seed samples. Briefly, the resuspended pellet was streaked onto 3 plates of TWZ semiselective media (0.5% peptone, 0.025% CaCl_2 , 1% Tween-80, 50 mg/liter Berberine, 50 mg/liter cycloheximide, 50 mg/liter 2, 3, 5-triphenyltetrazolium chloride). After incubation at 28°C for 48–72 h, 15 cream-colored colonies with red centers were arbitrarily selected from 3 plates for colony PCR. If all three plates had less than 15 colonies, all putative *A. citrulli* colonies were selected for PCR assay.

Table 1. Results of tests on samples of cucurbit seed lots tested for *Acidovorax citrulli* collected from various provinces in China in 2010 to 2018

Years	Provinces	No. of seed lots surveyed	Positive seed lots (frequency %)	Positive seed lots/total samples (frequency %)		
				Watermelon	Muskmelon	Rootstock
2010	Xinjiang	176	38	36/164	2/12	/
	Subtotal	176	38 (21.59)	36/164 (21.95)	2/12 (16.67)	/
2011	Xinjiang	168	32	25/132	7/36	/
	Ningxia	4	1	1/4	/	/
2012	Subtotal	172	33 (19.19)	26/136 (19.12)	7/36 (19.44)	/
	Xinjiang	739	155	140/658	8/40	7/41
2013	Ningxia	34	8	8/34	/	/
	Gansu	140	51	34/80	/	17/60
2014	Subtotal	913	214 (23.44)	182/772 (23.58)	8/40 (20.00)	24/101
	Xinjiang	417	184	150/364	34/52	0/1
2015	Ningxia	40	23	20/32	3/8	/
	Gansu	39	21	20/26	0/12	1/1
2016	Heilongjiang	8	1	/	1/8	/
	Jilin	6	1	/	1/6	/
2017	Inner Mongolia	8	1	/	1/8	/
	Hebei	3	0	/	0/3	/
2018	Shandong	17	1	/	/	1/17
	Anhui	11	6	/	/	6/11
2019	Shanghai	56	8	/	/	8/56
	Fujian	1	1	/	/	1/1
2020	Subtotal	606	247 (40.76)	190/422 (45.02)	40/97 (41.24)	17/87 (19.54)
	Xinjiang	345	42	22/264	20/81	/
2021	Ningxia	61	12	12/53	0/8	/
	Gansu	52	9	8/38	1/8	0/6
2022	Heilongjiang	15	2	0/6	2/8	0/1
	Jilin	11	4	3/8	1/3	/
2023	Inner Mongolia	15	3	2/6	1/8	0/1
	Hebei	25	4	4/23	0/1	0/1
2024	Shandong	34	1	/	/	1/34
	Shānxi	11	3	0/1	/	3/10
2025	Anhui	50	5	3/28	/	2/22
	Shanghai	21	0	/	/	0/21
2026	Subtotal	640	85 (13.28)	54/427 (12.65)	25/117 (21.37)	6/96 (6.25)
	Xinjiang	312	48	31/211	13/81	4/20
2027	Ningxia	71	16	12/35	4/26	0/10
	Gansu	50	9	8/20	1/20	0/10
2028	Heilongjiang	25	4	3/12	1/13	/
	Jilin	10	4	1/2	3/8	/
2029	Inner Mongolia	19	2	2/5	0/14	/
	Shandong	30	5	/	0/2	5/28
2030	Shānxi	10	0	0/2	/	0/8
	Anhui	70	3	3/44	/	0/26
2031	Shanghai	20	4	0	/	4/20
	Subtotal	617	95 (15.40)	60/331 (18.13)	22/164 (13.41)	13/122 (10.66)

(Continued on next page)

