Biological Characterization of Australian Isolates of Citrus Tristeza Virus and Separation of Subisolates by Single Aphid Transmissions

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ABSTRACT

Broadbent, P., Brlansky, R. H., and Indsto, J. 1996. Biological characterization of Australian isolates of citrus tristeza virus and separation of subisolates by single aphid transmissions. Plant Dis. 80:329-333.

Citrus tristeza virus in Australian citrus is a complex of isolates that differ in biological properties and rates of aphid transmission. Biological characterization of isolates on citrus indicators produced a range of symptoms varying in type and severity depending on the field host and geographical location. Single aphid transmissions with the vector *Toxoptera citricida* separated some of these subisolates based on biological indexing on three citrus indicators and the numbers of inclusion bodies produced. Inclusion numbers produced in *Citrus aurantiifolia* were positively related to isolate and subisolate severity in that host.

Additional keywords: seedling yellows, stem pitting

Citrus tristeza virus (CTV) is the cause of severe stem pitting and decline diseases of citrus and is one of the most economically important citrus pathogens worldwide (4). There are numerous isolates of CTV, which cause different disease syndromes (13,18,20,24,26,28). CTV is believed to have become established in Australia about 1860 in the Sydney area (12), and in the irrigation settlements of the Murray River in the 1940s (17). Almost every citrus tree in Australia is infected with CTV, and the most efficient vector of CTV, the brown citrus aphid (*Toxoptera citricida* (Kirkaldy)), is abundant. The use of decline tolerant stock–scion combinations has limited economic losses. Stem pitting disease of Marsh and Thompson grapefruits, which devastated plantings in the 1950s, has been controlled by mild strain protection (7). However, severity of CTV isolates in field mother trees (budwood source trees) has increased with tree age and subpropagations. In 1990, stem pitting of navel oranges was identified in Queensland (6,21). This outbreak of orange stem pitting (OSP) has increased the need for more rapid and reliable CTV isolate identification to detect budwood source hosts latently infected with OSP isolates. Various biochemical and serological methods for CTV isolate differentiation have shown promise, but biological indexing remains the only consistently reliable method for identifying severe isolates of CTV (14,26). Enumeration of inclusion bodies can also be used to determine isolate severity (5). Recently, restriction analysis of the CTV coat protein gene amplified by the polymerase chain reaction has distinguished mild from severe Australian CTV isolates in grapefruit (15).

In this study, we attempted to discriminate among Australian isolates and subisolates of CTV by using single aphid transmissions, biological indexing on at least three citrus indicators, and by determining numbers of inclusion bodies.

Florida Agricultural Experiment Station Journal Series No. R-04587.

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Accepted for publication 12 December 1995.

Publication no. D-1996-0110-06R

MATERIALS AND METHODS

Virus isolates. In this paper, CTV field sources are referred to as isolates and single aphid transmissions from these are referred to as subisolates. The term subisolate rather than strain is used because these may still be a mixture of strains. Two hundred and fifty isolates and subisolates of CTV from a range of citrus species or clones growing in diverse geographic locations in Australia were maintained by budding into glasshouse-grown sweet orange (*Citrus sinensis* (L.) Osbeck) or West Indian lime (*C. aurantiifolia* (L.) Swingle) seedlings. In this study, 18 field isolates and 50 subisolates were chosen for further study. Field symptoms of CTV isolates ranged from those causing severe stem pitting in grapefruit or sweet orange to those causing no apparent adverse effects on the health of field trees on decline-tolerant rootstocks. Apparently healthy mature Valencia orange (*C. aurantium* L.) and Fewtrell mandarin (*C. reticulata* Blanco) trees on Appleby Seville orange rootstock (normally intolerant combinations to CTV-induced decline) were also studied because they were survivors and may contain valuable cross-protecting strains.

