

Diversity and distribution of *Alternaria brassicicola* isolated from broccoli in Kagawa Prefecture, Japan

Fumihiro Nishimura

xf1540@pref.kagawa.lg.jp

Kagawa Prefecture: Kagawa-ken <https://orcid.org/0000-0001-9509-0536>

Takahiro Katayama

Kagawa Prefecture: Kagawa-ken

Mitsutaka Mori

Kagawa Prefecture: Kagawa-ken

Kyoko Murakami

Kagawa Prefecture: Kagawa-ken

Chatchawan Jantasuriyarat

Kasetsart University - Bangkhen Campus: Kasetsart University

Naoki Mori

Kobe University Faculty of Agriculture Graduate School of Agricultural Science: Kobe Daigaku Daigakuin Nogaku Kenkyuka Nogakubu

Kenichi Ikeda

Kobe University Faculty of Agriculture Graduate School of Agricultural Science: Kobe Daigaku Daigakuin Nogaku Kenkyuka Nogakubu

Research Article

Keywords: Broccoli, Vegetative compatibility group (VCG), UP-PCR, Nitrate-nonutilizing mutant, Genetic diversity

Posted Date: September 8th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10793849/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Alternaria brassicicola is a major pathogen causing *Alternaria* sooty spot in broccoli, leading to significant economic losses. To understand the population structure of this pathogen in Kagawa Prefecture, Japan, 36 isolates were collected from 29 broccoli fields during 2014 and 2015. All isolates were identified as *A. brassicicola* based on morphology and sequencing of the rDNA-internal transcribed spacer (rDNA-ITS) region. Genetic diversity was evaluated by polymorphisms using universally primed polymerase chain reaction (UP-PCR) and vegetative compatibility groupings (VCGs) using nitrate-nonutilizing (*nit*) mutants. While rDNA-ITS sequences showed no polymorphism, UP-PCR showed various polymorphisms and the *A. brassicicola* isolates were divided into several clusters. Using a modified chlorate-amended medium supplemented with Rose Bengal, 372 *nit* mutants were generated, which were classified into 18 VCGs. Most of these VCGs were field-specific. However, VCG12, VCG13, VCG14, and VCG18 were distributed across multiple fields and nurseries. No clear correlation was observed between virulence levels and genetic clusters or VCGs, suggesting that phylogenetic differentiation is not progressing, likely due to differences in pathogenicity. Since no correlation was found between the UP-PCR analysis and the VCG analysis, it was suggested that the majority of *A. brassicicola* isolates in Kagawa Prefecture are in a differentiated state after only a few invasions.

Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is an essential vegetable in Japan, with an annual production of approximately 160,500 tons (E-stat 2024). Due to its high consumption and importance, it was recognized as a "Designated Vegetable" (Shitei Yasai) by the Japanese government in 2026, to ensure a stable supply. Unfortunately, broccoli production is threatened by various diseases, most notably *Alternaria* sooty spot caused by *Alternaria brassicicola* (Urushibara and Sakai 2006) and *A. brassicae* (Mori and Nishiwaki 2022). Both of these pathogens cause characteristic circular lesions on leaves and, more critically, black spots on flower buds. Even minor symptoms on the flower buds significantly reduce the market value. At present, control of the disease relies solely on fungicides applied throughout the growing season. However, the intensive use of these fungicides has led to the emergence of strains of *A. brassicicola* that are resistant to the succinate dehydrogenase inhibitors they contain (Nishimura et al. 2025). This highlights the urgent need for integrated pest management that does not rely solely on fungicides to control this disease.

While seeds (Kaur and Dutta 2024; Kohl et al. 2010), weeds (Chupp and Sherf 1960; Nishikawa and Nakashima 2020), and crop residues (Nishimura et al. 2026) are likely the primary sources of inoculum, their relative importance in field epidemics remains unclear. To understand the population structure of such pathogens, vegetative compatibility grouping (VCG) analysis using nitrate non-utilizing (*nit*) mutants has been a powerful tool. This approach has been successfully applied to various fungi, including *Fusarium oxysporum* (Correll et al. 1987; Puhalla 1985; Takehara and Kuniyasu 1994), *Verticillium dahliae* (Korolev et al. 2000), *V. lecanii* (Sugimoto et al. 2003) and *A. brassicicola* on cauliflower in India (Javeria et al. 2018). Although previous studies have attempted to generate *nit* mutants in *Alternaria* species, similar detailed information on the genetic and physiological diversity of *A. brassicicola* populations in specific production areas is limited.

In this study, we investigated the diversity of *A. brassicicola* isolates collected from broccoli fields in Kagawa Prefecture in Japan. We used a multifaceted approach combining rDNA-internal transcribed spacer (rDNA-ITS) region sequencing, universally primed polymerase chain reaction (UP-PCR) polymorphism analysis, and VCG analysis, to characterize the population structure and discuss the potential primary inoculum sources for more effective disease management.

Furthermore, we found that all *A. brassicicola* isolates used in this study exhibited resistance to KClO₃, which prevented the obtain of *nit* mutants using conventional methods. However, we overcame this technical challenge and successfully obtained *nit* mutants by optimizing the culture medium conditions.