Each PCR reaction mixture contained 1.5 mM MgCl₂, 0.2 mM dNTP, 300 nM of each primer, and 1 U of *Taq* polymerase (TaKaRa, Dalian, China) in 25 µl PCR reaction mixture. Each colony (approximately 1 mm in diameter) was picked up with a sterilized toothpick and directly transferred to a PCR tube. PCR was conducted in an Eppendorf Mastercycler System 2700 (Eppendorf, Germany) using the following thermal cycle program: one cycle of 95°C for 1 min; 35 cycles of 94°C for 35 s, 68.1°C for 35 s, and 72°C for 45 s, and incubation at 72°C for 5 min. The reaction was then immediately cooled to 10°C, and 10 µl of each PCR reaction mixture was analyzed by electrophoresis on a 2.5% agarose gel at 120 V for 10 min. A seed sample was considered to be positive for *A. citrulli* if one or more colonies were positive by PCR (i.e., yielded the expected amplicon). If no *A. citrulli*-like colonies developed on the three TWZ plates, or if none of the selected colonies were positive by PCR assay, the seed sample was considered to be negative for *A. citrulli*.

Detection of *A. citrulli* within seeds. In order to determine if *A. citrulli* cells were present inside or on the surface of seeds in PCR-positive cucurbit seedlots, lots (one each of watermelon, muskmelon, and pumpkin or bottle gourd rootstock) that tested positive for *A. citrulli* were selected. Samples ($n = 100$ seeds/lot) from each lot were surface disinfested as described below to eradicate surface microflora. Briefly, each seedlot was soaked in 30% HCl for 10 min, washed three times in ddH₂O for 5 min, and then air-dried at room temperature for 1 h. Each seed was cracked open with a fingernail clipper as described above, and incubated in 500 µl of PBST in a sterilized microcentrifuge tube for 4 h at room temperature. Subsequently, the seed wash was pelleted by centrifugation at $6,000 \times g$ for 3 min, resuspended in 10 µl of PBST, and spread onto one plate of TWZ semiselective media. Putative *A. citrulli* colonies were tested by colony PCR assay as described above.

Results

Prevalence of *A. citrulli* in commercial cucurbitaceous seeds in China from 2010 to 2018. Out of 4,839 commercial cucurbit seed samples tested for *A. citrulli* between 2010 and 2018, 68.71% were watermelon, 19.37% were muskmelon, and 11.92% were rootstock (Table 1, Fig. 1). Overall, 18.00% (871/4,839) of the samples were positive for *A. citrulli* when tested by the semiselective agar enrichment followed by colony PCR assay. Based on cucurbit species tested, 18.92% (629/3,325), 17.93% (168/937), and 12.82% (74/577) of watermelon, muskmelon, and rootstock seedlots, respectively, tested positive for *A. citrulli* (Table 1, Fig. 2). The prevalence of *A. citrulli*-infested seedlots was highest in 2013 (40.76%, 247/606). Between 2010 and 2012, 3 years showed a 19.19–23.44% prevalence of *A. citrulli*-infested seedlots. Interestingly, between 2014 and 2018, 5 years showed a 6.41–15.40% prevalence of *A. citrulli*-infested seedlots.

Fig. 1. Prevalence of *Acidovorax citrulli* in commercial cucurbitaceous seeds in China from 2010 to 2018.

Table 1. (Continued from previous page)