Biological indexing. The citrus indicators chosen to identify and assess severity of symptoms of the CTV isolates and subisolates were sweet orange (cv. Ruby Blood or Symons), bittersweet seville (*C. aurantium*), Duncan grapefruit (*C. paradisi* Macf.), Eureka lemon (*C. limon* (L.) Burm f.), and West Indian lime (14). When the indicators were 15 cm high, seedlings were selected for uniformity and trueness-to-type, and potted in UC mix (50 parts peat and 50 parts sand with added nutrients) (1), five per 15-cm pot. Four of the seedlings were inoculated by side-grafting with buds from the donor plant, with one seedling left as an uninoculated control. Sweet orange seedlings maintained at a maximum daytime temperature of >30°C were used to identify the orange stem pitting strain of CTV. The seedling yellows reaction (11) in Eureka lemon, bittersweet seville orange, or Duncan grapefruit and the vein clearing reaction in West Indian lime were evaluated in indicator seedlings held at ambient glasshouse temperatures (20 to 30°C). Foliar symptoms in West Indian lime of vein clearing and leaf cupping, chlorosis, and reduction in plant height were recorded periodically after major growth flushes during a 12-month period. Seedling yellows reactions in grapefruit, Eureka lemon, and bittersweet seville were evaluated 4 months after inoculation. Stem pitting was determined by peeling the stems of sweet orange and West Indian lime above the point of inoculation at the final reading.

Glasshouse-grown virus-free trees of Hamlin orange on Warnock sour orange rootstock were inoculated with 18 field isolates of CTV to assess the quick decline reaction.

Numbers of inclusion bodies. Leaves of CTV-infected and healthy West Indian lime were marked and measured for length and width daily in order to harvest plant

tissues at a uniform stage of development. When the measurement was the same on 2 consecutive days, a leaf was considered fully expanded and was harvested. Three leaves from the growth flush were harvested for each inoculated and control plant. Petiole samples, approximately 1 cm long, were excised from the leaves at the abscission zone, dried over silica gel, and stored. Samples were rehydrated in phosphate-buffered saline, pH 7.4, and 30- to 40-μm transverse sections were prepared using a Harris freezing microtome. The sections were stained with azure A (5). The total number of inclusions was determined in 10 randomly selected sections, and the means were calculated for each CTV isolate.

Aphid transmissions. Apterous brown citrus aphids (*T. citricida*) were maintained on virus-free sweet orange seedlings. In transmission studies, groups of 20 to 40 aphids were fed on CTV bud-inoculated sweet orange seedlings for a virus acquisition access period of 24 h. Aphids were then placed singly on virus-free sweet orange seedlings. After an inoculation access period of 24 h, the aphids were killed by spraying with an aphicide (Malathion). Plants were then grown in an insect-free greenhouse at ambient temperatures. Transmission efficiency was defined as the ratio between the number of infected plants and the total number of aphid-inoculated plants.

Enzyme-linked immunosorbent assay (ELISA). CTV infection resulting from aphid transmission was determined after 2 months by double antibody sandwich ELISA (3), using CTV polyclonal antiserum (Sanofi Phyto-Diagnostics, Libourne, France). Leaf tissue was taken at or before the full expansion stage and processed immediately. Sap was expressed using a Pollahne leaf press (Erich Pollähue Meku, 3015 Wennigsen A.M., Germany) and diluted in phosphate-buffered saline, pH 6.4, containing 0.05% Tween at a ratio of 1 g of tissue to 10 ml of buffer. Extracts of healthy and CTV-infected tissues were included in all tests. Samples were replicated twice per plate (two wells per sample), giving a total of four values per plant. Reactions were read spectrophotometrically at 405 nm on a Titertek plate reader (Pathtech Diagnostics Pty Ltd., Balwyn Victoria 3101, Australia). A positive reaction was defined as an optical density reading more than twice that of the healthy control.

RESULTS

Biological indexing. CTV field isolates obtained from the major citrus growing areas in Australia and also from Carnarvon (Western Australia) and Darwin (Northern Territory) caused a wide range of symptom intensities on biological indicators. Only two trees (Ruby grapefruit from Darwin) were CTV negative by biological indexing and ELISA. Indexing results showed that the tristeza virus complex differed with the citrus species and geographical location (Table 1).

The virus complex occurring Australia-wide in oranges, tangors, and mandarins generally induced a moderate to severe vein clearing reaction in West Indian lime, with or without a corking of veins, and always induced a seedling yellows reaction in seedlings of Smooth Seville orange, Duncan grapefruit, or Eureka lemon. Vein corking, a severe reaction of West Indian lime, was more common in autumn flushes. Seedlings with a severe yellows reaction rarely recovered and generally did not show stem pitting symptoms. The isolates tested produced a range of symptoms on the sweet orange indicator plants ranging from (i) no symptoms, to (ii) size reduction to varying degrees with smaller leaves and a zinc-manganese-like deficiency pattern, and (iii) occasional vein clearing and occasional pits in the stem. Budwood from some tangor and orange trees in Queensland caused a severe reaction in sweet orange indicative of OSP. The extreme symptoms associated with OSP are reduced new growth, severe deficiency-like patterns in leaves that are reduced in size, and a fine stem pitting with associated gum that results in a brittle honeycombing of the stem.