Materials and methods

Isolation of *Alternaria brassicicola*

From November 2014 to March 2015, a total of 36 isolates of *A. brassicicola* were collected from 29 broccoli fields in Kagawa Prefecture (Table 1). All isolates were collected using the method described by Nishimura et al. (2025). Specifically, a 5 mm² portion of diseased flower buds was surface-sterilized in 80% ethanol for 15 s, 1% aqueous Antiformin for 1 min, and then washed with sterilized distilled water for 10 s. The sample was then placed on potato dextrose agar (PDA; Shimadzu Diagnostics Corp., Kyoto, Japan) and incubated at 25°C for 4–5 days in the dark. All isolates were examined under a microscope and identified as *A. brassicicola* based on their conidial shape. Conidia were dark brown, cylindrical to obclavate with an indistinct beak, and ca. 16–55 × 6–17 µm in size. This morphology was consistent with the descriptions of Ellis (1971) and Simmons (2007). These isolates were deposited in the Genbank Project database of the National Agriculture and Food Research Organization (Tsukuba, Japan).

Table 1

Isolates of *Alternaria brassicicola* collected in Kagawa Prefecture, Japan, organized by vegetative compatibility group (VCG), with relative pathogenicity

Isolate	MAFF No.	Collection date	Nursery	Field	Host cultivar ^{a)}	Nit mutant classes ^{b)}				VCG	Relative pathogenicity ^{c)}					
						nit1	nit3	NitM	Total		Conidia			Mycelium		
BK-1	102101	20-Nov-14	Nakatado	A	Ohayo	6	0	3	9	1	1.0	±	0.1	1.0	±	0.1
BK-3	102107	20-Nov-14	Nakatado	C	Ohayo	17	2	4	23	2	1.0	±	0.0	1.0	±	0.1
BK-7	101942	1-Dec-14	Nakatado	F	-	6	8	0	14	3	1.0	±	0.0	0.9	±	0.1
BK-8	248323	1-Dec-14	Nakatado	G	-	4	0	2	6	4	1.0	±	0.0	1.0	±	0.0
BK-11	101938	18-Dec-14	Takamatsu	I	Ohayo	4	0	1	5	5	1.0	±	0.1	0.8	±	0.1
BK-19	248328	24-Dec-14	Nakatado	M	Ohayo	6	2	4	12	6	0.5	±	0.1	0.7	±	0.0
BK-23	247986	6-Jan-15	Ayasaka	Q	-	2	0	1	3	7	1.1	±	0.1	0.9	±	0.1
BK-26	101960	21-Jan-15	Seisan	-	-	7	2	1	10	8	1.0	±	0.1	1.1	±	0.2
BK-35	101973	20-Feb-15	Ayasaka	Y	-	4	1	1	6	9	1.0	±	0.1	1.0	±	0.2
AL-1	248334	1-Nov-14	Ayagawa	AA	Yoshimidori	1	0	1	2	10	1.0	±	0.1	1.0	±	0.0
OK-1	248336	3-Jan-15	Ayagawa	AB	Okumidori105	24	9	0	33	11	1.0	±	0.0	0.9	±	0.0
BK-2	102102	20-Nov-14	Nakatado	B	Ohayo	3	0	2	5	12	1.1	±	0.1	1.1	±	0.1
BK-5	248322	1-Dec-14	Nakatado	E	-	22	1	8	31	12	0.9	±	0.1	1.0	±	0.2
BK-13	101939	18-Dec-14	Takamatsu	J	Ohayo	8	0	2	10	12	1.0	±	0.1	0.9	±	0.1
BK-14	248326	18-Dec-14	Takamatsu	J	Ohayo	0	2	2	4	12	1.0	±	0.2	0.9	±	0.1
BK-20	248329	24-Dec-14	Ayasaka	N	Ohayo	3	0	4	7	12	1.0	±	0.1	0.9	±	0.1
BK-24	101967	6-Jan-15	Ayasaka	R	-	26	0	2	28	12	0.9	±	0.0	0.9	±	0.2
AL-2	248335	1-Nov-14	Ayagawa	AA	Yoshimidori	15	5	1	21	12	1.0	±	0.1	1.0	±	0.1
BK-4	102108	1-Dec-14	Ayasaka	D	-	7	0	2	9	13	1.1	±	0.2	1.0	±	0.2
BK-12	248325	18-Dec-14	Takamatsu	I	Ohayo	7	2	3	12	13	1.0	±	0.1	0.9	±	0.0
BK-32	248332	5-Feb-15	Nakatado	W	-	1	3	1	5	13	1.0	±	0.2	1.0	±	0.2
BK-33	101972	6-Feb-15	Ayasaka	X	-	1	0	1	2	13	1.1	±	0.1	1.0	±	0.1
BK-9	101941	4-Dec-14	Takamatsu	H	-	2	0	1	3	14	0.9	±	0.0	0.8	±	0.0
BK-10	248324	4-Dec-14	Takamatsu	H	-	4	0	2	6	14	0.9	±	0.1	0.9	±	0.1
BK-27	101968	24-Jan-15	Ayasaka	S	-	6	0	1	7	14	1.1	±	0.4	0.9	±	0.2
BK-15	106186	18-Dec-14	Takamatsu	K	Ohayo	5	0	1	6	15	1.2	±	0.1	1.0	±	0.1
BK-28	101968	24-Jan-15	Takamatsu	T	-	4	1	2	7	15	1.2	±	0.2	1.0	±	0.1
BK-17	106187	24-Dec-14	Nakatado	L	Ohayo	2	0	2	4	16	1.2	±	0.2	1.0	±	0.1
BK-18	248327	24-Dec-14	Nakatado	M	Ohayo	2	0	1	3	16	1.0	±	0.2	1.0	±	0.0
BK-21	248330	24-Dec-14	Ayasaka	O	Ohayo	5	0	2	7	17	1.0	±	0.0	1.0	±	0.0
BK-22	106188	6-Jan-15	Ayasaka	P	-	15	3	2	20	17	0.8	±	0.1	1.1	±	0.1
BK-36	101974	7-Mar-15	-	Z	-	3	2	1	6	17	1.0	±	0.1	1.1	±	0.1
BK-37	248333	7-Mar-15	-	Z	-	5	2	8	15	17	1.1	±	0.1	1.0	±	0.0
BK-29	101970	5-Feb-15	Seisan	U	-	14	0	4	18	18	-			0.9	±	0.1
BK-30	248331	5-Feb-15	Seisan	U	-	1	1	1	3	18	1.0	±	0.2	1.0	±	0.1
BK-31	101971	5-Feb-15	Nakatado	V	-	8	0	2	10	18	1.1	±	0.1	1.0	±	0.1
Total			-	-	-	250	46	76	372	-	-			-		