Years	Provinces	No. of seed lots surveyed	Positive seed lots (frequency %)	Positive seed lots/total samples (frequency %)		
				Watermelon	Muskmelon	Rootstock
2016	Xinjiang	302	42	25/201	15/80	2/21
	Ningxia	66	13	7/31	5/25	1/10
	Gansu	60	6	2/30	3/20	1/10
	Heilongjiang	27	2	0/12	2/15	/
	Jilin	14	2	0/2	2/12	/
	Inner Mongolia	22	3	1/11	2/11	/
	Anhui	50	4	2/24	/	2/26
	Shanghai	10	1	/	/	1/10
	Subtotal	551	73 (13.25)	37/311 (11.90)	29/163 (17.79)	7/77 (9.09)
2017	Xinjiang	342	27	15/271	10/61	2/10
	Ningxia	56	5	2/35	3/20	0/1
	Gansu	20	2	1/10	1/10	/
	Heilongjiang	26	3	1/10	2/16	/
	Jilin	20	1	1/12	0/8	/
	Liaoning	56	4	2/17	2/39	/
	Inner Mongolia	30	2	1/15	1/12	0/3
	Shandong	18	2	0/2	/	2/16
	Anhui	25	2	1/5	/	1/20
	Shanghai	5	0	/	/	0/5
	Subtotal	598	48 (8.03)	24/377 (6.37)	19/166 (11.45)	5/55 (9.09)
2018	Xinjiang	302	14	7/249	7/51	0/2
	Ningxia	50	4	2/28	2/20	0/2
	Gansu	30	5	2/18	3/10	0/2
	Heilongjiang	29	2	1/16	1/13	/
	Jilin	30	3	2/12	1/18	/
	Liaoning	36	3	1/16	2/20	/
	Inner Mongolia	32	3	3/19	0/10	0/3
	Shandong	22	2	1/12	/	1/10
	Anhui	35	2	1/15	/	1/20
	Subtotal	566	38 (6.71)	20/385 (5.19)	16/142 (11.27)	2/39 (5.13)
	Total	4839	871 (18.00)	629/3325 (18.92)	168/937 (17.93)	74/577 (12.82)

Prevalence of *A. citrulli* in commercial cucurbitaceous seeds in different seed production areas in China. The majority of cucurbit seeds tested in this survey were produced in Xinjiang Province (64.12%); while 8.08% were produced in Gansu Province, 7.89% were produced in Ningxia Province, 2.60% were produced in Inner Mongolia, and 17.31% were produced in 9 other provinces (Fig. 3, Table 1). The prevalence of *A. citrulli*-infested seedlots was highest in Gansu Province (26.34%, 391/1,031) followed by Ningxia (21.47%, 82/382), Xinjiang (18.76%, 582/3103), and Inner Mongolia (11.11%, 14/126). The prevalence of *A. citrulli* in seedlots from the other 9 provinces combined was 10.75% (90/837) (Fig. 3, Table 1).

Frequency of detection of *A. citrulli* in internal cucurbits seed tissues. The frequencies with which we detected *A. citrulli* in the internal tissues of seeds were 2% (2/100), 6% (6/100), and 3% (3/100) for watermelon, muskmelon, and pumpkin and bottle gourd rootstock samples, respectively.

Discussion

BFB has heavy potential for economic losses in Chinese cucurbit fruit and seed production systems. While infested seeds represent a critically important source of primary inoculum for BFB outbreaks, there have been no previous attempts to determine the prevalence of *A. citrulli* in commercial cucurbit seedlots in China, nor to determine if this inoculum source is correlated to the frequency of BFB outbreaks. Accordingly, this is the first study to survey the prevalence of *A. citrulli* in commercial cucurbit seeds in China. Overall, we determined that between 2010 and 2018, 18.00% of cucurbit seed samples were infested with the pathogen.

In addition to watermelon and muskmelon, other cucurbitaceous crop species are susceptible to *A. citrulli* infection, and their seeds can be infested with, and transmit, BFB to seedlings (Hopkins and Thompson 2002). Of the 577 pumpkin and bottle gourd rootstock seed samples collected, 10.74% were positive for *A. citrulli*, respectively. In July 2012, we demonstrated that *A. citrulli* transmitted from pumpkin rootstock seeds by grafting can cause watermelon seedling blight (Tian et al. 2013). These results indicated that infected rootstock cucurbits could be significant inoculum sources of *A. citrulli*.