Field symptoms of OSP in Washington navel oranges were reproduced by graft transmission in seedlings of sweet orange cultivar Symons and in Newhall navel or Hamlin oranges on rough lemon stocks. Symptoms were produced earlier and were more severe on Symons sweet orange seedlings. Symptom expression was best in 2-year-old wood of indicators held at high ambient summer temperatures (>30°C) in the glasshouse. Field trees from Queensland (OSP and symptomless) caused severe symptoms of vein clearing, leaf cupping and yellowing, extreme stunting, vein corking, and stem pitting when graft-inoculated to West Indian lime seedlings. Swingle citrumelo (*C. paradisi* × *Poncirus trifoliata* (L.) Raf.), Rangpur lime (*C. limonia* Osbeck), Orlando tangelo (*C. reticulata* × *C. paradisi*), and Emperor mandarin did not develop stem pitting when inoculated with buds from OSP trees.

CTV field isolates from grapefruit or Appleby Seville orange (Table 1) usually did not produce a seedling yellows reaction. Shoot growth of indicator plants was checked temporarily, but leaf size and appearance were not affected. However, in a few cases, CTV inoculum from Eureka lemon, Ruby, or Marsh grapefruit trees produced a mild, often transitory seedling yellows (SY) reaction in Eureka lemon, with no symptoms appearing after the second growth flush. This mild SY reaction was usually not observed in corresponding bittersweet seville indicators. Grapefruit (Thompson, Marsh, Ruby) isolates from temperate and tropical climates in Australia varied in their effects on West Indian lime. There was a direct relationship between severity of stem pitting and fruit symptoms of the field tree and

Table 1. Biological index and transmissibility by single aphids of *Toxoptera citricida* of Australian field isolates of citrus tristeza virus (CTV)

CTV accession no.	Geographical location[x]	Origin	Biological index[y]	Transmission (%)[z]
PB 61	NSW	Marsh grapefruit	Mild GF-SP	15
PB 64	Vic	Marsh grapefruit	Severe GF-SP	15
PB 209	Vic	Marsh grapefruit	Mild GF-SP	0
PB 67	Vic	Marsh grapefruit	Mild GF-SP	5
PB 23	Vic	Marsh grapefruit	Mild GF-SP	0
PB 44	NSW	Marsh grapefruit	Severe GF-SP	0
PB 47	Vic	Marsh grapefruit	Severe GF-SP	0
PB 66	Vic	Marsh grapefruit	Severe GF-SP	5
PB 65	Vic	Valencia orange/ Appleby Seville	SY, no decline	11.8
PB 63	SA	Wilson navel orange	SY, no OSP	20
PB 100	Qld	Benyenda navel orange	SY, no OSP	20
PB 75	Qld	Benyenda navel orange	SY, OSP	55
PB 87	Qld	Benyenda navel orange	SY, no OSP	10
PB 72	Qld	Ortanique tangor	SY, OSP	25
PB 90	Qld	Ortanique tangor seedling	SY, OSP	30
PB 62	Vic	Appleby Seville	Mild SP-WIL	25
PB 68	NSW	Hickson mandarin	SY	15
PB 212	Vic	Fewtrell mandarin/ Appleby Seville	SY, no decline	0

[x] NSW = New South Wales; SA = South Australia; Vic = Victoria; Qld = Queensland.

[y] SY = Seedling yellows reaction in Eureka lemon indicators. OSP = Orange stem pitting reaction. GF-SP = Grapefruit stem pitting; mild = no field symptoms and a mild reaction on West Indian lime seedlings; severe = trunk pitting of field trees and a severe reaction on West Indian lime seedlings. SP-WIL = stem pitting West Indian lime.

