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Detached leaf inoculation of germplasm for rapid screening of resistance to citrus canker and citrus bacterial spot

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Abstract A detached leaf protocol for rapid screening of germplasm for resistance to citrus canker (*Xanthomonas citri* subsp. *citri*, *Xcc*) and citrus bacterial spot (*Xanthomonas alfalfae* subsp. *citrumelonis*, *Xac*) was developed to evaluate limited quantities of leaf material. Bacterial inocula of *Xcc* or *Xac* at 10^4, 10^5, or 10^8 cfu ml^{-1} were injection-infiltrated into the abaxial surface of disinfested, immature leaves of susceptible and resistant genotypes. Inoculated detached leaves were placed on the surface of 0.5% water agar plates and incubated at 28°C under a 12 h photoperiod. Likewise, inocula were infiltrated into attached leaves of greenhouse plants. At high inoculum concentrations of *Xcc* or *Xac* (10^8 cfu ml^{-1}), resistant cultivars of kumquat developed a hypersensitive-like reaction within 3 days post inoculation (dpi). At 10^5 cfu ml^{-1}, populations 14 dpi were $<10^4$ per inoculation site. In canker-susceptible *Citrus* spp. ('Duncan' grapefruit and 'Rough' lemon), water-soaked areas occurred by 3 dpi and typical canker lesions developed by 7 to14 dpi. Concentration of *Xcc* recovered from inoculation sites was approximately 10^5 cfu ml^{-1} by 14 dpi. In citrus bacterial spot-susceptible citrus ('Swingle' citrumelo and grapefruit), symptoms developed within 7 dpi. Populations of *Xac* after inoculation at 10^5 cfu ml^{-1} were comparable to *Xcc* in susceptible hosts 14 dpi ($>10^5$). The detached leaf assay is useful for the characterization and differentiation of lesion phenotype for each *Xanthomonas* pathogen permitting rapid screening of germplasm resistance based on the quantification of number of lesions and bacterial concentration.

Keywords Citrus germplasm screening · Bacterial disease resistance · *Xanthomonas alfalfae* subsp. *citrumelonis* · *Xanthomonas citri* subsp. *citri*

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Introduction

Xanthomonas citri subsp. *citri*, (*Xcc*) causes citrus canker, a serious citrus leaf and fruit spotting disease (Graham et al. 2004). *Xanthomonas alfalfae* subsp. *citrumelonis* (*Xac*) is a less virulent pathogen, known only in Florida citrus nurseries, that causes citrus bacterial spot (CBS; Gottwald and Graham 1990; Graham and Gottwald 1991). Canker strain A of *Xcc* is pathogenic on all citrus species and produces disease reactions that range from highly susceptible to moderately resistant. 'Key' or 'Mexican' lime (*Citrus aurantifolia* Swingle), grapefruit (*C. paradisi* Macf.), lemon (*C. limon* (L.) Burm. f.), and selected sweet orange cultivars (*C. sinensis* (L.) Osb.) are

among the most susceptible commercial varieties (Gottwald et al. 1993; Koizumi 1981). The host range of *Xac* is quite different and much narrower (Gottwald et al. 1993; Graham et al. 1990). *Xac* primarily affects rootstocks in nurseries, such as trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and its hybrids 'Swingle' citrumelo (*P. trifoliata*×*C. paradisi*) and 'Carrizo' citrange (*P. trifoliata*×*C. sinensis* L.). Grapefruit, sweet orange, and lemon (*C. limon* (L.) Burm. f.) are more resistant to CBS. Lesions caused by *Xcc* develop a hypertrophic and hyperplastic proliferation of cells resulting in raised, callus-like lesions on the leaf and fruit surface, whereas lesions caused by *Xac* are necrotic surrounded by water-soaking that are flat or slightly sunken (Graham and Gottwald 1991).

Mesophyll susceptibility to *Xcc* and *Xac* varies widely among citrus types as measured in host range studies involving inoculation of immature tissues, although artificial inoculations produce more susceptible reactions in cultivars than is observed in the field (Gottwald et al. 1993). This is because expanding leaves are most vulnerable to infection and field resistance to *Xcc* is directly related to tissue juvenility (Gottwald and Graham 1992; Graham et al. 1992). *Citrus* cultivars and species with greater frequency, size, and duration of leaf flushes and duration of fruit growth are more field-susceptible to *Xcc* than less vigorous cultivars or those whose foliage matures more rapidly (Gottwald et al. 1993; Koizumi 1981). To measure mesophyll tissue susceptibility to *Xcc* (Stall et al. 1982), inoculation of leaves and fruit without wounding is best assessed by injection-infiltration of bacteria through the stomates (Gottwald and Graham 1992; Graham et al. 1992; Koizumi 1976).

