Check for updates

Plant Pathology

WILEY

ORIGINAL ARTICLE OPEN ACCESS

Plasmid Copy Number Variation Impacts Pathogenicity and Quantification of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* Infecting Mung Bean

Ahmed Saad[1,2] | Niloofar Vaghefi[1,3] | Barsha Poudel[1] | Anthony J. Young[4] | Lisa A. Kelly[5] | Noel L. Knight[1,4,6]

[1]Centre for Crop Health, University of Southern Queensland, Toowoomba, Queensland, Australia | [2]Department of Primary Industries and Regional Development, South Perth, Western Australia, Australia | [3]School of Agriculture, Food and Ecosystem Sciences, University of Melbourne, Parkville, Victoria, Australia | [4]School of Agriculture and Food Sustainability, The University of Queensland, Gatton, Queensland, Australia | [5]Department of Primary Industries, Queensland Government, Toowoomba, Queensland, Australia | [6]Centre for Crop and Disease Management, School of Molecular and Life Sciences, Curtin University, Bentley, Western Australia, Australia

Correspondence: Noel L. Knight (noel.knight@unisq.edu.au)

Received: 4 March 2025 | **Revised:** 4 July 2025 | **Accepted:** 9 July 2025

Funding: This reaseach was funded by the Broadacre Cropping Initiative (Project 36), a joint investment of the University of Southern Queensland and the Queensland Government Department of Primary Industries.

Keywords: bacterial wilt | diagnostics | digital PCR | quantitative PCR | tan spot

ABSTRACT

In bacteria, plasmids can confer the ability to cause disease. Although they can potentially vary in copy number, little has been reported on the dynamics of plasmids in plant-pathogenic bacteria. Pathogenicity of the bacterium *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff), which causes foliar disease in leguminous crops, including mung bean (*Vigna radiata*), has previously been linked to a plasmid. This study explored the variation in plasmid copy number among a genetically diverse collection of 25 Cff isolates using purposely designed quantitative PCR assays for chromosomal and plasmid DNA targets. Pathogenicity and virulence of six Cff isolates, including one plasmid-free isolate, were assessed on the susceptible mung bean cultivar Opal-AU using visual symptoms, trifoliate leaf dry weights and Cff DNA quantities following stem inoculation. Plasmid copy numbers varied significantly ($p < 0.001$) among plasmid-carrying isolates, ranging from 1.37 to 2.74. For subsequent quantification in planta, the single-copy chromosomal PCR target gene, *gyrase b*, was selected. Mung beans inoculated with plasmid-carrying isolates exhibited significant variation in visual symptoms ($p < 0.001$), while trifoliate leaf dry weights and Cff DNA quantities did not significantly differ. The treatment using a plasmid-free isolate was not significantly different from the negative control for each of the disease traits. Knowledge of plasmid dynamics in Cff populations lays a foundation for improved understanding of the inheritance and impact of plasmid-related traits. The quantification assays will be useful for monitoring Cff populations, and the demonstrated variation in pathogenicity and virulence can assist efforts to breed host plant germplasm with reduced susceptibility to Cff.

1 | Introduction

Bacterial diseases of plants are a persistent and evolving threat to crop production (Sharma et al. 2022). Disease management strategies rely on tactics to reduce pathogen populations in the environment and genetic improvement programmes to breed less susceptible crop varieties. The effectiveness of these strategies requires comprehensive knowledge of the factors affecting bacterial pathogenicity and their interactions with the host and environment. The genetic features conferring

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2025 The Author(s). *Plant Pathology* published by John Wiley & Sons Ltd on behalf of British Society for Plant Pathology.

pathogenicity may be located outside of the bacterial chromosome, on self-replicating, extrachromosomal DNA molecules known as plasmids (Clark et al. 2019). The dynamics of plasmids among isolates of the same bacterial species has had limited investigation, with examples of the presence or absence of a plasmid determining pathogenic or beneficial traits (Bultreys and Gheysen 2023; Melnyk and Haney 2017), the proficiency of the plasmid host species affecting the maintenance of the plasmid in a population (Kottara et al. 2021), and the copy number of plasmids playing a role in the transmission to new bacterial generations (Hernández-Beltrán et al. 2021; Ilhan et al. 2018).

Curtobacterium flaccumfaciens pv. *flaccumfaciens* (Cff), a gram-positive, vascular pathogen that systemically colonises leguminous plants (Osdaghi et al. 2020), has had its pathogenicity linked to the presence of a linear plasmid, which contains a suite of pathogenicity-related genes (Chen et al. 2021; Evseev et al. 2022; O'Leary and Gilbertson 2020; Sparks et al. 2025; Vaghefi et al. 2021). The bacterium causes tan spot disease, also referred to as bacterial wilt or bacterial scorch (Osdaghi et al. 2020). Symptoms include leaf chlorosis and subsequent necrosis, typically initiating from leaf margins, leading to wilting, stunting and death (Diatloff and Imrie 2000; Vaghefi et al. 2021). Disease progression is most rapid at temperatures greater than 30°C and when plants are stressed (Osdaghi et al. 2020). Seed-borne inoculum is a major source of disease; however, in-field inoculum harboured on crop debris or alternative hosts may also play a role in disease epidemiology (EPPO 2023; Gonçalves et al. 2017; Júnior et al. 2012; Osdaghi et al. 2020).

The potential for Cff to persist on a range of crops, and its risk as an emerging pathogen (EPPO 2023; Kizheva et al. 2024; Tokmakova et al. 2024), has demonstrated the need to investigate the genetic diversity among different populations, develop tools for efficient detection and explore the resources available to effectively control or reduce disease outbreaks. To support this goal, genome sequencing and population genetic studies have been conducted on Cff collections from Australia (Sparks et al. 2025; Vaghefi et al. 2021). These have complemented similar projects on the genetic variation within Cff across collections from Brazil, Iran, Russia, Spain and the United States, indicating separation into several distinct genetic clusters (Agarkova et al. 2012; Evseev et al. 2022; Gonçalves et al. 2019; McDonald and Wong 2000; Osdaghi et al. 2018). Conserved genetic regions have been identified and used for detecting the pathogen.

Species-specific PCR is an important tool for not only detecting pathogens but also quantifying pathogen load in plant tissues or field environments (Knight et al. 2022; Ophel-Keller et al. 2008; Schaad and Frederick 2002). Quantitative approaches using real-time or digital PCR platforms can support visual disease rating and provide objective measures of in planta pathogen populations (Knight et al. 2012; Utami et al. 2024). Applying this technique to screening plant germplasm for susceptibility to bacterial pathogens, or comparing isolate virulence, can also provide useful insights into disease traits and assist breeding programmes (Garces et al. 2014; Tang et al. 2020).

Interestingly, two DNA targets for detecting Cff, originally reported as a restriction digestion fragment (Messenberg Guimaraés et al. 2001) or a fragment from repetitive-sequence-based PCR (rep-PCR) (Tegli et al. 2002), were based on genes now known to be in the plasmid. Studies have since reported the use of the rep-PCR sequence, predicted to be a trypsin-like serine protease gene, for Cff detection across several platforms (Puia et al. 2021; Tegli et al. 2020, 2022). The relevance of the plasmid as a target for detection is clear; however, reports of isolates lacking the plasmid suggest the potential to under-report the presence of Cff in plant or environmental samples. Isolates carrying the plasmid also have the potential to vary in the number of plasmid copies (Clark et al. 2019), further complicating quantification of bacterial numbers. This issue may be alleviated by the use of PCR assays targeting conserved chromosomal regions in the *Curtobacterium* genus (Evseev et al. 2022).

The impact of Cff on crop production has predominantly focused on common beans (*Phaseolus vulgaris*); however, its effects and survival in other crop species can have important implications for production and trade. Mung bean (*Vigna radiata*), for example, is an increasingly popular crop due to its protein and nutrient content, short cropping duration, and potential export value (Batzer et al. 2022). While mung bean production is greatest across south and southeast Asia (Pandey et al. 2018), it is emerging as a valuable crop in new regions. In Australia, mung bean is valued at an annual AUD118 million and is an expanding export commodity for the Queensland and northern New South Wales growing regions (AMA 2023; DAF 2018). Low and variable yields are a major obstacle for mung bean production in Australia, generally attributed to adverse environmental conditions and impacts of diseases, such as halo blight, tan spot and powdery mildew (Batzer et al. 2022; Chauhan and Williams 2018; Kelly et al. 2021; Noble et al. 2019). Among these, tan spot has the greatest impact when the environment is hotter and drier (Osdaghi et al. 2020), conditions that are increasingly prevalent and are already impacting agricultural production (Singh et al. 2023; Trancoso et al. 2020).