^a All hosts are broccoli (*Brassica oleracea* var. *italica*); a dash indicates the variety is unspecified.^c Multiple isolates were inoculated onto harvested broccoli leaves, and isolate BK-8 was inoculated onto all leaves as a check among different leaves. After 7 days at 20°C in the dark, the diameter of the lesions was measured, and the relative pathogenicity and standard deviation were calculated.

Isolate	MAFF No.	Collection date	Nursery	Field	Host cultivar ^{a)}	Nit mutant classes ^{b)}				VCG	Relative pathogenicity ^{c)}	
						<i>nit1</i>	<i>nit3</i>	NitM	Total		Conidia	Mycelium
^b Nit mutant phenotypes were determined according to growth on basal medium amended with different sources of nitrogen (Table 3).												
^c Multiple isolates were inoculated onto harvested broccoli leaves, and isolate BK-8 was inoculated onto all leaves as a check among different leaves. After 7 days at 20°C in the dark, the diameter of the lesions was measured, and the relative pathogenicity and standard deviation were calculated.												

Polymorphism analysis of isolates

Genomic DNA was extracted from all 36 isolates using the CTAB method (Porebski et al. 1997) from PDA-grown mycelia, and was then used as a template for the PCR testing. For the rDNA-ITS sequencing, the reaction mixture (20 µl) contained 10 µl polymerase mix (AmpliTaq Gold 360 Master Mix, Thermo Fisher Scientific, Waltham, MA, USA), 0.2 µM of ITS4 and ITS5 primer (White et al. 1990), and 25 ng of extracted template DNA. A Takara PCR Thermal Cycler Dice Touch (Takara Bio Inc., Kusatsu, Japan) was used with the following program: polymerase activation at 95°C for 9 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 40 s; and final extension at 72°C for 7 min. The amplified products were purified using a MonoFas DNA Purification Kit I (Animos Inc., Kawaguchi, Japan) and sequenced by a DNA sequencing service (Eurofins Scientific SE, Luxembourg City, Luxembourg). The obtained sequences for ITS region from the 36 isolates were analyzed using MEGA v.12.1.2. Nucleotide sequences for the ITS fragments were deposited in the DNA Data Bank of Japan.

For the UP-PCR polymorphism analysis, each reaction mixture (25 µl) contained 12.5 µl polymerase mix (AmpliTaq Gold 360 Master Mix); 0.2 µl GC enhancer; 0.2 µM of one of three UP-PCR primers, either L21 (Bulat et al., 2000) or [GTG]5 or [GCC]5 (Zhou et al. 1999) (Table 2); and 50 ng of the extracted template DNA. The PCR was performed using a Takara PCR Thermal Cycler Dice Touch (Takara Bio Inc.) with the following program: polymerase activation at 95°C for 9 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s (for the L21 and [GTG]5 primers) or 62°C for 30 s (for the [GCC]5 primer), and extension at 72°C for 5 min; and final extension at 72°C for 7 min. The amplified products were then separated by electrophoresis on a 2.0% agarose gel (Type Ⅱ, Sigma-Aldrich, St. Louis, MO, USA) in 0.5 × TBE buffer at 100 V for 70 min using a Mupid-2plus electrophoresis system (Advance, Tokyo, Japan). A DNA ladder (FastGene 1 kb DNA Marker Pro; Nippon Genetics, Tokyo, Japan) was used of each gel as a reference. The gels were stained with GelRed (Biotium, Inc., Fremont, CA, USA) and visualized under UV light. The bands obtained from the three UP-PCR primers were compiled and scored as a binary matrix, where the presence or absence of bands at particular positions were recorded as 1 and 0, respectively. The similarity coefficient was estimated using Nei’s formula (Nei and Li 1979). A dendrogram based on the similarity coefficient was constructed using the unweighted pair-group method with arithmetic average (UPGMA; Sneath and Sokal 1973). The robustness of clusters was assessed by bootstrap analysis with the WINBOOT program and 1000 repeated samplings with replacement (Yap and Nelson 1996).

Table 2
Nucleotide sequences of the primers used for UP-PCR

Primer	Sequence (5'-3')	Length (bp)	GC (%)	Tm (°C)	Reference
L21	GGATCCGAGGGTGGCGTTCT	21	67	74	Bulat et al. (2000)
[GTG]5	GTGGTGGTGGTGGTG	15	67	50	Zhou et al. (1999)
[GCC]5	GCCGCCGCCGCCGCC	15	100	60	Zhou et al. (1999)

Pathogenicity of isolates

The pathogenicity of the *A. brassicicola* isolates was determined by the detached leaf method, using broccoli as the host. Conidia and mycelia were used as two distinct types of inoculum.