Detached leaf assays are often employed to evaluate resistance of germplasm to bacterial pathogens (Moragrega et al. 2003). For citrus bacterial diseases, a detached leaf assay based on wound inoculation was developed to evaluate the host range and virulence of *Xac* strains infecting citrus in Florida (Graham and Gottwald 1990; Graham et al. 1990). This protocol used a high inoculum concentration (10^8 cfu ml^{-1}) for needle-prick inoculation of detached leaves to rapidly differentiate the aggressiveness of CBS strains by measuring the diameter of lateral development of the flat, necrotic lesions caused by these strains (Graham et al. 1990). However, needle prick inoculation was unsuitable for measuring differences in development of raised lesions caused by *Xcc* strains for various citrus species, cultivars and citrus relatives to canker (Gottwald et al. 1993). For citrus production areas where *Xcc* is not present, an in vitro method using needle inoculation of citrus seedlings grown in a test tube was developed (López and Navarro 1981) as a standardized procedure for quarantine diagnosis of *Xcc* on citrus fruits entering the European Union (OEPP/EPPO 2005).

Highest resistance to canker occurs in kumquats (*Fortunella* spp.), and some other citrus relatives (Gottwald et al. 1993; Viloria et al. 2004). One approach for developing more resistant varieties is introgression of canker resistance from such citrus relatives into susceptible commercial cultivars. Conventional sexual hybridization with citrus relatives for disease resistance in scion germplasm is difficult (Viloria et al. 2004). Therefore, alternative strategies utilize in vitro protoplast fusion for somatic hybridization or introduction of canker resistance genes via *Agrobacterium* transformation and regeneration of in vitro plants from tissue culture (Grosser et al. 2000, 2008). For resistance screening purposes, a more definitive inoculation method for *Xcc* was sought for screening citrus germplasm and newly developed candidates via tissue culture propagation. Detached leaf inoculation could provide for more rapid and early screening of limited quantities of leaf material for resistance to canker under pathogen containment conditions.

The objectives of the present study were: i) to evaluate injection-infiltration of detached leaves with *Xcc* and *Xac* by characterizing the reactions of known resistant and susceptible citrus hosts to canker and CBS, and ii) to compare the disease phenotype and bacterial development on detached leaves in petri dishes with that on attached leaves of greenhouse-grown plants.

Materials and methods

Bacterial strains and plant material

The *Xcc* strain X2002-0014 used in this study was isolated in 2002 from sweet orange (*C. sinensis*) in Dade County, FL and the *Xac* strain F-1 was isolated in 1984 from 'Swingle' citrumelo in Polk County, FL.

Strains were stored in glycerol under −80°C conditions in an ultra low freezer.

The cultivars chosen for comparison of resistance to *Xcc* were 'Meiwa' kumquat (*Fortunella crassifolia* Swingle),'Nagami' kumquat (*F. margarita* [Lour.] Swingle), 'Duncan' grapefruit and 'Rough' lemon (*C. jambhiri* Lush). Cultivars for comparison of resistance to *Xac* were the kumquats cultivars, 'Duncan' grapefruit and 'Swingle' citrumelo. Plants of each type were grown in soil-less medium (The Scotts Co., Marysville, OH, USA) contained in 3.8 l pots and maintained in the greenhouse between 20 to 30°C temperature. Plants were fertilized every 2 weeks with Peters 20-10-20 (0.5 g l^{-1}), and supplemented with Essential Minor Elements (5 g per pot; The Scotts Co.).

Detached leaf assay

For assays, *Xcc* and *Xac* strains were streaked on nutrient agar and single colonies seeded into nutrient broth and grown at 28°C for 24 h to log phase. The bacterial suspension was centrifuged at 10,000 g for 20 min, re-suspended in sterile saline phosphate buffer (PBS; 40 mM Na_2HPO_4+25 mM KH_2PO_4), and adjusted to 0.1 OD at 620 nm, equivalent to 10^8 colony-forming units (cfu) per ml. Bacterial cell density was adjusted to 10^4, 10^5, or 10^8 cfu ml^{-1} for inoculations.