Limited information is available for Cff on mung bean. The earliest report of possible Cff in Australia occurred in 1934 on common beans (*P. vulgaris*) in Victoria (Adam and Pugsley 1934). Subsequently, tan spot of mung bean in Australia was first detected in 1984 across central and southern Queensland, with later reports indicating a widespread distribution (Wood and Easdown 1990). Investigations of mung bean susceptibility to tan spot, using a collection of mung bean cultivars and Cff isolates, demonstrated that disease expression among the cultivars and virulence (degree of damage) of isolates varied (Diatloff and Imrie 2000). With all but one cultivar reported to be susceptible, breeding efforts to introduce less susceptible germplasm continue to be a priority.

The current study aimed to explore the dynamics of the pathogenicity-related plasmid among a collection of genetically diverse Australian Cff isolates, including isolates not carrying the plasmid. Quantitative real-time (qPCR) and droplet digital PCR (ddPCR) assays were designed to detect and quantify the chromosome and plasmid of Cff, facilitating the determination of plasmid copy numbers. This knowledge was used to determine the appropriateness of the plasmid for quantitative comparisons and to explore the effect of plasmid copy number variation on virulence. The disease impact of a selection of isolates was assessed using a single susceptible mung bean cultivar.

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

The results offer valuable insights into the role of plasmids in pathogenicity and virulence, while also assisting with screening mung bean and related germplasm for susceptibility to Cff.

2 | Materials and Methods

2.1 | PCR Assay Design

Four probe-based PCR assays for qPCR or ddPCR platforms were developed to amplify a plasmid-specific region in Cff (assay Cff_141), a species-specific region of the Cff gyrase B (*gyrB*) gene (assay Cf_180_*gyrB*), a genus-specific region of the Cff *gyrB* gene (assay Curto_57_*gyrB*) and the elongation factor 1-α (*EF1α*) gene of mung bean (assay Vr_157_*EF1α*) (Table 1). Alignments and primer/probe design were performed against reference sequences as described by Knight et al. (2022).

The plasmid-specific assay was a modified design based on a region targeted by Tegli et al. (2002). Modification included reducing the amplicon size and designing a probe. The targeted sequence was aligned across five published plasmid sequences (GenBank accessions CP080396, CP041260, CP045288, CP071884 and CP074440).

Due to reports of Cff isolates without a plasmid (Sparks et al. 2025; Vaghefi et al. 2021) and variation in plasmid copy numbers among isolates, the single-copy phylogenetically informative chromosomal gene *gyrB* was selected for assay design. Sequences of 117 Cff isolates from Australia (Sparks et al. 2025), Cff type strain DSM 20129 (GenBank accession: CP080395) and closely related species and pathovars were used for primer and probe alignment. Sequence variation within *gyrB* from Cff led to the design of two assays, one focusing on *C. flaccumfaciens* as a species and one focusing on the *Curtobacterium* genus, allowing DNA amplification from all isolates in the collection.

Amplification of mung bean DNA was performed using primers described by Kundu et al. (2013) and a probe designed against the *EF1α* sequence of *V. radiata* (GenBank accession XM_014656474). Mung bean DNA detection acted as an internal positive control in PCR tests of plant DNA templates.

2.2 | Plasmid Copy Number Assessment

The copy number of the linear plasmid (pCff119) (Vaghefi et al. 2021) was assessed for 25 Cff isolates (Table S1) using a duplex PCR of the Cff_141 (plasmid) and Curto_57_*gyrB* (chromosome) assays in a QX200 ddPCR platform (Bio-Rad), following the manufacturer's instructions. Each assay was initially optimised in uniplex across a temperature gradient (55.5°C to 65.5°C) before combining as a duplex. Each 22 μL ddPCR contained 11 μL of 2× ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 0.25 μM of each forward and reverse primer (Integrated DNA Technologies), 0.15 μM of each probe (Integrated DNA Technologies) and 5 μL of template. Thermal cycling conditions consisted of 95°C for 10 min, followed by 50 cycles of 94°C for 30 s and 58°C for 60 s, with a final denaturation at 98°C for 10 min, followed by a hold step at 4°C. Copy numbers for each target were calculated in the QX Manager Standard Edition software v. 1.2 (Bio-Rad).

Control templates included Cff DNA (0.5 ng) as a positive control and sterile water as a no-template control (NTC). The experimental samples were Cff cell suspensions of 1.0 optical density (OD) at 600 nm, grown as described for inoculum production

TABLE 1 | Primer/probe sets for detection and quantification of DNA sequences in the *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) plasmid, the gyrase B (*gyrB*) gene of *C. flaccumfaciens* (Cf) and the *Curtobacterium* genus (Curto), and the elongation factor 1-α (*EF1α*) gene of mung bean (*Vigna radiata*; Vr).

Target	Primer/probe[a]	Sequence (5′–3′)	Product (bp)
Cff_plasmid	Cff_141_F	CAAACGACCGATAGCTCCAT	141
	Cff_141_R[b]	GATGTTCCCGGTGTTCAG	
	Cff_141_P[c]	AGCAGCAACTATCGCCACGC	
Cf_species	Cf_180_*gyrB*_F	CGACATCCGCGAAGGG	180
	Cf_180_*gyrB*_R	CGGACGACGTCGCGT	
	Cf_180_*gyrB*_P[c]	CGGCACCGAGCTGACCCACTG	
Curto_genus	Curto_57_*gyrB*_F	AGCCTAGCTGGCCACATGG	57
	Curto_57_*gyrB*_R	TGGGTGAGGTTGTCAGCATTT	
	Curto_57_*gyrB*_P[c]	ACATGCCGTCCAAATTTCCTCTACCATC	
Vr	Vr_157_*EF1α*_F[d]	CCTCTTGGACGTTTTGCTGT	157
	Vr_157_*EF1α*_R[d]	TGTAACCATCCCCTGATGA	
	Vr_157_*EF1α*_P[c]	CACCAAGGCTGCCCAGAAGA	

[a]Suffix: F, forward primer; R, reverse primer; P. hydrolysis probe.
[b]Primer sequence published in Tegli et al. (2002).
[c]Cff_141_P, Cf_180_*gyrB*_P and Curto_57_*gyrB*_P labelled with 6-carboxyfluorescein (FAM; uniplex) or hexachlorofluorescein (HEX; duplex) and *Vr*_157_*EF1a*_P labelled with FAM on the 5′ terminus. HEX and FAM labelled probes contained internal ZEN and 3′ terminus Iowa Black FQ quenchers.
[d]Primer sequence published in Kundu et al. (2013).

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

below, and adjusted with a 10^5-fold dilution. Cell suspensions were stored overnight at −20°C before testing. Representative DNA samples from the plant inoculation experiments described below were also assessed. Each template was assessed in duplicate.

A BLAST search of the Cff genome (taxid: 138532) confirmed the presence of only one sequence target for each PCR assay in the plasmid and chromosome, respectively. Therefore, for each ddPCR assay, the relative copy number of the Cff_141 (plasmid) PCR target was calculated by dividing the ddPCR copy numbers of the Cff_141 assay by the ddPCR copy numbers of the Curto_57_*gyrB* assay. The four values (two technical replicates of two biological replicates) were used to produce a boxplot with the package *ggplot2* v. 3.4.2 in RStudio v. 2023.06.0 (R Core Team 2017).

Plasmid copy numbers were also assessed based on relative read depths to the single-copy genes *gyrB* and *rpoB* in Illumina paired-end sequences of 119 isolates of Cff from Australia (Sparks et al. 2025). Sequence reads for each isolate were independently aligned to sequences of a complete plasmid (pCFF113; NCBI accession: CP041260.1), a partial *gyrB* gene (NCBI accession: MW732638.1) and a *rpoB* gene (NCBI accession: KT935421.1) using the short-read aligner Burrows–Wheeler Aligner (bwa-mem) v. 0.7.15 with default settings (Li and Durbin 2009). SAMtools v. 1.11 (Li et al. 2009) was used to sort and convert data from Sequence Alignment Map into Binary Alignment Map formats. The 'depth' function in SAMtools (with the -a option for plasmid/gene sequences, or the -a and -r options for PCR amplicon regions) was used to calculate read depth (the number of sequence reads covering each specific base pair position in the reference sequence) across the plasmid/gene sequences and the Cff_141 and Curto_57_*gyrB* amplicon sequences. Finally, the plasmid size and total read depth were calculated, and the 'statistics' function in Python was used to determine the mean read depth. The relative copy numbers of the plasmid were calculated by dividing the read depth of the plasmid sequence by the read depths of the *gyrB* or *rpoB* gene regions.

2.3 | Quantitative Real-Time PCR Conditions

Quantitative real-time PCR was conducted on a CFX Opus 96 or CFX384 platform (Bio-Rad). Assays were initially assessed in uniplex, across a gradient of annealing temperatures (48°C to 66°C) and with target and non-target species DNA (0.5 ng/μL) (Table S2). Duplex assays of Cff_141, Cf_180_*gyrB* or Curto_57_*gyrB* and Vr_157_*EF1α* were also assessed. In uniplex reactions, all probes were labelled with a 5′ terminus 6-carboxyfluorescein (FAM) fluorophore, while in duplex reactions, the Vr_157_*EF1α* probe remained labelled with a FAM fluorophore and each bacterial probe was labelled with a 5′ terminus hexachlorofluorescein (HEX) fluorophore. All probes contained internal ZEN and 3′ terminus Iowa Black RQ quenchers. Each 20 μL reaction consisted of 10 μL of ImmoMix (Bioline), 0.25 μM of each forward and reverse primer (Integrated DNA Technologies), 0.15 μM of probe (Integrated DNA Technologies) and 5 μL of template DNA. Optimal thermal cycling conditions consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 62°C for 60 s.