In China, some cucurbit varieties are produced specifically for domestic markets. For example, in Gansu and Ningxia provinces, Lvbaio and Jincheng varieties of watermelon are heavily produced for local consumption. Since there is high demand for these varieties, it is difficult to implement the 3- to 5-year crop rotations that are recommended for BFB management in these regions. Unfortunately, watermelon seedlots of Lvbaio and Jincheng varieties were both produced in regions of Gansu (Xue et al. 2009) and Ningxia (Jin et al. 2014) that have a history of BFB outbreaks. In regions where *A. citrulli* is prevalent, the risk of BFB development is high when environmental conditions are favorable for disease development. In this

Fig. 2. Prevalence of *Acidovorax citrulli* in different commercial cucurbitaceous seeds in China.

study, the majority of watermelon seed samples collected from Gansu and Ningxia provinces was comprised of Lvbaio (102/144, 70.83%) and Jincheng (106/123, 86.18%) varieties, respectively. The frequencies of *A. citrulli*-infested seedlots of watermelon varieties Lvbaio and Jincheng were 35.29% (36/102) and 33.02% (35/106), which were more than those observed in Xinjiang Province. Data from the current study suggest that cucurbit seeds produced in regions with a history of BFB have increased risk of seed infestation with *A. citrulli*. Therefore, crop rotations with noncucurbit hosts, in addition to seed testing, should be strictly implemented to mitigate the effect of *A. citrulli* in these regions.

We observed that the frequency of *A. citrulli*-infested cucurbit seedlots increased between 2010 and 2013, but decreased by more than 40% after 2014. Many factors may have contributed to the low incidence of *A. citrulli*-infested seedlots after 2014. However, the most important factor may have been a combination of the recognition of the economic impact of BFB and use of antimicrobial seed treatments. In recent years, many seed producers relied on local farmers to produce cucurbit seeds. However, all of the fruits, including those showing BFB symptoms, were used by some farmers to increase seed yield and profit. In 2013, (40.76%) (247/606) of the commercial cucurbit seedlots collected in China were positive for *A. citrulli*. At the same time, BFB outbreaks were reported in many regions of China, including Liaoning (An et al. 2013), Ningxia (Jin et al. 2014), and Shandong (Zhang et al. 2014). At this time, many small seed companies and farmers acknowledged the importance of BFB and subsequently started discarding rotten and symptomatic fruits from seed production fields and implementing seed treatments, including thermotherapy, NaOCl, HCl, and peroxyacetic acid. During 2014 to 2018, the prevalence of *A. citrulli*-infested seeds was dramatically reduced, and we speculate that improved sanitation and seed treatments were in part responsible for this reduction.

We observed that 2 to 6% of the tested cucurbit seed samples collected in China were internally infected with *A. citrulli*. This suggests that externally applied seed treatments alone will not completely eliminate seedborne *A. citrulli* inoculum in China. Highly effective treatments must be able to penetrate the seed coat to completely eliminate deep-seated bacterial cells (Burdman and Walcott 2012; Dutta et al. 2016). Hopkins et al. (1996) reported that fermentation of seeds in watermelon juice for 24–48 h, followed by treatment with 1% HCl for 15 min, eliminated BFB seedling transmission. Additionally, they reported that treatment with peroxyacetic acid completely eliminated *A. citrulli* from naturally infested watermelon and melon seeds (Hopkins et al. 2003). On the other hand, Dutta et al. demonstrated that externally applied peroxyacetic acid could not eliminate *A. citrulli* from seeds that were infected by pistil invasion, which led to the deposition of bacterial cells in the embryo of the seed (Dutta et al. 2012, 2016). More detailed studies are needed to prove that seedlots

Fig. 3. Prevalence of *Acidovorax citrulli* in commercial cucurbitaceous seeds in different seed production areas in China.

infected/infested with *A. citrulli* are not due to cross contamination during harvesting or processing in China. While no chemical or physical seed treatments are 100% effective at eradicating *A. citrulli*, seed treatments can significantly reduce BFB seed-to-seedling transmission. Nonetheless, producing *A. citrulli*-free cucurbit seed is the first step toward effective BFB management in China. Therefore, to reduce the risk of seed infestation in commercial seed production, only symptomless cucurbit fruits should be harvested, and in fact, seeds should not be harvested from fields in which symptomatic fruits and foliage have been observed. In summary, based on our survey results, *A. citrulli*-infested seed is a serious threat to the watermelon and muskmelon fruit and seed industries in China and must be reduced through comprehensive, zero-tolerance strategies in seed production fields, seed health testing, and seed treatments.

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