[z] Percentage of infected plants using 20 to 40 aphids on source plant and single aphids on receptor plants.

severity of vein clearing and stem pitting in the West Indian lime indicator. Inoculations from symptomless grapefruit consistently produced the mildest and most transitory symptoms on indicators—a few vein flecks in the leaves of West Indian lime formed immediately after inoculation, with few or none on subsequent leaves and with only an occasional mild pit after one or more season's growth. The most severe isolates caused extensive vein clearing, leaf cupping, severe overlapping or grouped pits, and ultimately, cracking of the bark, corking, and splitting of veins in the West Indian lime indicator seedlings. The mildest isolates did not restrict growth of West Indian lime indicators, whereas the most severe ones restricted growth.

Decline of virus-free sweet/sour orange trees was more rapid and severe when the CTV inoculum was from Meyer lemon, sweet orange, or mandarin. However, decline did occur following inoculation with most CTV isolates from grapefruit, regardless of the severity of the stem pitting reaction.

The tolerance of Australian CTV isolates to high temperatures appears to be increasing. Many Queensland isolates, including the OSP ones, gave better symptoms in flushes produced at temperatures >30°C. Some isolates from the budwood multiplication blocks at Dareton, NSW, were also heat tolerant and interfered with indexing for citrus exocortis viroid on Etrog citron (*C. medica* L.).

Numbers of inclusion bodies. CTV inclusion bodies in West Indian lime were observed regardless of severity of isolate or subisolate. Inclusions were not found in any healthy control plant. The number of inclusions was significantly higher for severe isolates and subisolates than for the mild ones, regardless of the presence or absence of a seedling yellows reaction (Table 2). The exception was for Wilson navel, where numbers of inclusion bodies were similar regardless of severity of reaction in West Indian lime. No apparent differences were noted in the types of inclusions formed by the various isolates.

Aphid transmissibility. Table 1 summarizes the transmissibility under uniform conditions (sweet orange to sweet orange) of different field isolates of CTV by single aphids of *T. citricida*. Transmission efficiency varied from 0 to 55%. Field isolates from orange or mandarin carrying SY were more readily transmitted (20.7%) than CTV isolates from grapefruit (5%). Orange stem pitting isolates were transmitted at a higher rate (36.7%) than endemic isolates from orange or mandarin (12.8%).

Transmission from Australian field sources, using single aphids, revealed that most contained a mixture of CTV subisolates varying in severity, as evidenced by reaction in biological indicators (Table 2). For example, field isolate PB 64 from a Marsh grapefruit, with field symptoms of stem pitting, was found to contain CTV subisolates that were mild and severe in host reaction and in numbers of inclusions produced in West Indian lime. The absence of the SY-reaction in indicators inoculated with field isolates of CTV did not confirm the absence of SY strains. CTV isolate PB 62 from a 40-year-old Appleby Smooth

Table 2. Symptom expression of Australian citrus tristeza virus (CTV) field isolates (b) and subisolates obtained by single aphid transmissions (a1) in citrus indicators