The duplex of Cf_180_*gyrB* and Vr_157_*EF1α* was used to test the inoculated mung bean DNA samples (see Section 2.5). For quantification, a 10-fold dilution series of Cff DNA ranging from 25 ng to 0.025 pg per reaction (final volume) was assessed in triplicate. A standard curve was generated using the CFX Maestro Software v. 1.1 (Bio-Rad) by plotting the logarithm of Cff DNA concentrations against the quantification cycle (C_q). Pure Cff and mung bean DNA templates were included as positive and negative controls, with sterile water included as an NTC. A mixed DNA template containing both Cff and mung bean DNA (extracted from Opal-AU tissue inoculated with isolate BRIP 70623) was also included in each run. Each sample template was assessed in duplicate.

Mean Cff DNA values were transformed to indicate the total DNA quantity in the entire dry trifoliate leaf sample for each plant (Knight et al. 2012). Briefly, for each inoculated mung bean DNA template, the mean DNA quantity value was multiplied by 10 (to account for the 10-fold dilution of the original DNA sample), divided by 5 to obtain the ng/μL value, then multiplied by 100 (microlitre volume of eluted DNA) to determine the total quantity of Cff DNA extracted from the subsample. The total Cff DNA quantity was divided by the subsample weight to determine the Cff DNA quantity per milligram, then multiplied by the total trifoliate leaf dry weight to determine the Cff DNA quantity in the combined trifoliate leaf sample.

For the Cf_180_*gyrB* and Vr_157_*EF1α* duplex reaction, intra- and inter-assay variation was reported as the mean DNA quantity and coefficient of variation (CV) of replicate samples of a mixed Cff and mung bean DNA template.

2.4 | Planting and Glasshouse Conditions

Two replicated glasshouse trials were conducted at the University of Southern Queensland, Australia in 2022 and 2023. Mung bean cultivar Opal-AU, considered susceptible to tan spot (Douglas 2021), was grown for pathogenicity and virulence experiments. Two seeds were planted into 1.5 L pots (14 cm diameter) containing steam-sterilised (75°C for 45 min) potting mix (Scotts Osmocote Premium Plus Superior). Pots were placed on metal trays and kept under natural light conditions, with day/night temperatures of 27°C to 33°C and 20°C to 25°C, respectively. Pots were manually watered at the soil surface prior to inoculation, with watering post-inoculation via submersion of the pot bases in approximately 1 cm of water twice a week. After germination, each pot was thinned to one plant. At 7 days after planting, pots were fertilised with Complete Garden Health Treatment (Seasol International Pty Ltd) at 0.7% (vol/vol) and at 2 weeks with PowerFeed (Seasol International Pty Ltd) at 1.1% (vol/vol) in rainwater (Vaghefi 2022).

2.5 | Inoculum Production, Inoculation and Harvest

Six Cff isolates, collected in a previous study (Sparks et al. 2025), were selected for pathogenicity and virulence assessment (Table 2). Selection was informed by genetic clustering (Sparks et al. 2025) and temporal and geographic origin. Isolates were grown on nutrient agar (NA; Amyl Media) for 3 days, and a single colony was transferred to 10 mL of nutrient broth (NB; Amyl

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 | Metadata for six *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolates selected for mung bean inoculation experiments.

Isolate	Year	State[a]	Location	Genetic cluster[b]	Plasmid haplotype[b]
DAR 56672	1986	Qld	Biloela	1	pCff119-H1
CffT13932	2015	NSW	Moree	1	pCff119-H2
BRIP 70606	2018	NSW	Mullaley	Admixed (4 and 6)	pCff119-H1
Cff089	2018	Qld	Brookstead	Admixed (2 and 5)	No plasmid
BRIP 70614	2018	Qld	Wooroolin	1	pCff119-H1
BRIP 70623	2019	Qld	Brookstead	2	pCff119-H1

Note: Each isolate was originally isolated from mung bean.
[a]Queensland (Qld); New South Wales (NSW).
[b]Genetic clusters and plasmid haplotypes (H) reported by Sparks et al. (2025).

Media) in a 50 mL tube before placing in a shaker-incubator at 180 rpm and 27°C for 48 h. On the day of inoculation, each culture was centrifuged for 10 min at 11,200 *g*; the supernatant was discarded, and the bacterial pellet was resuspended in 8 mL of sterile water. Each cell suspension was diluted to an OD of 1.0 at 600 nm (DS-11FX spectrophotometer, DeNovix Inc.), which is equivalent to 10^8 CFU mL^{-1}.

The inoculation experiment included seven treatments, consisting of the six isolates and a sterile water control. Inoculations were conducted after the emergence of the first trifoliate leaf, approximately 15 days after planting. For each treatment, 10 μL of cell suspension or water was placed on the stem between the unifoliates and the base of the first trifoliate leaf petiole. An acupuncture needle (0.3 mm diameter, Dongbang Medical Co. Ltd) was used to pierce through the inoculum droplet and stem three times. The experiment was performed twice; each was arranged in a randomised complete block design with four replicates for each of the seven treatments.

At 15 days after inoculation, the first (oldest) three trifoliate leaves were visually rated for symptom expression. Visual rating used a 0 to 100% scale (5% increments) to measure the chlorosis and necrosis visible on each leaf, where 0 indicates no symptom development and 100 indicates expression of chlorosis and/or tan-brown necrosis across the entire leaf area. The sum of the three trifoliate leaf visual ratings for each plant was used for subsequent analyses. The first three trifoliate leaves of each plant were harvested and placed in a paper bag. Tissues were dried for 3 days at 55°C and weighed.

2.6 | Tissue Preparation and DNA Extraction

Dried leaf tissues were placed into 15 mL tubes with two steel balls (4.5 mm diameter) and ground at 6.5 m/s for 60 s in a FastPrep-24 instrument (MP Biomedicals). A subsample of 15 to 20 mg was taken and DNA extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. To reduce the possible impact of inhibitors, each sample was diluted 10-fold in sterile water.

Pure Cff DNA was extracted from cells grown in NB (as described above) using the GenElute Bacterial Genomic DNA Kit (Merck) according to the manufacturer's instructions. Pure mung bean DNA was extracted from 1-week-old leaf tissue using the DNeasy Plant Pro Kit (Qiagen). DNA of non-target organisms (Table S2), used for PCR optimisation, was extracted from pure tissues using the DNeasy Plant Mini Kit (Qiagen). DNA characteristics were measured using a DS-11FX spectrophotometer (DeNovix Inc.) and quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific).

2.7 | Statistics

All statistical analyses were performed in RStudio v. 2023.06.0 (R Core Team 2017; Wickham 2016). To determine the appropriate statistical tests, the normality and homogeneity of variances of the data was assessed. The Shapiro–Wilk test ($\alpha = 0.05$) ('shapiro.test' function in package *stats*) was used to test for normality. Homogeneity of variances ($\alpha = 0.05$) across treatments was evaluated using Levene's test ('leveneTest' function in package *car*). Based on these results, nonparametric methods were appropriate for analysis of plasmid copy number, visual symptom, Cff DNA quantity, and trifoliate leaf dry weight data. Due to the wide range of Cff DNA quantity values, a $\log(x+1)$ transformation was applied. Independent replicated experiments were compared using the Wilcoxon rank-sum test ('wilcox.test' function in package *stats*). To compare measurements for multiple isolates or treatments, the Kruskal–Wallis test was conducted ('kruskal.test' function in package *stats*). This nonparametric method tests whether samples originate from the same distribution. Following a significant Kruskal–Wallis test ($\alpha = 0.05$), Dunn's test was performed for the visual symptom, Cff DNA quantity, and trifoliate leaf dry weight data to conduct pairwise comparisons ('dunn.test' function in package *dunn.test*). For the plasmid copy number data, Conover's test was used to generate pairwise comparisons ('conover.test' function in package *conover.test*). These tests identify specific group differences and adjust for multiple comparisons using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Significant differences identified by Dunn's test or Conover's test were summarised using a compact letter display ('cldList' function in package *multcompView*), which assigns letters to groups based on their statistical significance. Boxplots were generated to visualise the distribution of each data set (function 'geom_boxplot' in package *ggplot2*). Correlations between visual symptoms, Cff DNA quantity, and trifoliate leaf dry weight were assessed using Spearman's rank correlation coefficient ('cor.test' function in package *stats*).