Detached leaves and attached leaves were utilized at the optimal stage of susceptibility for bioassay. Four to five immature leaves (75% expanded) were collected from shoots (20–25 cm long) induced by pruning vigorous plants. Leaf samples were collected in the morning from greenhouse-grown plants (0.5–1 m in height) of each type and placed inside sealable plastic bags on ice for transport to the lab. Leaves were rinsed three times with sterile distilled water in the same plastic bags to remove any debris or spray residues, dipped in 70% ethanol for 30 s, and immersed in 0.5% sodium hypochlorite for 30 s. The leaves were immediately rinsed three times with sterile distilled water (SDW). Three to four leaves were assayed per treatment and the experiment was repeated three times.

Disinfested leaves handled by the petiole end were placed on a sterile paper towel and using a 1 cm^3 needleless tuberculin syringe, bacterial suspension was loaded and the syringe tip pressed against the abaxial surface of the leaf with the index finger of a latex gloved hand supporting the leaf from behind. Bacterial suspension (approximately 2 μl) was infiltrated into the leaf until the water-soaked area reached about 6 mm in diameter. Three areas on each side of the leaf mid-vein were infiltrated. Excess inoculum was wiped from the leaf surface with a sterile paper towel. Inoculated leaves were placed on the surface of soft water agar (0.5%) with the abaxial side up. The petiole was removed and the leaf pressed onto the agar surface with a plastic spreader to obtain as much contact as possible. Petri dishes were immediately sealed with parafilm and the plates incubated in an environmentally controlled growth chamber (28°C; fluorescent light at 60 μmol m^{-2} s^{-1} for 12-h photoperiods). Symptoms on the inoculated detached leaves were assessed 2, 3, 7 and 21 days post inoculation (dpi). The number of stomatal lesions per injection-infiltration site was counted under a stereo microscope at 6X magnification.

The bacterial population in the inoculated areas was estimated at 14 dpi. Leaf disks (6 mm diameter), circumscribing each infiltrated area, were excised and ground with 1.0 ml of PBS buffer using a glass homogenizer. Serial dilutions of suspension were plated on kasugamycin-cephalexin-chlorothalonil (KCC; nutrient agar plus kasugamycin 16.0 mgl^{-1}, cephalexin 16.0 mg l^{-1}, and chlorothalonil 12.0 mgl^{-1}) agar medium (Viloria et al. 2004). Total bacterial colonies per inoculation site were expressed as log cfu per inoculation site. Symptoms on detached leaves were compared to those on attached leaves after inoculation in the greenhouse by injection-infiltration as described below.

Attached leaf assay

Immature leaves (75% expanded) on greenhouse seedlings of the same types used for the detached leaf assay were inoculated using a 1 cm^3 tuberculin syringe (Viloria et al. 2004). In brief, *Xcc* inoculum adjusted to 10^4 , 10^5, and 10^8 cfu ml^{-1} was infiltrated by pressing the needleless syringe tip against the abaxial leaf surface to produce a zone of water-soaked tissue about 6 mm in diameter. Three injection infiltrations were performed on each side of the mid-vein. At least three leaves were inoculated per plant,

and three plants were inoculated per assay. The inoculated shoot was covered with a plastic bag for 24 h to maintain high relative humidity conducive for bacterial growth in the leaves. Development of symptoms on leaves was evaluated periodically up to 21 dpi. Lesion counts were performed at 14 dpi.

Data analysis

The log transformation of bacterial concentration per inoculation sites and the mean number of lesions per inoculation site at different inoculum concentrations were analyzed using the General Linear Models procedure (SAS Institute, Cary, NC). Mean separation was performed according to the Waller's *K*-ratio *t*-test ($P \leq 0.05$).