To prepare the conidial suspension, 8-mm-diameter plugs were excised from 7-day-old cultures on PDA using a sterile cork-borer. Each plug was placed in a 2.0 ml tube containing sterile distilled water and vortexed to release the conidia. The concentration of the conidial suspension was standardized to 10⁵ conidia /ml using a hemocytometer. To prepare the mycelial inoculum, 6-mm-diameter agar plugs were excised from the growing edge of a 7-day-old culture on PDA, using a sterile cork-borer.

Leaves of broccoli (cv. Ohayo) were harvested approximately 30 days after planting. The leaves were washed with running tap water and surface-sterilized with 80% ethanol using a hand sprayer. The leaves were air-dried and placed on aluminum trays with their adaxial surfaces facing upward. Five or six isolates were inoculated onto each leaf, both conidia and mycelia. For each isolate, an inoculum consisted of either a 10 µl droplet of conidial suspension or one mycelial plug placed with the mycelial side facing down, onto a non-wounded leaf. To maintain high humidity, moist paper towels were placed at the corners of each tray. The trays were covered with a lid and maintained at 20°C in the dark for 6 days, when the diameter of lesions was measured by ruler. In addition, isolate BK-8 was inoculated onto every leaf as a standard reference (positive control) to normalize the variations in susceptibility among individual leaves. Sterile distilled water and plugs of clean PDA were used as negative controls. The experiment was replicated three times.

Relative pathogenicity was calculated based on the lesion diameters, using the BK-8 isolate as the reference, according to the following formula: Relative pathogenicity = (Lesion diameter of the test isolate)/ (Lesion diameter of BK-8 on the same leaf). Statistical analysis was performed in RStudio (version 2023.09.0 + 463) and R (version 4.3.1). Data were subjected to the Kruskal–Wallis test at a significance level of *P* < 0.05.

Generation of nitrate-nonutilizing mutants

Nitrate-nonutilizing (*nit*) mutants were obtained following the procedure of Puhalla (1985), using chlorate-containing agar medium. Three types were used in this experiment. The minimal medium (MM) was composed of 20 g agar, 30 g sucrose, 2 g NaNO₃, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g KCl, and 0.2 ml of a sterile trace elements solution (95 ml distilled water, 5 g citric acid, 5 g ZnSO₄·7H₂O, 4.75 g FeSO₄·7H₂O, 1 g Fe(NH₄)₂(SO₄)₂·6H₂O, 250 mg CuSO₄·5H₂O, 50 mg MnSO₄·H₂O, 50 mg H₃BO₃, and 50 mg Na₂MoO₄·2H₂O) per liter of distilled water. The chlorate-amended medium (MMC) consisted of MM supplemented with 1.6 g L-asparagine and 15 g KClO₃ per liter. The Rose Bengal–amended medium (MMR) was based on the method of Rodrigues (2009), using MMC modified by increasing KClO₃ to 60 g/l, and adding 50 ppm Rose Bengal after autoclaving.

All isolates were initially cultured on PDA at 25°C in the dark for 7 days. They were then transferred to MM and culture was continued under the same conditions. After 7 days on MM, 4-mm-diameter plugs were excised using a sterile cork-borer. For each of the 36 isolates, five mycelial plugs were inoculated onto plates of MMC and MMR. At least, 10 plates were used for each isolate. The plates were cultured at 25°C in the dark for up to 9 weeks, and were inspected periodically for fast-growing, chlorate-resistant sectors (Fig. 1a). The sectors were transferred to MM media and cultured for an additional 7 days. Very thin expansive colonies with no aerial mycelium were considered *nit* mutants. These mutants were maintained on an ammonium medium, in which the NaNO₃ in MM was replaced with 1 g/l ammonium tartrate.

Nit mutant phenotypes

To determine physiological phenotypes, the *nit* mutants were characterized according to the modified procedure of Correll et al. (1987). For each *nit* mutant, 4-mm diameter plugs were excised from cultures on MM using a sterile cork-borer. These plugs were then placed onto plates of media with different nitrogen sources. These were nitrate medium (MM, as described above), nitrite medium (MM with the NaNO₃ replaced by 0.5 g/l NaNO₂), hypoxanthine medium (MM with the NaNO₃ replaced by 0.2 g/l hypoxanthine), and ammonium medium (as described above). The plates of media were incubated at 25°C in the dark for 4 days, and the resulting colony morphology was scored relative to the wild-type parent, to assign the different phenotypic classes, i.e., *nit1*, *nit3*, and NitM (Table 3).

Table 3
Nitrate-nonutilizing (*nit*) mutants of *Alternaria brassicicola*, grown on media containing various nitrogen sources

Nitrogen source	Growth ^{a)}			
	WT ^{b)}	<i>nit1</i>	<i>nit3</i>	NitM
Nitrate (MM)	+	-	-	-
Nitrite	+	+	-	+
Ammonium	+	+	+	+
Hypoxanthine	+	+	+	-
^a +, typical wild-type dense growth; -, thin growth with no aerial mycelia				
^b Wild type				

Compatibility tests

To determine VCGs, mycelial plugs from *nit1* and either NitM or *nit3* mutants were paired on MM media and cultured at 25°C in the dark for 7 days. Each pairing was replicated at least three times. Vegetative compatibility was assessed visually at 3–5 day intervals for up to 4 weeks. Paired mutants were considered compatible and assigned to the same VCG when dense, wild-type (prototrophic) growth, indicative of heterokaryon formation, developed at the zone of contact between two colonies (Fig. 1b). The analysis of the distribution and richness of the VCGs was based on the Chao index. This index was calculated in RStudio (version 2023.09.0 + 463) and R (version 4.3.1).