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

3 | Results

3.1 | PCR Design

Assay Cff_141 targeted a conserved region among the available plasmid sequences for Cff (Table 1; Figure S1). A BLAST search indicated the primer and probe sites targeted a conserved and specific sequence for Cff. The assay was redesigned from published assays to amplify a shorter fragment to ensure efficient amplification in qPCR, and to include a hydrolysis probe which enabled multiplexing with other PCR targets.

The Cf_180_*gyrB* assay target (a chromosomal species-specific region of the Cff gyrase B [*gyrB*] gene; Table 1) was conserved for 112 of 117 Australian Cff isolates, with five isolates (BRIP 70602, Cff063, Cff064, BRIP 70610 and Cff080) containing single-nucleotide polymorphisms in the primer binding regions. A BLAST search indicated the primer and probe sites were partially conserved within the *C. flaccumfaciens* species. Identical primer binding site sequences were detected in other species, indicating potential cross-amplification; however, the probe provided useful discrimination between *C. flaccumfaciens* and other species. Alignments across *C. flaccumfaciens* accessions suggested the assay may amplify other pathovars (Figure S2).

The Curto_57_*gyrB* assay target (a genus-specific region of the Cff *gyrB* gene; Table 1) was conserved for 117 Australian Cff isolates and most GenBank accessions for Cff. A BLAST search indicated the primer and probe sites were partially conserved among the *Curtobacterium* genus; however, the primer binding site sequences were identical in many other species. The probe binding site sequence was identical to predominantly the *Curtobacterium* genus. This demonstrates that the Curto_57_*gyrB* assay can be an alternative detection or quantification tool for Cff in controlled experiments, and potentially allow detection of diverse *Curtobacterium* species in mixed samples (Figure S3).

The Vr_157_*EF1α* assay (targeting the elongation factor 1-α (*EF1α*) gene of mung bean; Table 1) combined published primers for *Vigna mungo* (Kundu et al. 2013) with the design of a probe (Figure S4). A BLAST search indicated that the primer and probe binding sites were shared among many species, including the single accession available for the *EF1α* gene of *V. radiata* (GenBank accession: XM_014656474). The reverse primer provided the greatest discrimination among species.

3.2 | Plasmid Copy Numbers

Based on the relative quantities of ddPCR targets in the pCff119 plasmid and the *gyrB* gene, there were 1.37 to 2.74 plasmid copies present in plasmid-carrying isolates (Figure 1; Table S1). Two isolates (Cff089 and BRIP 70624) contained no detectable plasmid DNA. The plasmid copy number was significantly different among the 25 Cff isolates ($p < 0.001$; Figure 1, Table S3). The five plasmid-carrying isolates used in inoculation experiments had plasmid copy numbers of 2.38 (BRIP 70623), 2.43 (BRIP 70614), 2.45 (BRIP 70606), 2.72 (CffT13932) and 2.74 (DAR 56672). Plasmid copy numbers in DNA extracted from diseased leaves occurred across a range similar to the pure isolates (data not shown).

Relative read depth comparisons from Illumina sequencing data for the pCff119 plasmid and single-copy *gyrB* and *rpoB* genes produced plasmid copy number values ranging from 1 to 5.9. Mean estimated plasmid copy numbers were similar among the ddPCR ($\bar{x} = 2.0$, $n = 23$), Cff_141 to Curto_57_*gyrB* sequenced amplicons ($\bar{x} = 2.4$, $n = 113$), Cff plasmid to *gyrB* sequenced DNA ($\bar{x} = 2.2$, $n = 113$) and Cff plasmid to *rpoB* sequenced DNA ($\bar{x} = 2.1$, $n = 113$) analyses (Table S1). The relative sequenced read depth of *gyrB* to *rpoB* ranged from 0.7 to 1.4 ($\bar{x} = 1.0$, $n = 119$), indicating each chromosomal target is present at the same number of copies.

3.3 | Quantitative PCR Characteristics

The Cf_180_*gyrB* assay was able to detect Cff DNA from 0.25 pg to 25 ng, and the Vr_157_*EF1α* assay was able to detect mung bean DNA from 25 pg to 25 ng. A standard curve was calculated for the Cf_180_*gyrB* assay ($R^2 = 0.99$, $y = -3.6x + 15.7$, $E = 89.9$) and the Vr_157_*EF1α* assay ($R^2 = 0.99$, $y = -3.2x + 28.1$, $E = 105.0$). Based on these results, a cut-off point, or limit of quantification, was set at 31.6 cycles for Cff DNA. The respective mean values ($n = 8$) and coefficient of variation (CV) of a mixed DNA sample assessed for intra-assay variability were 1.8 ng Cff DNA (CV = 10.2%) for Cf_180_*gyrB* and 31.0 ng mung bean DNA (CV = 21.8%) for Vr_157_*EF1α*. The respective mean values ($n = 4$) and CV of the mixed DNA sample assessed for inter-assay variability were 2.0 ng Cff DNA (CV = 11.1%) for Cf_180_*gyrB* and 29.2 ng mung bean DNA (CV = 13.0%) for Vr_157_*EF1α*.

3.4 | Tan Spot Disease Symptoms

Mung bean plants were inoculated with seven treatments, including five plasmid-carrying isolates, one plasmid-free isolate, and a water control (Table 2). Initial disease symptoms were observed on the trifoliate leaves 3 days after inoculation, with the timing of symptom expression dependent on isolate. Typical tan spot symptoms are shown in Figure 2. Visual disease rating at 15 days after inoculation was based on summed values for three trifoliate leaves, with potential scores ranging from 0 to 300%. Significant variation in the expression of visual disease symptoms occurred among the Cff isolates ($p < 0.001$) (Figure 3A, Table S4). For the plasmid-carrying isolates, the mean visual symptoms ranged from 266.1% for the DAR 56672 treatment to 127.5% for the BRIP 70623 treatment. No symptoms were observed on the mung bean plants inoculated with the plasmid-free isolate Cff089 or the water treatment.

3.5 | Trifoliate Leaf Dry Weight

Inoculation with each of the five plasmid-carrying isolates significantly reduced the trifoliate leaf dry weight compared to the control plants ($p < 0.002$). Visible stunting in the height of these plants was also observed. Among these plants, the mean trifoliate leaf dry weights ranged from 571 mg for the BRIP 70606 treatment to 520 mg for the DAR 56672 treatment, with no significant