Results

Comparison of lesions phenotypes from detached leaf and attached leaf assays

Inoculation of detached 'Duncan' grapefruit or 'Rough' lemon leaves with *Xcc* at 10^8 cfu ml^{-1} resulted in slight water-soaking by 72 h after inoculation (Fig. 1a and b), tissue hyperplasia and hypertrophy at 7 dpi (Fig. 1e and f), with the formation of raised, callus-like lesions typical of canker in compatible hosts. Inoculation of detached leaves of 'Duncan' grapefruit or 'Swingle' citrumelo with *Xac* at 10^8 cfu ml^{-1} induced little or no water-soaking after 72 h (Fig. 2a and b), by 7 dpi, flat, brownish-gray lesions in the center of the inoculation site with small irregular lesions developed at the edge (Fig. 2e and f). In contrast, when detached leaves of 'Meiwa' and 'Nagami' kumquat were inoculated with 10^8 cfu ml^{-1} *Xcc* or *Xac*, extensive tissue necrosis developed 48 or 72 h after inoculation, respectively (Figs. 1c, d and 2c, d). At 7 dpi, the tissue within the infiltrated area completely collapsed (Figs. 1g, h and 2g, h). At 21 dpi, lesions on detached leaf and attached leaves of 'Duncan' grapefruit and 'Rough lemon' or 'Swingle' citrumelo inoculated with *Xcc* or *Xac* at 10^4 cfu ml^{-1} were erumpent and callus-like or flat, gray-brown and necrotic, respectively (Figs. 3a, b and 4a, b). The lesions produced on detached and attached leaves 21 dpi with *Xcc* or *Xac* resembled those of canker or CBS formed by natural stomatal infections in the field. In contrast, inoculation of attached leaves of kumquat cultivars at 10^4 cfu ml^{-1} induced either a few small (<0.3 mm diameter) brown, necrotic spots or no visible symptoms (Figs. 3c, d, g, h and 4c, d, g, h).

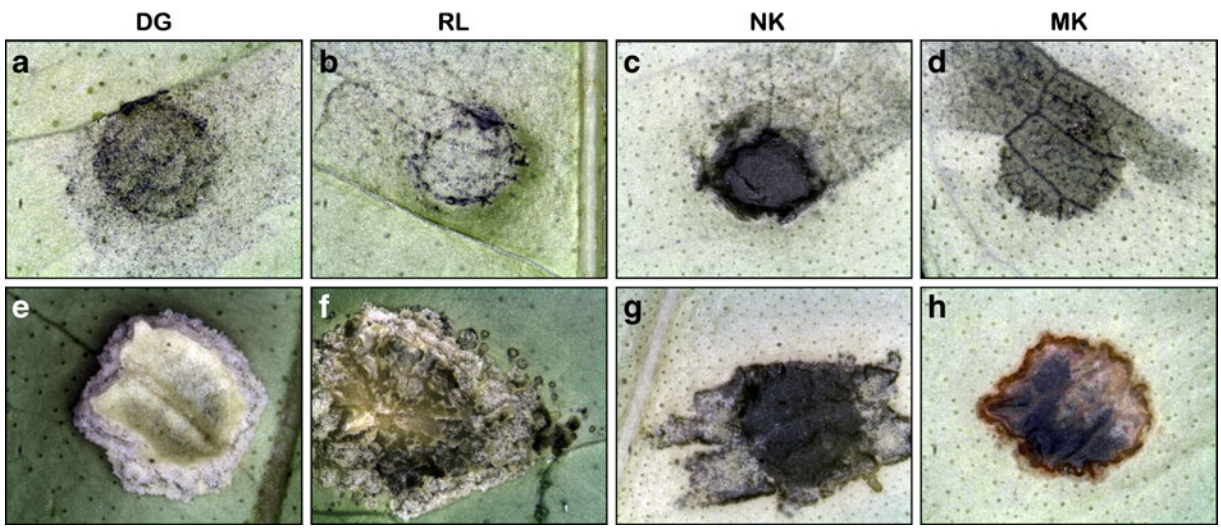

Fig. 1 Symptoms on detached leaves of 'Duncan' grapefruit (DG), 'Rough' lemon (RL), 'Nagami' kumquat (NK), and 'Meiwa' kumquat (MK) following inoculation with *Xanthomonas citri* subsp. *citri* (*Xcc*) at 10^8 cfu ml^{-1}. At 3 days post inoculation (dpi), DG (**a**) and RL (**b**) develop water-soaking within the infiltrated area ; NK (**c**) and MK (**d**) produce a rapid tissue collapse similar to a hypersensitive response within the infiltrated area. At 7 dpi, DG (**e**) and RL (**f**) produce proliferation of callus-like tissue; NK (**g**) and MK (**h**) develop more extensive tissue collapse within the infiltrated area