Accession number	Isolate origin	Trans.[w]	Inclusion bodies[x]	Biological indexing[y]		
				Vein clearing (A)	Seedling yellows (B)	Orange stem pitting (C)
PB 61	Marsh grapefruit	b	12.1e[z]	Mild	Neg	Neg
PB 5	3970-New S. Wales	a1	36.6de	Mild	Neg	Neg
PB 6		a1	15.8de	Mild-mod	Neg	Neg
PB 18		a1	26.7de	Mild-mod	Neg	Neg
PB 58		a1	30.9de	Mild-mod	Neg	Neg
WIL 43		a1	16.1de	Mild	Neg	Neg
WIL 45		a1	11.3e	Mild	Neg	Neg
PB 62	Appleby Smooth	b	2.2e	Mild	Neg	Neg
PB8	Seville-Victoria	a1	14.9de	Mild	Pos	Neg
PB 12		a1	185.8a	Severe	Pos	Neg
PB 14		a1	2.1e	Mild-mod	Pos	Neg
PB 56		a1	155.6ab	Severe	Pos	Neg
PB 57		a1	125.9bc	Severe	Pos	Neg
PB 63	Wilson navel-	b	34.9de	Severe	Pos	Neg
PB 11	South Australia	a1	16.7de	Severe	Pos	Neg
PB 16a		a1	17.5de	Mild	Neg	Neg
PB 16b		a1	49.4d	Mod-sev	Neg	Neg
PB 17		a1	12.5de	Mild	Pos	Neg
PB 64	Marsh grapefruit-	b	24.6de	Mod-sev	Neg	Neg
PB 50	Victoria	a1	14.2de	Mild	Neg	Neg
PB 51		a1	7.8e	Mild	Neg	Neg
PB 52		a1	153.1ab	Severe	Neg	Neg
PB 53		a1	184.5a	Severe	Neg	Neg
PB 70		a1	...	Mod	...	...
PB 71		a1	...	Severe	...	...
PB 65	Valencia/Appleby	b	10.3e	Mild-mod	Pos	Neg
PB 54	Smooth Seville-Vic.	a1	100.8c	Severe	Pos	Neg
PB 55		a1	(>400)	Severe	Pos	Neg
PB 67	Marsh grapefruit-	b	19.4de	Mild	Neg	Neg
PB 60	Victoria	a1	20.8de	Mild-mod	Neg	Neg
PB 68	Hickson mandarin-	b	88.7c	Mod	Pos	Neg
PB 7	New South Wales	a1	1.1e	Mild-mod	Pos	Neg
PB 10		a1	25.5de	Sev-mild	Pos	Neg
PB 9		a1	...	Mod-sev	Pos	Neg
PB 72	Ortanique tangor-	b	...	Severe	Pos	Pos
PB 73	Queensland	a1	...	Severe	Pos	Pos
PB 74		a1	...	Severe	Pos	Pos
PB 91		a1	...	Severe	Pos	Neg
PB 92		a1	...	Severe	Pos	Neg
PB 93		a1	...	Severe	Pos	Neg
PB 94		a1	...	Mod	Pos	Neg
PB 95		a1	...	Severe	Pos	Neg
PB 96		a1	...	Severe	Pos	Pos
PB 75	Benyenda navel-	b	...	Severe	Pos	Pos
PB 76	Queensland	a1	...	Severe	Pos	Pos
PB 77		a1	...	Severe	Pos	Pos
PB 78		a1	...	Severe	Pos	Pos
PB 79		a1	...	Severe	Neg	Pos
PB 80		a1	...	Severe	Pos	Pos
PB 81		a1	...	Severe	Pos	Pos
PB 82		a1	...	Severe	Neg	Neg
PB 83		a1	...	Severe	Neg	Pos
PB 84		a1	...	Severe	Pos	Pos
PB 85		a1	...	Severe	Neg	Pos
PB 86		a1	...	Severe	Neg	Pos
PB 90	Ortanique tangor seedling-Queensland	b	...	Severe	Pos	Pos
PB 100	Benyenda navel-	b	...	Severe	Pos	Neg
PB 101	Queensland	a1	...	Severe	Pos	Neg
PB 102		a1	...	Mod	Neg	Neg
PB 103		a1	...	Severe	Pos	Pos
PB 104		a1	...	Mod	Neg	Neg

[w] b = bud; a = single aphid transmission.
[x] Mean separation by Waller-Duncan *k*-ratio *t* test, $P \leq 0.05$.
[y] Indicator seedlings, A = West Indian lime, B = Eureka lemon or bittersweet seville, C = sweet orange.
[z] Average number of inclusions calculated from total in 10 sections randomly selected from three petioles from each of three plant-virus combinations.

Seville tree did not give an SY reaction, but five subisolates obtained by single aphid transmission from an inoculated sweet orange seedling to sweet orange seedlings all indexed positively for SY (Table 2). Even the absence of an SY reaction (>3 months after inoculation feeding) following a primary single aphid transmission did not indicate its failure to be transmitted. Positive SY reactions sometimes occurred following a second single aphid transmission (data not shown). For example, PB 86 (negative SY reaction but an isolate derived from PB 75 positive SY) has produced single aphid-transmitted subisolates with positive SY reactions.

However, isolate PB 61, used for preimmunizing of grapefruit against stem pitting in Australia, appeared to contain a stable mixture of mild subisolates, as revealed by both biological indexing and numbers of inclusion bodies. Comparisons of these aphid-separated isolates in Duncan grapefruit seedlings showed uniformity in plant size and an absence of stem pitting symptoms.