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

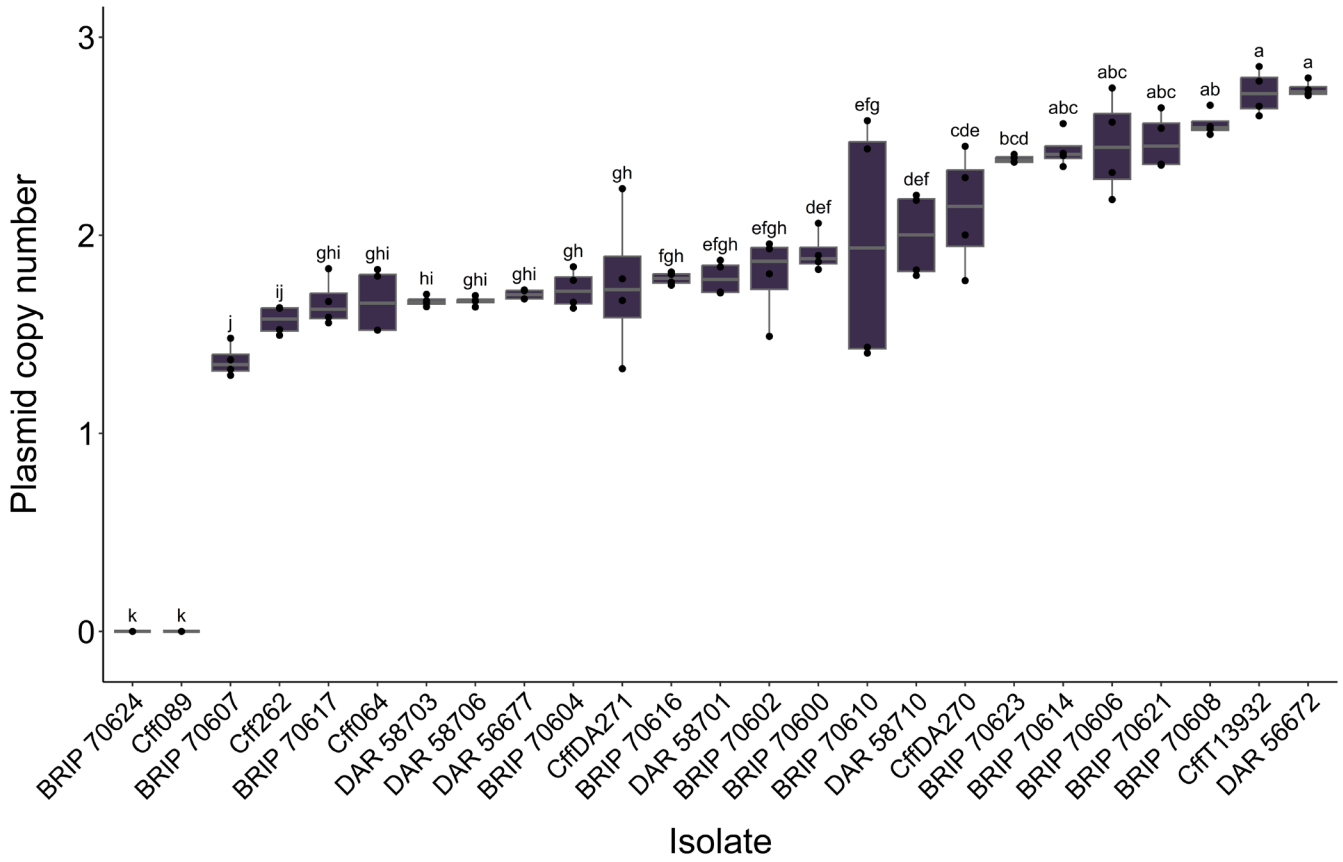


FIGURE 1 | Estimated plasmid copy numbers for 25 *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolates. The copy numbers were determined by the ratio of plasmid DNA to *gyrB* DNA copies in diluted cell suspensions in droplet digital PCR. The grey centre line denotes the median value (50th percentile), while the purple boxes contain the 25th to 75th percentiles of the data. The black whiskers denote the minimum and maximum values and black dots indicate the individual data points. Isolates with the same letter are not significantly different in their plasmid copy number ($n = 4$, $p < 0.05$ significance level). Mean separations were calculated on plasmid copy number values using Conover's test.



FIGURE 2 | Example of tan spot symptoms on the first trifoliate leaf of mung bean cultivar Opal-AU. Leaf responses range from no symptoms to 100% symptomatic leaf tissues (left to right). Visual rating accounted for both chlorosis and necrosis across the leaf area. Images were edited in PowerPoint, Microsoft 365.

difference among the five plasmid-carrying isolate treatments. Treatment BRIP 70623 (735 mg) was not significantly different from the control (1244 mg) or the other plasmid-carrying isolate treatments. In contrast, the trifoliate leaf dry weights of plants inoculated with the plasmid-free isolate Cff089 (1544 mg) were 19.7% greater than the control plants. However, this difference was not significant at $\alpha = 0.05$ (Figure 3B, Table S5).

3.6 | *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* DNA Quantities

Significant variation in the Cff DNA quantities was reported among the seven treatments ($p < 0.001$) (Figure 3C, Table S6). This was predominantly due to the Cff089 and control treatments, for which only one sample in each contained a detectable quantity

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

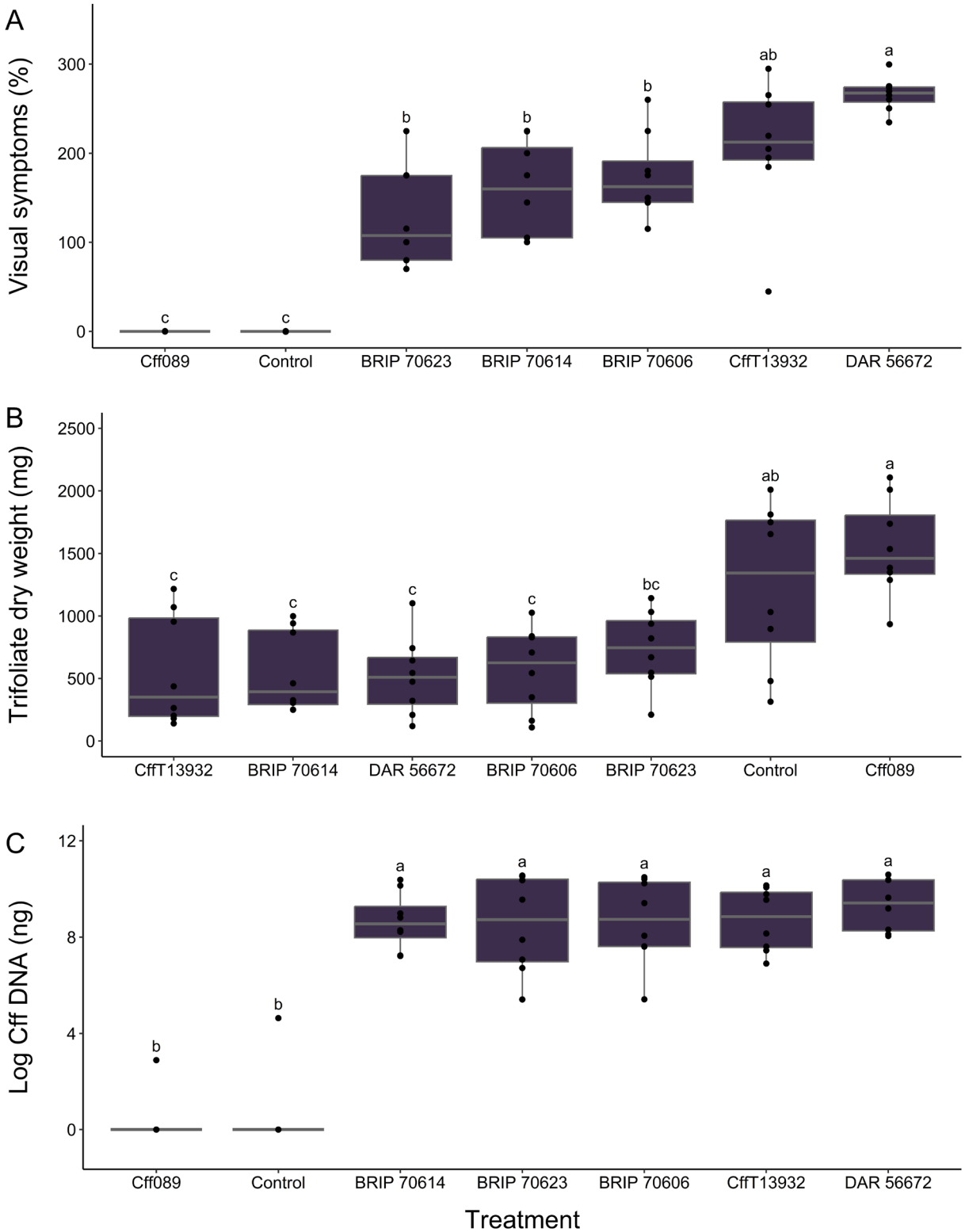


FIGURE 3 | Disease responses caused by six *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) isolates and a water control treatment at 15 days after inoculation of mung bean plants (cv. Opal-AU, $n = 6$). Responses include visual leaf disease symptoms (A), trifoliate leaf dry weights (B) and Cff DNA ($\log[x+1]$) in total leaf tissue samples (C). Different letters represent significant differences among the treatments at $\alpha < 0.05$. Mean separations were calculated using Dunn's test.

of Cff DNA (17 and 102 ng, respectively). The remaining Cff089 samples had quantification cycle (C_q) values outside the range of quantification (31.87 to 36.69). For the remaining control samples, two had no detection, with the other five samples exhibiting C_q values outside the range of quantification (31.71 to 38.02). The control sample with detectable Cff DNA was associated with a plant observed to have mild visible symptoms, not initially ascribed to tan spot disease but likely due to inoculum crossover in the

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

randomised blocks. No significant difference was observed for the Cff DNA quantity among the plasmid-carrying isolate treatments, with the mean values ranging from 17,509 ng for the DAR 56672 treatment to 10,376 ng for the BRIP 70614 treatment.

3.7 | Correlation of Disease Traits

A significant positive correlation occurred between visual disease symptoms and Cff DNA quantity ($\rho = 0.66$, $p < 0.001$). A significant negative correlation occurred between trifoliate leaf dry weights and visual disease symptoms ($\rho = -0.61$, $p < 0.001$) and Cff DNA quantity ($\rho = -0.41$, $p = 0.002$), respectively.

4 | Discussion

Tools for detecting and quantifying genetic characteristics of pathogens are important for disease assessment and understanding pathogen dynamics. This study reports the development of PCR-based assays for the quantification of chromosomal- and plasmid-associated regions of the Cff genome. These assays were used to provide the first demonstration of variation in the plasmid copy number among Cff isolates. The chromosome-based PCR assay also allowed detection and quantification of a single-copy chromosomal region in all Cff isolates, regardless of plasmid presence or absence. These tools demonstrated variation in disease traits on mung bean among a selection of six Cff isolates. This approach and information will be beneficial for assessing and managing disease on crops affected by Cff.