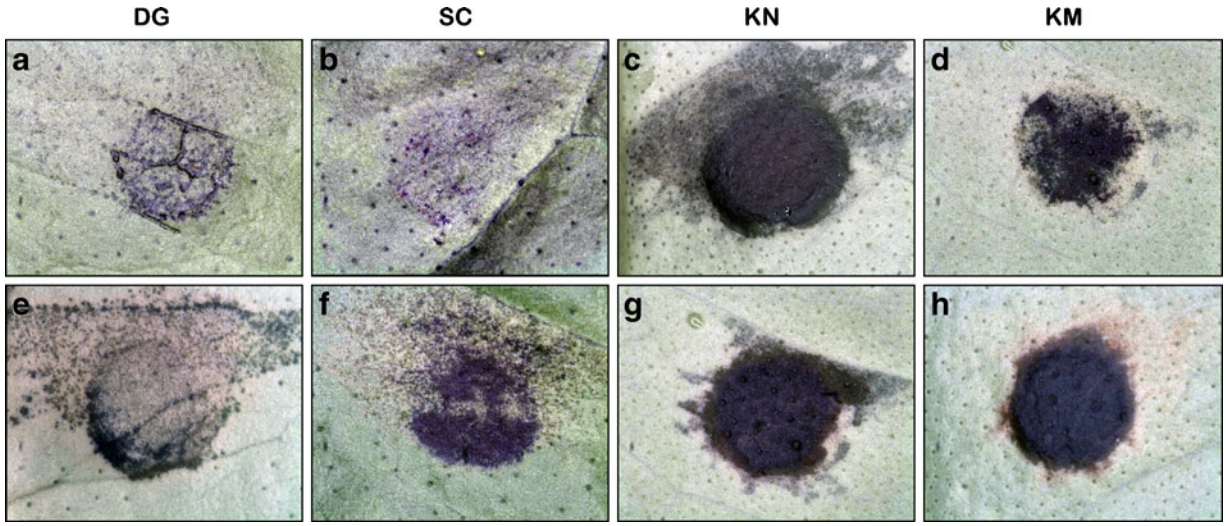

Fig. 2 Symptoms on detached leaves of 'Duncan' grapefruit (DG), 'Swingle' citrumelo (SC), 'Nagami' kumquat (NK), and 'Meiwa' kumquat (MK) following inoculation with *Xanthomonas alfalfae* subsp. *citrumelonis* (*Xac*) at 10^8 cfu ml^{-1}. At 3 days post inoculation (dpi), DG (**a**) and RL (**b**) develop very slight water soaking within the infiltrated area; NK (**c**) and MK (**d**) show collapse of the tissue in the inoculated area, similar to a hypersensitive response. At 7 dpi, DG (**e**) and SC (**f**) develop CBS lesions that are necrotic in the center with individual watersoaked lesions at the edge of the inoculation site; NK (**g**) and MK (**h**) develop extensive tissue collapse within the infiltrated area

Comparison of bacterial concentrations from detached leaf and attached leaf assays

Concentrations of *Xcc* in lesions following inoculation of detached leaf and attached leaves of 'Meiwa' or 'Nagami' kumquat were low to very low (<2.9 log cfu ml^{-1}) compared with 'Duncan' grapefruit and 'Rough' lemon (up to 5.3 and 5.2 log cfu ml^{-1}, respectively; Table 1). Similarly, concentrations of *Xac* in lesions after inoculation of attached or detached