Results

Morphology and phylogenic taxonomy of the isolates

All 36 collected isolates were confirmed as *A. brassicicola* based on their morphology. The rDNA-ITS sequences of all isolates were 100% identical to those of *A. brassicicola* from Canada (AF392985), China (OQ405067), and Japan (LC440585) in the NCBI database. Phylogenic analysis of rDNA-ITS sequences showed that all isolates formed a single cluster (Fig. 2).

The UP-PCR using three primers identified 38 bands among the 36 tested isolates (Fig. S1). The UPGMA phylogenic tree of these bands showed that OK-1 occupied an independent position among *A. brassicicola*, with extremely low genetic similarity to the other isolates (Fig. 3). Although the other isolates were divided into several clades, their bootstrap values at the branching points were all relatively low. Since OK-1 was isolated from only a single cultivar ('Okumidori105') in a single field (Table 1), this suggests host specificity and a potential correlation between genetic clusters and host cultivars.

Generation and characterization of nit mutants

Initially we attempted to obtain *nit* mutants of *A. brassicicola* from broccoli following the method described by Puhalla (1985), but this method failed to yield the characteristic fast-growing sectors. Instead, the colonies covered the entire MMC surface, indicating resistance to KClO₃. However, the MMR medium, which contained Rose Bengal and four times as much KClO₃ as the MMC, successfully induced sector formation and revealed multiple *nit* mutants (Fig. 1a). A total of 372 *nit* mutants were found on the MMR media, including 250 *nit1*, 46 *nit3*, and 76 NitM (Table 1).

No NitM mutants were obtained from isolates BK-7 and OK-1, so the only comparison in the search for VCGs was *nit3* with *nit1*. No prototrophic growth was observed among these mutants, and they were each assigned different VCGs. A total of 18 VCGs were identified among the 36 isolates (Table 1). Most of these VCGs were specific to a single field within one nursery, with the exception of VCG12, VCG13, VCG14, and VCG18.

Interestingly, VCG12 included seven isolates (BK-2, BK-5, BK-13, BK-14, BK-20, BK-24, and AL-2) which were obtained from four different nurseries (Nakatado, Ayasaka, Takamatsu, and Ayagawa; Tables 1 and S1). VCG13 included four isolates (BK-4, BK-12, BK-32, and BK-33) from three nurseries (Nakatado, Ayasaka, and Takamatsu). VCG14 included three isolates (BK-9, BK-10, and BK-27) from two nurseries (Ayasaka and Takamatsu). VCG18 included three isolates (BK-29, BK-30, and BK-31) from two nurseries (Nakatado and Seisan; Tables 1 and S1). The Chao index values for VCG richness among different nurseries ranged from 0 to 0.47, indicating low similarity in VCG composition among the nurseries (Table 4).

Table 4
Similarity^a of the vegetative compatibility groups of *Alternaria brassicicola* isolates from different nurseries

Nursery	Nakatado	Ayasaka	Seisan	Takamatsu	Ayagawa	Unknown
Nakatado	1.00	-	-	-	-	-
Ayasaka	0.22	1.00	-	-	-	-
Seisan	0.09	0.00	1.00	-	-	-
Takamatsu	0.22	0.47	0.00	1.00	-	-
Ayagawa	0.16	0.19	0.00	0.20	1.00	-
Unknown	0.00	0.22	0.00	0.00	0.00	1.00
^a Chao index using the "vegan" package in R.						

Pathogenicity of *Alternaria brassicicola* isolates

Pathogenicity was tested using conidial suspensions, and also mycelial plugs because BK-29 failed to produce conidia. Although the one-way ANOVA indicated significant differences in virulence among the 36 isolates (*P* < 0.05, data not shown), subsequent Steel–Dwass multiple comparison tests failed to identify any significant differences between individual isolates or VCGs (Table 1). Consequently, no clear correlation was observed between pathogenicity levels and VCG groupings in this study.

Discussion

Although *Alternaria* sooty spot can be caused by both *Alternaria brassicicola* and *A. brassicae*, only *A. brassicicola* was isolated from broccoli fields in Kagawa Prefecture. Several reasons for this can be considered. *A. brassicicola* has higher virulence and sporulation ability than *A. brassicae* (Blagojević et al. 2020). Moreover, *A. brassicicola* is capable of mycelial growth at higher temperatures than *A. brassicae*, whereas the growth of *A. brassicae* is drastically inhibited at temperatures above 25°C (Blagojević et al. 2020). In Kagawa Prefecture, most broccoli seedlings are grown during August and September, when the monthly average temperature is over 26°C. This high-temperature climate during the early growth stage creates an ecological disadvantage for *A. brassicae*, while favoring the proliferation of *A. brassicicola*. Additionally, seed-borne inocula may play a critical role. Nishikawa et al. (2014) demonstrated that the dominant *Alternaria* spp. on cruciferous seeds varies by host plant, with *A. brassicicola* being the most frequently detected. Therefore, *A. brassicicola* may be becoming the dominant species.