The presence of a plasmid containing pathogenicity-related genes has been linked to pathogenicity in Cff (Chen et al. 2021; Evseev et al. 2022; O'Leary and Gilbertson 2020; Osdaghi et al. 2020; Sparks et al. 2025; Vaghefi et al. 2021). However, nonpathogenic isolates without the plasmid have also been isolated from diseased plant tissues (Vaghefi et al. 2021). Bacteria can exchange genetic material, which may include the loss or gain of a plasmid and subsequent alterations of fitness in different environments (Vivian et al. 2001). The existence of Cff isolates without the plasmid demonstrated a need for alternative PCR detection methods beyond those which have been reported for Cff detection based on a region found in the plasmid (Messenberg Guimaraés et al. 2001; Tegli et al. 2002). This led to the development of PCR assays based on the *gyrB* gene, which successfully amplified for the majority of Cff isolates, while also theoretically discriminating against other bacteria. The focus on nucleotide-specific discrimination during PCR assay design constrained the optimisation of primer and probe characteristics, potentially affecting the PCR efficiency of the Cf_180_*gyrB* assay. While it is unlikely that an efficiency of 89.9% would substantially affect relative quantification, it does fall outside the optimal efficiency range of 90% to 110%. Due to DNA sequence variability, a single assay capable of detecting all Cff isolates, while also discriminating against other related bacteria, could not be generated.

Plasmid copy number was not fixed among the isolates. This was determined by directly testing bacterial suspensions in ddPCR, thus avoiding bias due to DNA extraction procedures (Plotka et al. 2017). The findings were confirmed with read depth analyses of genome sequence data. This provided further justification for the development of alternative PCR assays. Single-copy chromosomal genes, such as *gyrB*, are preferred targets for DNA quantification via PCR; however, the plasmid and chromosome of Cff are physically independent, and the observation suggested they did not occur in a 1:1 ratio. This has implications for reporting DNA quantities and estimating bacterial load. For example, a 2:1 plasmid to chromosome ratio may result in twice as much bacterial load being reported when using a plasmid-related PCR assay. If the plasmid copy number varies among isolates, then relevant comparisons would be impossible.

The multicopy nature of the plasmid lends itself to a greater chance of detection, providing a benefit for presence/absence screening. However, comparative PCR quantification based on a plasmid target should be avoided. The epidemiological role of variation in plasmid copy number among Cff isolates is not yet established but has potential implications for disease expression. Interestingly, isolate DAR 56672 exhibited the greatest plasmid copy number, while also being associated with the greatest values for visual symptom expression, bacterial biomass production and plant biomass reduction. It is possible that greater plasmid copy numbers may influence the strength of plasmid-borne characteristics (Clark et al. 2019; Friehs 2004; Schierstaedt et al. 2019) or increase the rate of plasmid transmission (Dimitriu et al. 2021; Williams and Thomas 1992), although plasmids may also confer a fitness cost (San Millan and MacLean 2017; Williams and Thomas 1992). Virulence in the human pathogen *Yersinia pestis* has been linked to up-regulated plasmid copy numbers during infection; however, plasmid copy numbers in plant pathogens have received limited attention (Coplin 1989; Schierstaedt et al. 2019; Vivian et al. 2001). Plasmid copy numbers may also be linked to the process of bacterial replication, with greater plasmid copy numbers increasing the chance of transferral to daughter cells and the inheritance of pathogenicity (Jahn et al. 2015). Retention of plasmids occurring at lower numbers may also involve active partitioning to decrease the chance of a plasmid-free cell developing (Williams and Thomas 1992).

Plasmid-free isolates and their lack of pathogenicity have been documented in previous reports (Sparks et al. 2025). The pathogenicity function of the plasmid was further evidenced in the current study by the nonpathogenic plasmid-free isolate Cff089. Not only did this isolate not induce visual disease symptoms or reduce trifoliate leaf dry weights, it also did not proliferate in the mung bean tissues asymptomatically. This leads to questions regarding the role of such isolates in the environment and whether they may pose a disease risk if the plasmid is acquired, while also providing opportunities to study the influence of the plasmid on bacterial growth characteristics. It is possible that the population from which isolate Cff089 was derived had the plasmid and was pathogenic, but the plasmid was lost during cell division, either in the plant tissue or during successive culturing following the concomitant relaxation of selection pressures for its retention (Smith and Bidochka 1998). The complex situation of Cff isolates ranging from no plasmid to variation in plasmid copy numbers, along with variation in pathogenicity and virulence, suggests a need to elucidate the role of the genes in the plasmid.

Disease reactions to Cff have traditionally been assessed based on visual inspections (Diatloff and Imrie 2000; Harveson et al. 2015;

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Wood and Easdown 1990). While these can be relatively rapid and cheap to perform, they are subjective and limited by the ability to visually characterise precise disease symptoms, which can lead to variation among experiments. Image-based analysis has also been applied to Cff disease symptoms (Vaghefi 2022), improving the precision of ratings but increasing the time to assess symptoms. In contrast, PCR-based methods of quantification provide objective measures of pathogen numbers and can be standardised by using a consistent tissue weight for DNA extraction or quantifying a host DNA target (Knight et al. 2012; Utami et al. 2024). Plant tissue dry weights also provide an objective assessment of the impact of a pathogen on plant growth, offering a further perspective on disease impacts. While useful to apply, both PCR-based and plant tissue dry weight methods require more resources than traditional visual rating. Strategic application of alternative disease assessment methods can support visual assessments and offer further insights into plant–pathogen interactions.

The assessment of tan spot disease traits on mung bean cultivar Opal-AU suggested variation among the plasmid-carrying isolates for visual symptoms. A trend was observed where isolate DAR 56672 caused the most severe disease responses. In contrast, Cff DNA quantities and trifoliate leaf dry weights provided no significant distinction. Sparks et al. (2025) similarly reported significant differences in disease severity among 11 Cff isolates on the moderately resistant mung bean cultivar Jade-AU. A previous study by Diatloff and Imrie (2000) identified mild and severe disease-causing isolates of Cff; however, upon inoculation across a range of mung bean cultivars, it was reported that their relative virulence varied. This isolate by cultivar interaction suggests that the use of a single susceptible cultivar in the current study may have limited the potential to discriminate among isolates for their disease impacts.

Assessment of visual symptoms, Cff DNA quantities and trifoliate leaf dry weights facilitated consideration of a range of disease impacts. The consistent reduction of trifoliate leaf dry weights in response to isolates carrying the plasmid indicates a general plant reaction to each isolate, with a significant reduction in biomass suggested to correlate to reduced grain yields (Geetika et al. 2022). In contrast, a range of visual disease symptoms was observed, indicating variation in foliar responses to each isolate. Differences in trifoliate leaf dry weight and visual symptoms may reflect the combined effects of the stem inoculation method, which delivered an equal quantity of bacteria to each plant, and the subsequent ability of each isolate to move within the plant tissue.

The assessment of Cff DNA quantities provides another perspective of the disease process, with a focus primarily on the ability of the bacteria to move to, colonise and reproduce in the trifoliate leaf tissues. The Cff DNA quantities suggested a range among the isolates; however, no statistically significant differences occurred between them. While PCR-based biomass estimates may exhibit variation due to the rate of disease expression and pathogen reproduction, there is also a potential impact of Cff asymptomatically colonising mung bean tissues (Diatloff and Imrie 2000). Any asymptomatic colonisation may have reduced the ability to discriminate among the isolates based on DNA quantities. Assessing three disease traits provides a more comprehensive perspective of the interactions between the host and the pathogen, while also suggesting that objective continuous variables, such as Cff DNA quantities and trifoliate leaf dry weights, provide both challenges for comparative assessments and precise interpretations of biological variation.

The information from this study begins to unravel the plasmid dynamics in Cff and indicates a need to further investigate the regulation of plasmid replication, inheritance and transmission, along with the expression of genes in the plasmid, and the relationship between plasmid copy number and virulence. The alternative PCR tools for Cff detection and quantification may be used to explore disease across different crops affected by Cff and investigate inoculum sources. Furthermore, the classification of virulence among a group of genetically diverse isolates will be used to support mung bean breeding efforts to screen for reduced susceptibility to Cff.

Acknowledgements

This research was funded by the Broadacre Cropping Initiative (Project 36), a joint investment of the University of Southern Queensland and the Queensland Government Department of Primary Industries. The authors thank C. A. Douglas for discussions during project development and supplying Opal-AU seed, and K. Cowan (New South Wales Plant Pathology and Mycology Herbarium), Y. P. Tan (Queensland Department of Primary Industries Plant Pathology Herbarium), D. L. Adorada and E. E. Adorada (University of Southern Queensland) for supplying and creating bacterial and fungal isolates. Open access publishing facilitated by University of Southern Queensland, as part of the Wiley - University of Southern Queensland agreement via the Council of Australian University Librarians.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Adam, D. S., and A. T. Pugsley. 1934. "Bacterial Plant Diseases in Victoria." *Journal of Agriculture, Victoria* 32: 304–323.

Agarkova, I. V., P. A. Lambrecht, A. K. Vidaver, and R. M. Harveson. 2012. "Genetic Diversity Among *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* Populations in the American High Plains." *Canadian Journal of Microbiology* 58: 788–801.