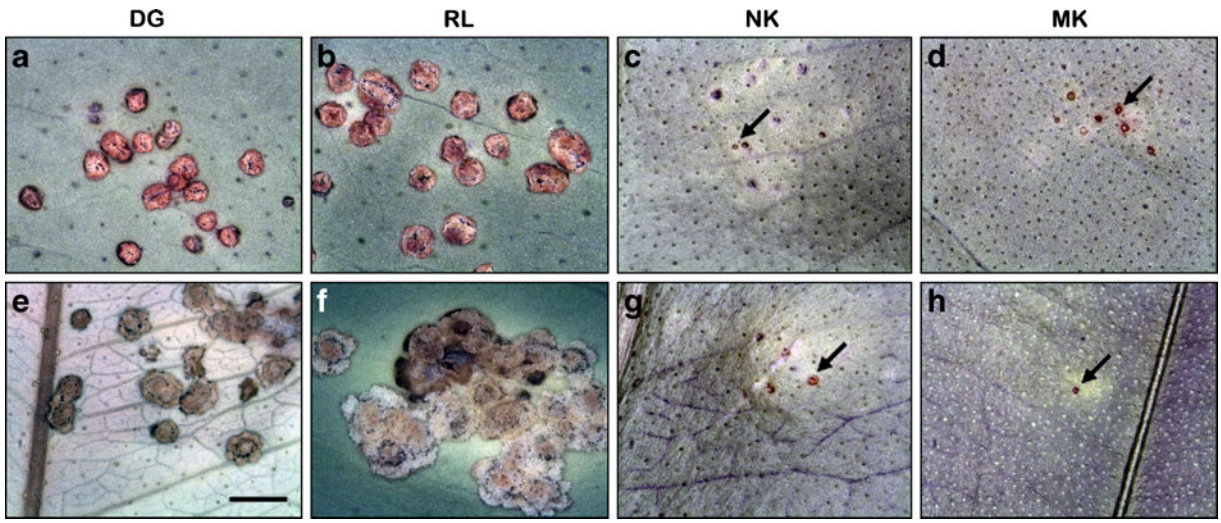

Fig. 3 Symptoms at 21 days post inoculation (dpi) on attached leaves (**a**–**d**) or detached leaves (**e**–**h**) following inoculation with *Xanthomonas citri* subsp. *citri* (*Xcc*) at 10^4 cfu ml^{-1} in 'Duncan' grapefruit (DG), 'Rough' lemon (RL), 'Nagami' kumquat (NK), and 'Meiwa' kumquat (MK). DG and RL develop erumpent callus-like lesions on attached leaf (**a**, **b**) and detached leaf (**e**, **f**); NK and MK develop brown, pin point size lesions, <0.3 mm in diameter (bar=2 mm) on attached leaves (**c**, **d**) and detached leaves (**g**, **h**)

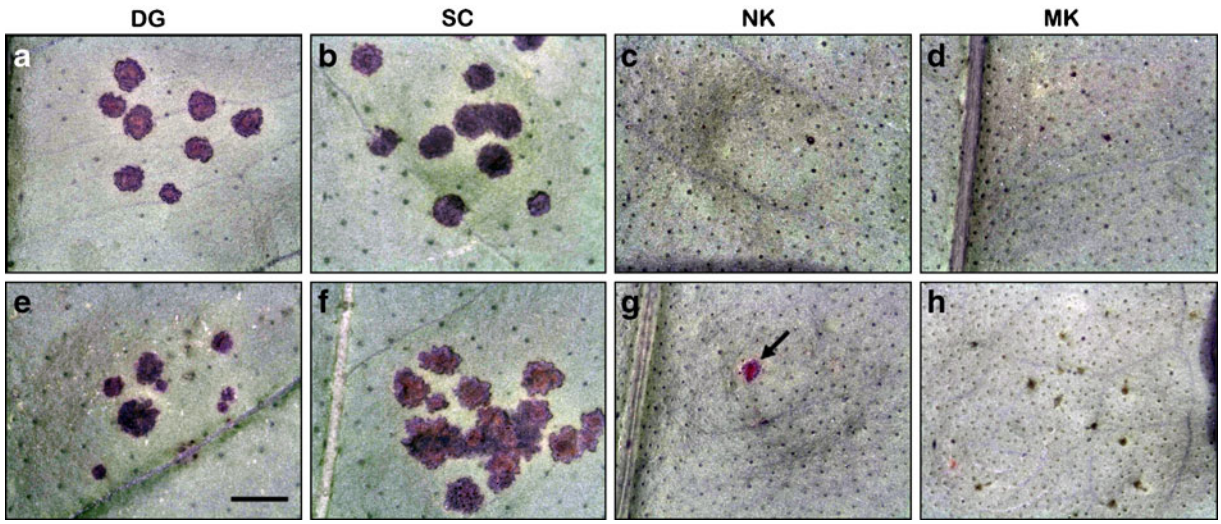

Fig. 4 Symptoms at 21 days post inoculation (dpi) on attached leaves (**a**–**d**) or detached leaves (**e**–**h**) following inoculation with *Xanthomonas alfalfae* subsp. *citrumelonis* (*Xac*) at 10^4 cfu ml^{-1} in 'Duncan' grapefruit (DG), 'Rough' lemon (RL), 'Nagami' kumquat (NK), and 'Meiwa' kumquat (MK). DG and SC develop flat, gray-brown lesions in attached leaves (**a**, **b**) and detached leaves (**e**, **f**). DG develop smaller lesions detached leaf (**e**) than attached leaf (**a**); NK and MK develop brown pin point size lesions, <0.3 mm in diameter (bar=2 mm) on attached leaf (**c**, **d**) and detached leaf (**g**, **h**)

leaves of 'Meiwa' or 'Nagami' kumquat were low (<3.6 log cfu ml^{-1}) or non-detectable. *Xac* concentrations in lesions on attached and detached leaves of 'Swingle' citrumelo were 5.5 and 5.1 log cfu ml^{-1}, respectively, and 'Duncan' grapefruit were 4.6 and 4.0 log cfu ml^{-1}, respectively.