Phylogenetic analysis of the rDNA-ITS region showed no polymorphisms among the isolates, which is consistent with previous findings in *A. brassicae* from India (Sharma et al. 2013). However, our subsequent analyses using UP-PCR and VCG revealed significant genetic diversity among the 36 isolates. Vegetative compatibility analysis using *nit* mutants has been widely used to investigate genetic relationships and physiological races in various phytopathogenic fungi, most notably in *Fusarium* species (Correll et al. 1987; Puhalla 1985; Takehara and Kuniyasu 1994). This approach has also been successfully applied to *V. dahliae* (Korolev et al. 2000), *V. lecanii* (Sugimoto et al. 2003), and *A. brassicicola* isolated from cauliflower in India (Javeria et al. 2018).

Our results suggest that *A. brassicicola* is less sensitive to chlorate than other fungi such as those in the *Fusarium* genus, necessitating the more stringent selection pressure provided by the modified MMR medium. The 36 isolates were classified into 18 VCGs, indicating high diversity within the Kagawa Prefecture population, and most of the VCGs were field-specific. *A. brassicicola* is known to survive on broccoli debris for at least 90 days (Nishimura et al. 2026) and to produce conidia on cabbage debris for up to 23 weeks (Humpherson-Jones 1989). This robust survival capability likely contributes to high diversity of VCGs observed within population. In contrast, VCG12, VCG13, VCG14, and VCG18 were distributed across multiple fields and nurseries. In Kagawa Prefecture, all of the broccoli seedlings are grown at a limited number of nurseries and then distributed to the various production fields. If the pathogen has infected the nurseries, common VCGs could be expected in multiple fields.

Although the VCGs indicated high diversity, integration of these results with the UP-PCR data suggests that some VCGs may represent derivative lineages within a larger group, such as VCG12. It is possible that minor genetic variations that hinder hyphal fusion have arisen among certain isolates within a major VCG cluster, potentially leading to an overestimation of VCG diversity. Further studies, including detailed genetic and compatibility analyses, are required to clarify these relationships.

The classification based on VCG did not necessarily correspond with the clusters identified by UP-PCR. Specifically, isolates belonging to VCG12 and VCG18 were scattered across different clusters and lineages. This inconsistency may be because UP-PCR reflects multiple genomic regions, whereas VCG is determined by specific vegetative incompatibility (*vic*) loci (Puhalla 1985). If different strains share the same alleles at all *vic* loci, they can fuse regardless of their overall genetic background. This also suggests that genetic recombination via the parasexual cycle may occur in field populations, which could explain the observed discrepancy between VCG and UP-PCR.

Although previous studies (e.g., Javeria et al. 2018) reported a correlation between pathogenicity and VCGs in certain fungal species, our results found no such relationship in *A. brassicicola*. Comparison of virulence among the 36 isolates revealed a lack of significant differences, contrasting with the diversity found in the UP-PCR and VCG analyses.

These results highlight the importance of integrated sanitary management within nurseries and fields to ensure consistent broccoli yields. Furthermore, our findings demonstrate that evaluating fungal diversity requires multifaceted approaches. The wide distribution of specific VCGs, combined with the genetic polymorphisms detected by UP-PCR, suggest that this pathogen was historically introduced through long-term broccoli cultivation in the region. Over time, these introduced strains likely became established locally and underwent gradual genetic diversification. While VCG analysis alone may not fully resolve fine-scale population dynamics due to possible overestimation, the VCG classification system used in this study may provide a powerful and strategic tool, which could facilitate future ecological analyses to track the long-term behavior and evolutionary dynamics of *A. brassicicola* in plant pathology.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies performed with human participants or animals.

Acknowledgments

We thank Mr. Gan Kisaki of Kagawa Prefecture for his help with sampling the isolates. We are also grateful to Dr. Toshiaki Takehara and Dr. Jouji Moriwaki of NARO for their detailed technical guidance on *nit* mutant analysis. We also appreciate Mr. Keizo Komobuchi of Kagawa Prefecture for his dedicated support during the VCG assays.

References

1. Blagojević JD, Vukojević JB, Ivanović ŽS (2020) Occurrence and characterization of *Alternaria* species associated with leaf spot disease in rapeseed in Serbia. *Plant Pathol* 69:883–900. <https://doi.org/10.1111/ppa.13168>
2. Bulat SA, Lübeck M, Alekhina IA, Jensen DF, Knudsen INMB, Lübeck PS (2000) Identification of a universally primed-PCR-derived sequence-characterized amplified region marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. *Appl Environ Microbiol* 66:4758–4763. <https://doi.org/10.1128/AEM.66.11.4758-4763.2000>
3. Chupp C, Sherf AF (1960) Crucifer diseases. Vegetable diseases and their control. Ronald Press Co., New York, pp 237–288
4. Correll JC, Kittich CJR, Leslie JF (1987) Nitrate non-utilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640–1646. [10.1094/Phyto-77-1640](https://doi.org/10.1094/Phyto-77-1640)
5. Ellis MB (1971) Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, UK, pp 467–468
6. E-stat (2024) Crop Statistics Survey: Vegetable production and shipping statistics for the 2024 harvest – broccoli. Available at <https://www.e-stat.go.jp/stat-search/files?page=1&layout=datalist&toukei=00500215&tstat=000001013427&cyccy=7&year=20240&month=0&tclass1=000001032288&tclass2=000001032753&tcl> 3 May 2026
7. Humpherson-Jones FM (1989) Survival of *Alternaria brassicae* and *Alternaria brassicicola* on crop debris of oilseed rape and cabbage. *Ann Appl Biol* 115:45–50. <https://doi.org/10.1111/j.1744-7348.1989.tb06810.x>
8. Javeria S, Deep S, Prasad L, Sharma P (2018) Vegetative compatibility grouping of *Alternaria brassicicola* causing black leaf spot in cauliflower. *Indian Phytopathol* 71:43–47. <https://doi.org/10.1007/s42360-018-0005-3>
9. Kaur N, Dutta B (2024) Characterization of seed-to-seedling transmission of *Alternaria brassicicola* in broccoli. *Plant Dis* 108:2046–2052. <https://doi.org/10.1094/PDIS-10-23-2002-RE>
10. Kohl J, Van Tongeren CAM, Groenenboom-de Haas BH, Van Hoof RA, Driessen R, Van Der Heijden L (2010) Epidemiology of dark leaf spot caused by *Alternaria brassicicola* and *A. brassicae* in organic seed production of cauliflower. *Plant Pathol* 59:358–367. <https://doi.org/10.1111/j.1365->