AMA. 2023. "Australian Mungbean Association, About Us." http://www.mungbean.org.au/about-us.html.

Batzer, J. C., A. Singh, A. Rairdin, K. Chiteri, and D. S. Mueller. 2022. "Mungbean: A Preview of Disease Management Challenges for an Alternative U.S. Cash Crop." *Journal of Integrated Pest Management* 13: 1–21.

Benjamini, Y., and Y. Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society. Series B, Statistical Methodology* 57: 289–300.

Bultreys, A., and I. Gheysen. 2023. "High Diversity of *Curtobacterium flaccumfaciens* in Poinsettia and Detection of Three Pathogenicity Plasmids for Identification of *C. flaccumfaciens* pv. *poinsettiae*." *Phytobiomes Journal* 8: 378–400.

Chauhan, Y. S., and R. Williams. 2018. "Physiological and Agronomic Strategies to Increase Mungbean Yield in Climatically Variable Environments of Northern Australia." *Agronomy* 8: 83.

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Chen, G., M. Khojasteh, A. Taheri-Dehkordi, S. M. Taghavi, T. Rahimi, and E. Osdaghi. 2021. "Complete Genome Sequencing Provides Novel Insight Into the Virulence Repertories and Phylogenetic Position of Dry Beans Pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*." *Phytopathology* 111: 268–280.

Clark, D. P., N. J. Pazdernik, and M. R. McGehee. 2019. "Chapter 23 - Plasmids." In *Molecular Biology*, edited by D. P. Clark, N. J. Pazdernik, and M. R. Mcgehee, 3rd ed., 712–748. Academic Cell.

Coplin, D. L. 1989. "Plasmids and Their Role in the Evolution of Plant Pathogenic Bacteria." *Annual Review of Phytopathology* 27: 187–212.

DAF. 2018. "Mungbean: The $70m Diversification Opportunity in North West Queensland." Queensland Government.

Diatloff, A., and B. Imrie. 2000. "Inoculation Techniques for Evaluating Resistance to *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in Mungbean Cultivars." *Australasian Plant Pathology* 29: 24–28.

Dimitriu, T., A. C. Matthews, and A. Buckling. 2021. "Increased Copy Number Couples the Evolution of Plasmid Horizontal Transmission and Plasmid-Encoded Antibiotic Resistance." *Proceedings of the National Academy of Sciences of the United States of America* 118: e2107818118.

Douglas, C. 2021. "Opal-AU Variety Management Package." Australian Mungbean Association.

EPPO. 2023. "*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*." EPPO Datasheets on Pests Recommended for Regulation. https://gd.eppo.int.

Evseev, P., A. Lukianova, R. Tarakanov, et al. 2022. "*Curtobacterium* spp. and *Curtobacterium flaccumfaciens*: Phylogeny, Genomics-Based Taxonomy, Pathogenicity, and Diagnostics." *Current Issues in Molecular Biology* 44: 889–927.

Friehs, K. 2004. "Plasmid Copy Number and Plasmid Stability." In *New Trends and Developments in Biochemical Engineering*, edited by T. Scheper, 47–82. Springer Berlin Heidelberg.

Garces, F. F., A. Gutierrez, and J. W. Hoy. 2014. "Detection and Quantification of *Xanthomonas albilineans* by qPCR and Potential Characterization of Sugarcane Resistance to Leaf Scald." *Plant Disease* 98: 121–126.

Geetika, G., G. Hammer, M. Smith, et al. 2022. "Quantifying Physiological Determinants of Potential Yield in Mungbean (*Vigna radiata* (L.) Wilczek)." *Field Crops Research* 287: 108648.

Gonçalves, R. M., M. I. Balbi-Peña, J. M. Soman, et al. 2019. "Genetic Diversity of *Curtobacterium flaccumfaciens* Revealed by Multilocus Sequence Analysis." *European Journal of Plant Pathology* 154: 189–202.

Gonçalves, R. M., C. A. Schipanski, L. Koguishi, et al. 2017. "Alternative Hosts of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, Causal Agent of Bean Bacterial Wilt." *European Journal of Plant Pathology* 148: 357–365.

Harveson, R. M., H. F. Schwartz, C. A. Urrea, and C. D. Yonts. 2015. "Bacterial Wilt of Dry-Edible Beans in the Central High Plains of the U.S.: Past, Present, and Future." *Plant Disease* 99: 1665–1677.

Hernández-Beltrán, J. C. R., A. San Millán, A. Fuentes-Hernández, and R. Peña-Miller. 2021. "Mathematical Models of Plasmid Population Dynamics." *Frontiers in Microbiology* 12: 606396.

Ilhan, J., A. Kupczok, C. Woehle, et al. 2018. "Segregational Drift and the Interplay Between Plasmid Copy Number and Evolvability." *Molecular Biology and Evolution* 36: 472–486.

Jahn, M., S. Günther, and S. Müller. 2015. "Non-Random Distribution of Macromolecules as Driving Forces for Phenotypic Variation." *Current Opinion in Microbiology* 25: 49–55.

Júnior, T. S., D. R. Negrão, A. T. Itako, and A. C. Maringoni. 2012. "Pathogenicity of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* to Several Plant Species." *Journal of Plant Pathology* 94: 427–430.

Kelly, L. A., N. Vaghefi, K. Bransgrove, et al. 2021. "One Crop Disease, How Many Pathogens? *Podosphaera xanthii* and *Erysiphe vignae* sp. nov. Identified as the Two Species That Cause Powdery Mildew of Mungbean (*Vigna radiata*) and Black Gram (*V. mungo*) in Australia." *Phytopathology* 111: 1193–1206.

Kizheva, Y., M. Pandova, M. Dimitrova, et al. 2024. "First Report of *Curtobacterium flaccumfaciens* in Bulgaria." *Pathogens* 13: 483.

Knight, N. L., A. Martin, M. W. Sutherland, and D. J. Herde. 2012. "Assessment of Infection by *Fusarium pseudograminearum* in Wheat Seedling Tissues Using Quantitative PCR and a Visual Discoloration Scale." *Plant Disease* 96: 1661–1669.

Knight, N. L., A. Moslemi, F. Begum, et al. 2022. "Detection of *Ramularia collo-cygni* From Barley in Australia Using Triplex Quantitative and Droplet Digital PCR." *Pest Management Science* 78: 1367–1376.

Kottara, A., J. P. J. Hall, and M. A. Brockhurst. 2021. "The Proficiency of the Original Host Species Determines Community-Level Plasmid Dynamics." *FEMS Microbiology Ecology* 97: fiab026.

Kundu, A., A. Patel, and A. Pal. 2013. "Defining Reference Genes for qPCR Normalization to Study Biotic and Abiotic Stress Responses in *Vigna mungo*." *Plant Cell Reports* 32: 1647–1658.

Li, H., and R. Durbin. 2009. "Fast and Accurate Short Read Alignment With Burrows-Wheeler Transform." *Bioinformatics* 25: 1754–1760.

Li, H., B. Handsaker, A. Wysoker, et al. 2009. "The Sequence Alignment/Map Format and SAMtools." *Bioinformatics* 25: 2078–2079.

McDonald, J. G., and E. Wong. 2000. "High Diversity in *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* Characterized by Serology and Rep-PCR Genomic Fingerprinting." *Canadian Journal of Plant Pathology* 22: 17–22.

Melnyk, R. A., and C. H. Haney. 2017. "Plasmid-Powered Evolutionary Transitions." *eLife* 6: e33383.

Messenberg Guimaraés, P., S. Palmano, J. J. Smith, M. F. Grossi de Sá, and G. S. Saddler. 2001. "Development of a PCR Test for the Detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*." *Antonie Van Leeuwenhoek* 80: 1–10.

Noble, T. J., A. J. Young, C. A. Douglas, B. Williams, and S. Mundree. 2019. "Diagnosis and Management of Halo Blight in Australian Mungbeans: A Review." *Crop and Pasture Science* 70: 195–203.

O'Leary, M. L., and R. L. Gilbertson. 2020. "Complete Genome Sequence Resource of a Strain of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, the Causal Agent of Bacterial Wilt of Common Bean, From Turkey." *Phytopathology* 110: 2010–2013.

Ophel-Keller, K., A. McKay, D. Hartley, and J. Curran. 2008. "Development of a Routine DNA-Based Testing Service for Soilborne Diseases in Australia." *Australasian Plant Pathology* 37: 243–253.

Osdaghi, E., S. M. Taghavi, S. Calamai, et al. 2018. "Phenotypic and Molecular-Phylogenetic Analysis Provide Novel Insights Into the Diversity of *Curtobacterium flaccumfaciens*." *Phytopathology* 108: 1154–1164.

Osdaghi, E., A. J. Young, and R. M. Harveson. 2020. "Bacterial Wilt of Dry Beans Caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*: A New Threat From an Old Enemy." *Molecular Plant Pathology* 21: 605–621.