Table 1 *Xanthomonas citri* subsp. *citri* or *X. alfalfae* subsp. *citrumelonis* concentration (log cfu per inoculation site) at 14 days following inoculation of 10^4 cfu ml^{-1} bacteria in attached or detached leaves of citrus and kumquat cultivars

Cultivar	Attached leaf	Detached leaf
Xanthomonas citri subsp. *citri* (*Xcc*)		
'Duncan' grapefruit	5.3 a[a]	4.9 a[a]
'Rough' lemon	5.2 a	4.6 a
'Nagami' kumquat	2.9 b	2.8 b
'Meiwa' kumquat	1.3 c	2.2 b
X. alfalfae subsp. *citrumelonis* (*Xac*)		
'Duncan' grapefruit	4.6 b	4.0 a
'Swingle' citrumelo	5.5 a	5.1 a
'Nagami' kumquat	0.0 c	1.1 b
'Meiwa' kumquat	0.0 c	3.6 ab

[a] Mean of the bacterial population per leaf disk. Means (n=16 attached leaf and n=30 detached leaf) followed by the same letter are not significantly different at $P \leq 0.05$ according to the Waller's K-ratio t-test

Comparison of lesion numbers from detached leaf and attached leaf assays

Lesion numbers per inoculation site on attached and detached leaves of 'Duncan' grapefruit after inoculation with *Xcc* at 10^4 cfu ml^{-1} were 8.9 and 7.5, respectively (Table 2). Inoculated 'Rough' lemon leaves developed 7.5 and 4.8 lesions per site on attached and detached leaves, respectively. Higher numbers of CBS lesions developed on 'Duncan' grapefruit after inoculation at 10^4 cfu ml^{-1} of attached leaves (21.2 lesions per site) compared to detached leaves (8 lesions per site). Similarly, 'Swingle' citrumelo inoculated at 10^4 cfu ml^{-1}, induced higher CBS lesion counts on attached leaves than on detached leaves (24 and 13 lesions per site, respectively).

Lesions on 'Meiwa' or 'Nagami' kumquat following inoculation with *Xcc* at 10^4 cfu ml^{-1} were not observed on attached leaves and were very low in number on detached leaves (0.9 and 1.2 lesions per site), hence inoculations of detached leaves were performed at 10^5 cfu ml^{-1} to quantify lesions for the kumquat cultivars (Table 2). Both cultivars developed higher numbers of lesions on detached leaves after inoculation at 10^5 than at 10^4 cfu ml^{-1}. By comparison, few or no CBS lesions developed on kumquat cultivars on attached or detached leaves irrespective

Table 2 Number of citrus canker lesions per inoculation site 14 days following inoculation with 10^4 or 10^5 cfu per ml^{-1} of *Xanthomonas citri* subsp. *citri* or *X. alfalfae* subsp. *citrumelonis* in attached or detached leaves of citrus and kumquat cultivars

Variety	Attached leaf	Detached leaf	
	10^4	10^4	10^5
Xanthomonas citri subsp. *citri* (*Xcc*)			
'Duncan' grapefruit	8.9 a[a]	7.5 a[a]	30.5 a[a]
'Rough lemon'	7.5 a	4.8 a	30.8 a
'Nagami' kumquat	0.0 b	0.9 b	7.2 b
'Meiwa' kumquat	0.0 b	1.2 b	2.6 b
X. alfalfae subsp. *citrumelonis* (*Xac*)			
'Duncan' grapefruit	21.2 a	7.8 b	21.4 b
'Swingle' citrumelo	24.0 a	13.0 a	48.1 a
'Nagami' kumquat	0.8 b	1.0 c	0.9 c
'Meiwa' kumquat	0.3 b	0.7 c	0.0 c

[a] Mean of lesions per inoculation site at different inoculum concentrations (10^4 and 10^5 ml^{-1}). Means (n=48 attached leaf, n=72 detached leaf for 10^4, and n=42 for 10^5) followed by the same letter are not significantly different at $P \leq 0.05$ according to the Waller's K-ratio t-test

of inoculum concentration (0 and 1.0 lesion per site on 'Nagami' or 'Meiwa', respectively).