Field isolate sources that produced OSP were found to contain subisolates that did not produce OSP (Table 2). For example, single aphid transmissions from PB 72 separated five of eight isolates that did not cause OSP. However, field source PB 100, which showed no OSP symptoms in either the field or the greenhouse tests, contained subisolate PB 103, which produced OSP in indicators. Primary single aphid transmissions from PB 75 (OSP and SY positive) contained six of 11 (54.5%) isolates that were OSP and SY positive and 91% that were OSP positive.

DISCUSSION

The tristeza virus complex in Australian citrus varies depending on geographical location and host. The damage in Australia caused by CTV includes the lethal (quick) decline of sweet orange on sour orange rootstock; wood pitting, vein flecking, and decline of acid lime; wood pitting and fruit distortion in grapefruit; and wood pitting of oranges. This is similar to that found in South Africa (18), but it is in marked contrast to Spain, Florida, California, and Israel, where damage is restricted to trees grafted on sour orange (2,26).

The host species exerts a sorting-out effect on the virus complex. Graft transmission of CTV from field sources of orange or mandarin to seedlings of Duncan grapefruit, bittersweet seville, and Eureka lemon resulted in a seedling yellows reaction. After an interval of a month or longer, some seedlings began to recover, and the remaining virus complex was no longer capable of inducing seedling yellows in subsequent transmissions. The assemblage of CTV strains in field trees of Smooth Seville, Eureka, or Lisbon lemon, or Marsh and Thompson grapefruits rarely caused even a mild seedling yellows reaction in seedlings of these varieties following graft transmission.

Stubbs (28) hypothesized that "strains having the greatest affinity for the host could be expected to multiply ... and impede or prevent the multiplication of less adapted strains." The continued presence of SY strains in the mixture is, however, demonstrated in our experiments by an SY reaction of receptor sweet orange seedlings following single aphid transmissions from field trees of Seville orange or Eureka lemon.

All isolates of CTV, irrespective of the citrus source, caused a vein clearing and stem pitting reaction in West Indian limes. No isolate, whether from a field source or obtained by single aphid transmission, caused a seedling yellows reaction in the absence of causing symptoms on West Indian lime.

Previously, aphid transmission rates with *Aphis gossypii* Glover varied with the acquisition host and with the severity of the isolate (25). In general, transmission from grapefruit or lemon to grapefruit and lemon was quite low. In our studies, Symons sweet orange seedlings were used as hosts for both acquisition and transmission feedings of *T. citricida*. So, transmission rates varied only with the severity of the CTV isolates. Isolates from orange or mandarin carrying SY were more readily transmitted than isolates from grapefruit. The highest rates of transmission were from plants containing isolates that caused OSP, which may account for the rapid field spread of OSP observed in Queensland. Costa and Grant (9) reported a transmission rate (9/55 or 17% of test plants infected) using single *T. citricida*. Later, studies by Costa et al. (10) showed no infection (0/30) by single *T. citricida*. Yokomi et al. (29) showed single aphid transmission rates up to 25% by *T. citricida*. Our results are consistent with the latter.

The separation of "strains" by single aphid transmissions has revealed severe strains or subisolates hidden within field isolates that index mild on West Indian lime (Table 2). The maintenance of the biological indexing profile (SY, OSP, West Indian lime reaction) of these primary single aphid transmissions when further single aphid transmissions are done may stabilize after the third time they are single aphid transmitted (unpublished data). Studies by Raccah et al. (24) and by Kano and Koizumi (16) have shown that tristeza variants are obtained from field samples using aphid vectors. However, both studies used multiple aphids as vectors. More information also is needed on what causes the SY reaction and its variability. However, the determination of whether an aphid has transmitted a single strain or an assemblage of strains will require the addition of other methodology to biological indexing. Additional techniques such as reaction to a battery of monoclonal antibodies (8,23), analysis of double-stranded RNA (19), DNA sequencing (22), and hybridization with cDNA probes (27) may provide the tools to identify specific CTV strains.

ACKNOWLEDGMENTS

Support for this cooperative research was provided by the Bilateral (U.S.–Australia) Science and Technology Collaborative Program, Australian Department of Industry, Technology and Commerce, the Australian Horticultural Research and Development Corporation, and a USDA-OICD International Collaborative Research Agreement 58-319R-2-038. We thank D. S. Howd, C. Dephoff, and N. Franks for technical assistance and P. Hicks for manuscript preparation.

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