Pandey, A. K., R. R. Burlakoti, L. Kenyon, and R. M. Nair. 2018. "Perspectives and Challenges for Sustainable Management of Fungal Diseases of Mungbean [*Vigna radiata* (L.) R. Wilczek var. *radiata*]: A Review." *Frontiers in Environmental Science* 6: 53.

Plotka, M., M. Wozniak, and T. Kaczorowski. 2017. "Quantification of Plasmid Copy Number With Single Colour Droplet Digital PCR." *PLoS One* 12: e0169846.

Puia, J. D., R. R. Murari, L. C. Borsato, et al. 2021. "Protocol for Detecting *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in the Leaves of Infected Bean Plants (*Phaseolus vulgaris* L.)." *Acta Scientiarum Agronomy* 43: e51031.

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

R Core Team. 2017. "R: A Language and Environment for Statistical Computing." R Foundation for Statistical Computing, Vienna, Austria.

San Millan, A., and R. C. MacLean. 2017. "Fitness Costs of Plasmids: A Limit to Plasmid Transmission." *Microbiology Spectrum* 5: 10. https://doi.org/10.1128/microbiolspec.mtbp-0016-2017.

Schaad, N. W., and R. D. Frederick. 2002. "Real-Time PCR and Its Application for Rapid Plant Disease Diagnostics." *Canadian Journal of Plant Pathology* 24: 250–258.

Schierstaedt, J., N. Bziuk, N. Kuzmanović, K. Blau, K. Smalla, and S. Jechalke. 2019. "Role of Plasmids in Plant-Bacteria Interactions." *Current Issues in Molecular Biology* 30: 17–38.

Sharma, A., P. Abrahamian, R. Carvalho, et al. 2022. "Future of Bacterial Disease Management in Crop Production." *Annual Review of Phytopathology* 60: 259–282.

Singh, B. K., M. Delgado-Baquerizo, E. Egidi, et al. 2023. "Climate Change Impacts on Plant Pathogens, Food Security and Paths Forward." *Nature Reviews Microbiology* 21: 640–656.

Smith, M. A., and M. J. Bidochka. 1998. "Bacterial Fitness and Plasmid Loss: The Importance of Culture Conditions and Plasmid Size." *Canadian Journal of Microbiology* 44: 351–355.

Sparks, A. H., D. L. Adorada, E. Colombi, et al. 2025. "Clonal Expansion From Standing Genetic Variation Underpins the Evolution of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in Australia." *Phytopathology*. https://doi.org/10.1094/PHYTO-01-25-0032-R.

Tang, Y., X. Wu, B. Lou, et al. 2020. "A Rapid Identification Method for Germplasm Materials Resistant to Citrus Huanglongbing Disease." *Agricultural Biotechnology* 9: 64–69.

Tegli, S., C. Biancalani, A. N. Ignatov, and E. Osdaghi. 2020. "A Powerful LAMP Weapon Against the Threat of the Quarantine Plant Pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*." *Microorganisms* 8: 1705.

Tegli, S., D. Gaudioso, and D. Stefanucci. 2022. "Innovative Detection of the Quarantine Plant Pathogen *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, Causal Agent of Bacterial Wilt of Leguminous Plants." In *Plant Pathology: Method and Protocols*, edited by N. Luchi, 251–261. Springer US.

Tegli, S., A. Sereni, and G. Surico. 2002. "PCR-Based Assay for the Detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in Bean Seeds." *Letters in Applied Microbiology* 35: 331–337.

Tokmakova, A. D., R. I. Tarakanov, A. A. Lukianova, et al. 2024. "Phytopathogenic *Curtobacterium flaccumfaciens* Strains Circulating on Leguminous Plants, Alternative Hosts and Weeds in Russia." *Plants* 13: 667.

Trancoso, R., J. Syktus, N. Toombs, D. Ahrens, K. K.-H. Wong, and R. D. Pozza. 2020. "Heatwaves Intensification in Australia: A Consistent Trajectory Across Past, Present and Future." *Science of the Total Environment* 742: 140521.

Utami, D., S. J. Meale, and A. J. Young. 2024. "Bacterial Leaf Spot Susceptibility Screening of Chili Pepper Cultivars Using qPCR Determination of *Xanthomonas euvesicatoria* pv. *euvesicatoria* Titers." *Phytopathology* 114: 681–689.

Vaghefi, N. 2022. "Inoculating Mungbean With *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) for Tan Spot Disease Assessment." *Protocols.Io*. https://doi.org/10.17504/protocols.io.bym17502pu17508e.

Vaghefi, N., D. L. Adorada, L. Huth, et al. 2021. "Whole-Genome Data From *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* Strains Associated With Tan Spot of Mungbean and Soybean Reveal Diverse Plasmid Profiles." *Molecular Plant–Microbe Interactions* 34: 1216–1222.

Vivian, A., J. Murillo, and R. W. Jackson. 2001. "The Roles of Plasmids in Phytopathogenic Bacteria: Mobile Arsenals?" *Microbiology* 147: 763–780.

Wickham, H. 2016. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag.

Williams, D. R., and C. M. Thomas. 1992. "Active Partitioning of Bacterial Plasmids." *Microbiology* 138: 1–16.

Wood, B. A., and W. J. Easdown. 1990. "A New Bacterial Disease of Mung Bean and Cowpea for Australia." *Australasian Plant Pathology* 19: 16–21.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Alignment of the *Cff*_141 plasmid assay primers (bold) and probe (bold and underlined) against the plasmid sequence of *C. flaccumfaciens* pv. *flaccumfaciens* (*Cff*), partially covering a trypsin-like serine protease gene sequence. GenBank accession number CP071884 was used for base pair reference positions. This sequence was conserved among *C. flaccumfaciens* pv. *flaccumfaciens* isolates containing the plasmid pCff119, pCFF113 or Cff1. **Figure S2:** Alignment of the *Cf*_180_*gyrB* assay primers (bold) and probe (bold and underlined) against gyrase b (*gyrb*) gene sequences of a group of *Curtobacterium* species. GenBank accession number CP071883 was used for base pair reference positions. Polymorphisms among the sequences are indicated by shading (black text on grey). **Figure S3:** Alignment of the Curto_57_*gyrB* assay primers (bold) and probe (bold and underlined) against gyrase b (*gyrb*) gene sequences of a group of *Curtobacterium* species and *Clavibacter michiganensis* subsp. *michiganensis* (related species). GenBank accession number CP071883 was used for base pair reference positions. Polymorphisms among the sequences are indicated by shading (black text on grey). **Figure S4:** Alignment of the *Vrr*_157_*EF1a* assay primers (bold) and probe (bold and underlined) against the elongation factor 1-alpha sequence of *Vigna radiata*. Uracil bases were changed to thymine for the alignment. GenBank accession number XM_014656474 was used for base pair reference positions. **Table S1:** ppa70037-sup-0005-TableS1.docx. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) plasmid pCff119 copy numbers estimated using duplex droplet digital PCR (ddPCR) ($n = 25$ isolates) and relative sequence read depths across PCR amplicons and entire plasmid and gene (*gyrB* and *rpoB*) regions ($n = 119$). ddPCR estimated plasmid copy numbers by dividing the *Cff*_141 by *Curto*_57_*gyrB* copies across four replicates, with the mean value and standard error (SE) calculated. For Illumina sequencing data either the target amplicon for the *Cff*_141 and *Curto*_57_*gyrB* assays or the entire plasmid and gene regions for *gyrB* and *rpoB* were assessed. Each were assessed for total read depth across the sequence and the mean read depth per base across the same sequence. The relative read depth, or relative copy number, was calculated using the mean read depth for the plasmid compared to both *gyrB* and *rpoB*. The *gyrB* and *rpoB* regions were also compared for relative copy number. Isolates are ordered by the relative read depth of *Cff* plasmid to *gyrB*. Table code ([a]) indicates isolates used in mung bean (cv. Opal-AU) inoculation experiments, (–) indicates isolates were not tested, (na) indicates the calculation was not applicable due to no plasmid being detected. Mean values were rounded to one decimal place. **Table S2:** Fungal and bacterial isolates used to provide non-target DNA for PCR assay optimisation. **Table S3:** Summary of statistics for the plasmid copy number of 25 *Curtobacterium flaccumfaciens pv. flaccumfaciens* isolates. **Table S4:** Summary of statistics for visual disease symptoms at 15 days after inoculation of mung bean plants (cv. Opal-AU) with six *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolates and a water control treatment. **Table S5:** Summary of statistics for trifoliate leaf dry weights at 15 days after inoculation of mung bean plants (cv. Opal-AU) with six *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolates and a water control treatment. **Table S6:** Summary of statistics for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) DNA (log[x + 1]) in total leaf tissue samples at 15 days after inoculation of mung bean plants (cv. Opal-AU) with six *Cff* isolates and a water control treatment.

13653059, 2025, 9, Downloaded from https://bsppjournals.onlinelibrary.wiley.com/doi/10.1111/ppa.70037 by Research Service - Research Information Service , Wiley Online Library on [09/02/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License