Symptoms of canker and CBS on attached leaves of susceptible cultivars after inoculation at higher inoculum concentrations (10^5 and 10^8 cfu ml^{-1}) were sometimes difficult to read due to the high density of lesions at 14 dpi; whereas, incipient lesions on detached leaves could be counted accurately as early as 10 dpi (data not shown). Reactions characteristic of a hypersensitive response developed on kumquat cultivars inoculated with *Xcc* and *Xac* at 10^8 cfu ml^{-1}. Inoculation of kumquat detached leaves with lower inoculum concentrations (10^4 or 10^5 cfu ml^{-1}) induced pin point, necrotic lesions, smaller than 0.3 mm diameter (Figs. 3 and 4).

Discussion

Evaluation of responses of resistant and susceptible citrus and kumquat cultivars to *Xcc* and *Xac* strains confirmed that injection-infiltration of detached leaves under controlled environmental conditions induces expression of host resistance similar to greenhouse inoculation of attached leaves. Moreover, lesions of canker and CBS on detached leaves resemble natural stomatal infections in the field. Previously, the method of attached leaf inoculation by injection-infiltration was used to screen resistance of triploid hybrids of 'Meiwa' kumquat with lemons and limes (Viloria et al. 2004). Hybrids of susceptible and resistant genotypes were separated based on lesion number and bacterial population per lesion. The comparison of attached and detached leaf assay in the present study validated the detached leaf assay for screening limited quantities of leaves of genotypes in vitro where regulatory policies constrain the inoculation of whole plants with a contagious bacterial pathogen (c.f. López and Navarro 1981). Similarly, Moragrega et al. (2003) reported that detached leaves of pear cultivars gave accurate and rapid symptoms for screening cultivar susceptibility to *Pseudomonas syringae* pv. *syringae.*

In the present study, the detached leaf assay provided a rapid quantitative assessment of resistance to bacterial spot diseases as measured by lesion number and bacterial concentration. Previously, detached and attached assays that relied on pinprick inoculations did not provide adequate assessment of quantitative resistance for erumpent canker lesions developing from wound sites (Graham and Gottwald 1990; Gottwald et al. 1993). Infiltration of bacteria through stomatal openings minimizes the wounding of the tissue and permits a more natural and reproducible assessment of leaf mesophyll resistance and lesion phenotype (Gottwald and Graham 1992; Graham et al. 1992).

Detached and attached leaves inoculated with *Xcc* or *Xac* at 10^8 cfu ml^{-1} provided unambiguous qualitative differences in the reactions for incompatible and compatible hosts based on phenotype and bacterial concentration recovered from inoculation sites after 14 days. This study further characterized the canker resistance in kumquat (Viloria et al. 2004) as a hypersensitive-like resistance associated with rapid necrosis and collapse of tissue after 48 to 72 h, and relatively low populations of both citrus bacterial spot pathogens at 14 dpi. Previously, the HR response in 'Nagami' kumquat could only be partially characterized using needle injection-infiltration of large areas of attached leaves because the severe reaction caused leaves to abscise within 5–7 dpi (Khalaf et al. 2007). Furthermore, the severe collapse could not be evaluated in any more detail than rapid cell collapse and death compared to the absence of this reaction in 'Duncan' grapefruit. The rapid collapse of tissue

limited the ability to follow bacterial populations in leaves beyond 6 days after inoculation.

With detached leaves, the progression of incompatible reactions in kumquats and compatible reactions of 'Duncan' grapefruit and 'Rough' lemon could be followed in detail for up to 21 dpi, from the initial stages of water-soaking or tissue collapse through to the development of erumpent callus-like lesions of typical canker in the field or the flat necrotic lesions of CBS affecting citrus nursery trees.

In summary, the detached leaf assay induced lesions and pathogen responses that could be used to reliably differentiate host-*Xanthomonas* spp. interactions for CBS and citrus canker on hosts of varying resistance. The detached leaf assay was accomplished with a small number of leaves at a development stage known to be susceptible (leaves 75% expanded). The detached leaf assay is amenable for screening of small quantities of leaf material for canker resistance in growth chambers where inoculation of plants is constrained by pathogen quarantine or in areas where seasonal weather might preclude year round growth flush, thus limited greenhouse-grown material could be used in the lab.

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