11. Korolev N, Katan J, Katan T (2000) Vegetative compatibility groups of *Verticillium dahlia* in Israel: their distribution and association with pathogenicity. *Phytopathology* 90:529–536. <https://doi.org/10.1094/PHYTO.2000.90.5.529>
12. Mori M, Nishiwaki Y (2022) *Alternaria* sooty spot of broccoli caused by *Alternaria brassicae* in Japan (Abstract in Japanese). *Jpn J Phytopathol* 88:71. <https://doi.org/10.3186/jjphytopath.88.71>
13. Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonuclease. *Proc Natl Acad Sci USA* 76:5269–5273
14. Nishikawa J, Kobayashi T, Natsuaki KT (2014) Seed-borne fungi on genebank-stored cruciferous seeds from Japan. *Seed Sci Technol* 42:47–59
15. <https://doi.org/10.15258/sst.2014.42.1.05>
16. Nishikawa J, Nakashima C (2020) Japanese species of *Alternaria* and their species boundaries based on host range. *Fung Syst Evol* 86:197–282. <https://doi.org/10.3114/fuse.2020.05.13>
17. Nishimura F, Katayama T, Ikeda K (2025) First report of SDHI–fungicide–resistant isolates of *Alternaria brassicicola* from broccoli in Japan. *J Gen Plant Pathol* 91:225–229. <https://doi.org/10.1007/s10327-025-01231-6>
18. Nishimura F, Katayama T, Nakanishi M, Nakai K, Yoshida M, Yamada T, Mori M (2026) Transmission via broccoli residue after harvesting and survival period of *Alternaria brassicicola* on it. *Jpn J Phytopathol* (in Japanese Engl abstract) 92:1–6
19. Porebski S, Bailey L, Baum BR (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide components. *Plant Mol Biol Rep* 15:8–15. <https://doi.org/10.1007/BF02772108>
20. Puhalla JE (1985) Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. *Can J Bot* 63:179–183. <https://doi.org/10.1139/b85-020>
21. Rodrigues TTMS (2009) Molecular and morphological characterization, with inference about recombination, for *Alternaria* species related to early blight of potato and tomato. Doctoral thesis, Federal University of Viçosa, Brazil. <http://locus.ufv.br/handle/123456789/1024> Viewed 3 May 2026
22. Sharma P, Deep S, Sharma M, Bhati DS (2013) Genetic variation of *Alternaria brassicae* (Berk.) Sacc., causal agent of dark leaf spot of cauliflower and mustard in India. *J Gen Plant Pathol* 79: 41–45. <https://doi.org/10.1007/s10327-012-0417-3>
23. Simmons EG (2007) *Alternaria*: an identification manual. CBS Biodiversity series 6. American Society of Microbiology, Washington DC, USA, pp 566–569. ISBN: 978-9070351687
24. Sneath PHA, Sokal RR (1973) Numerical taxonomy: The principles and practice of numerical classification. Freeman, WH & Co., San Francisco, California
25. Sugimoto M, Koike M, Hiyama N, Nagao H (2003) Genetic, morphological, and virulence characterization of the entomopathogenic fungus *Verticillium lecanii*. *J Invertebr Pathol* 82:176–187. [https://doi.org/10.1016/S0022-2011\(03\)00014-4](https://doi.org/10.1016/S0022-2011(03)00014-4)
26. Takehara T, Kuniyasu K (1994) Ecology of *Fusarium* diseases using *nit* mutants I. Production of *nit* mutants from several formae speciales of *Fusarium oxysporum*. *Jpn J Phytopathol* 60:699–704 (in Japanese with English abstract). <https://doi.org/10.3186/jjphytopath.60.699>
27. Urushibara T, Sakai H (2006) Occurrence of *alternaria* sooty spot of broccoli caused by *Alternaria brassicicola* (abstract in Japanese). *Jpn J Phytopathol* 72:52
28. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR protocols: a guide to methods and applications*. Academic, San Diego, pp 315–322. DOI:10.1016/B978-0-12-372180-8.50042-1
29. Yap I, Nelson RJ (1996) WINBOOT: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. In: *IRRI discussion papers series No. 14*, IRRI, Manila
30. Zhou Z, Miwa M, Hogetsu T (1999) Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). *New Phytol* 144:55–63. <https://doi.org/10.1046/j.1469-8137.1999.00504.x>

Figures

Fig. 1

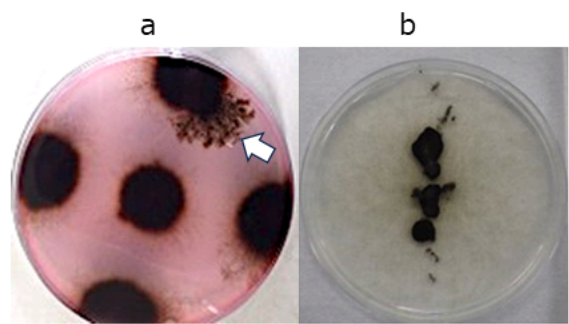


Figure 1

Alternaria brassicicola colonies. a, grown on MMR medium, which contains extra chlorate; the arrow indicates a chlorate-resistant sector; b, prototrophic growth induced by heterokaryon formation between *nit1* and *NitM* mutants of isolate BK-9, grown on MM medium.

Fig. 2

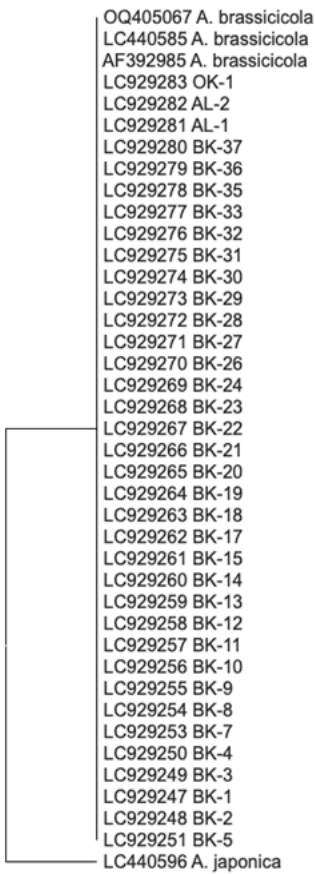


Figure 2

Maximum-likelihood phylogenetic tree based on the ITS regions of the 36 collected *Alternaria brassicicola* isolates.

Fig. 3

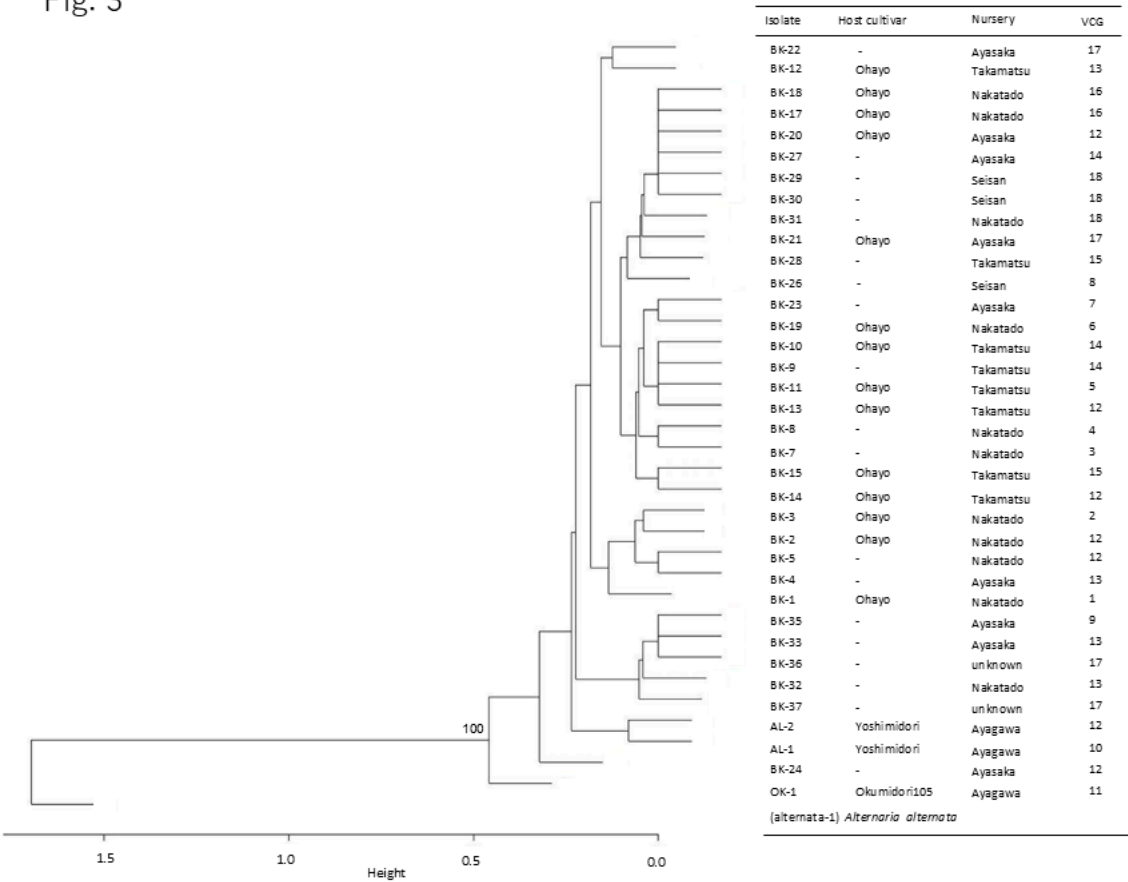


Figure 3

UPGMA phylogenetic tree of *Alternaria brassicicola* isolates from broccoli, by UP-PCR polymorphism. Numbers on branches are bootstrap probability values (>80) based on 1,000 replicates. *Alternaria cf. alternata*, isolated from broccoli and tentatively identified based on ITS region sequence, was used as an outgroup for comparison.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigSINishimura.pdf](#)
- [TableSINishimura.pdf](#)