Developing and communicating strategies for controlling virus diseases in vegetable cucurbit crops

Brenda Coutts
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Project Number: VG06022

VG06022

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for the vegetables industry.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the vegetables industry.

ISBN 0 7341 2866 5

Published and distributed by:
Horticulture Australia Ltd
Level 7
179 Elizabeth Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399

Horticulture Australia

FINAL REPORT

HORTICULTURE AUSTRALIA PROJECT VG06022

Developing and communicating strategies for controlling virus diseases in vegetable cucurbit crops

Brenda Coutts, *et al*.
Department of Agriculture and Food, Western Australia

June 2011

HORTICULTURE AUSTRALIA LIMITED PROJECT
VG06022

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This is the final report for the project VGO6022 'Developing and communicating strategies for controlling virus diseases in vegetable cucurbit crops'.

June 2011

This project was aimed at developing and communicating strategies for controlling virus diseases in vegetable cucurbit crops in Australia.

This project has been funded by HAL using the Vegetable Industry levy and matched funds from the Australian Government.

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CONTENTS

MEDIA SUMMARY

Virus diseases cause serious yield and quality losses in field grown cucurbit crops worldwide. In Australia, the main viruses of cucurbits are *Papaya ringspot virus* (PRSV), *Squash mosaic virus* (SqMV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV). Plants infected early have severely distorted fruit. High infection incidences, of ZYMV and PRSV in crops cause losses of marketable fruit of up to 100% and infected crops are often abandoned.

Two new alternative hosts of ZYMV were identified, the native cucurbit *Cucumis maderaspatanus* and wild legume *Rhyncosia minima*. No new alternative hosts of PRSV, SqMV or WMV were found in Western Australia or Queensland. Seed transmission of ZYMV (0.7%) was found in seedlings grown from ZYMV-infected fruit of zucchini but not of pumpkin. None was detected with PRSV or SqMV in zucchini or pumpkin seedlings, respectively.

ZYMV spread to pumpkins by aphids was greater downwind than upwind of a virus source. Delaying sowing by 2 weeks decreased ZYMV spread. Millet non-host barriers between pumpkin plantings slowed ZYMV infection. Host resistance gene (*zym*) in cucumber cultivars was effective against ZYMV. Pumpkin cultivars with resistance gene (*Zym*) became infected under high virus pressure but leaf symptoms were milder and infected plants higher yielding with more market-acceptable fruit than those without *Zym*. Most zucchini cultivars with *Zym* developed severe leaf and fruit symptoms.

ZYMV, PRSV, WMV and SqMV spread readily from infected to healthy cucurbit plants by direct leaf contact. ZYMV survives and remains infective on diverse surfaces for up to 6 hours but can be inactivated by some disinfectants.

Phylogenetic analysis indicates at least three separate introductions of ZYMV into Australia, with new introductions rarely occurring. ZYMV isolates clustered into three groups according to collection location i) Kununurra, ii) Northern Territory and iii) Carnarvon, Qld and Vic. A multiplex Real-Time PCR was developed which distinguished between the three groups of Australian isolates.

Integrated disease management (IDM) strategies for virus diseases of vegetable cucurbit crops grown in the field were improved incorporating the new information gathered. These strategies are aimed at causing minimal extra expense, labour demands and disruption to normal practices.

TECHNICAL SUMMARY

Virus diseases cause serious yield and quality losses in field grown cucurbit crops worldwide. In Australia, the main viruses of cucurbits are *Papaya ringspot virus* (PRSV), *Squash mosaic virus* (SqMV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV). All vegetable cucurbit crop types are susceptible to these viruses. Leaf symptoms include mosaic, deformation, blistering and reduced size. Plants become stunted. Fruits from plants infected early show severe distortion and knobbliness. High infection incidences in crops, especially of ZYMV and PRSV, cause losses of marketable fruit of up to 100% and infected crops are often abandoned.

Surveys to identify alternative hosts of ZYMV, PRSV, and WMV involved testing >5300 weed samples from a least 34 different species collected from northern Australia. Two new alternative hosts of ZYMV were identified, the native cucurbit *Cucumis maderaspatanus* and wild legume *Rhyncosia minima*. No new alternative hosts of PRSV, SqMV or WMV were found in Western Australia or Queensland. When seed collected from virus-infected zucchini and pumpkin fruit was tested, 0.7% ZYMV infection was found in zucchini seedlings but no seed transmission was detected in >9000 pumpkin seedlings. No seed transmission of PRSV or SqMV was detected in >1000 zucchini or >600 pumpkin seedlings, respectively.

The pattern of spread of ZYMV was examined in pumpkin plantings in which aphids spread the virus from internal or external infection foci. Spread to pumpkin was greater downwind than upwind of an internal source. When 25 m wide fallow or short non-host barriers separated external ZYMV sources from pumpkin plants, spread was smaller and more scattered with a non-host barrier than without. Tall non-host barriers (millet) between pumpkin plantings decreased ZYMV incidence by 34%. Delaying sowing by 2 weeks decreased ZYMV spread.

Under high ZYMV inoculum pressure, 2/14 cultivars zucchini cultivars with host resistance gene *Zym* had delayed infection (partial resistance), otherwise *Zym* did not diminish final ZYMV incidence. Zucchini cultivars carrying *Zym* often developed severe fruit symptoms, and only the two cultivars in which spread was delayed and one that was tolerant produced sufficiently high marketable yields to be recommended when ZYMV epidemics are anticipated. In three pumpkin cultivars with *Zym*, this gene was effective under low virus inoculum pressure, but not under high inoculum pressure. However, leaf and fruit symptoms were milder and marketable yields greater in cultivars with than without *Zym*. Resistance gene *zym* was effective against ZYMV in the five cucumber cultivars tested. Under high PRSV inoculum pressure, 5/14 zucchini cultivars with *Zym* produced at least 3 times the amount of marketable fruit of the other cultivars tested.

ZYMV, PRSV, WMV and SqMV were spread readily from infected to healthy cucurbit plants by direct leaf contact when leaves rubbed against each other. ZYMV was also transmitted when infected leaves were crushed or trampled, and, to a lesser extent on blades contaminated by infective sap. When infective sap containing ZYMV was applied to seven surfaces (cotton, plastic, leather, metal, tyre, rubber soled footwear and skin), it remained infective for 24 hrs on plastic, and up to 6 hrs on

the other surfaces. Disinfectants (nonfat milk powder, bleach, and Farmcleanse) were effective at inactivating ZYMV. Dipping ZYMV contaminated footwear in a footbath containing bleach prevented virus spread by trampling.

Phylogenetic analysis of ZYMV isolates from Australia and other countries indicated three separate introductions of the virus into Australia. Australian ZYMV isolates clustered into three groups according to collection location i) Kununurra, ii) Northern Territory and iii) Carnarvon, Qld and Vic. Also, once established in an isolated growing area, few further sequence changes were evident indicating that new introductions occur rarely. A multiplex Real-Time PCR was developed using dual-labelled probes which distinguished between the Australian isolates within the different groups.

The major achievements of the project are (i) achievement of considerably greater understanding of the epidemiology and control of virus diseases of field grown vegetable cucurbits, and (ii) the use of this new information to improve integrated management strategies for cucurbit virus disease management. The improved IDM strategies have been delivered to the vegetable cucurbit industry and awareness of the virus problem in cucurbits has increased.

Further studies should investigate how ZYMV is reintroduced to crops after breaks between cucurbit growing seasons including the role of native plant species as virus and vector hosts, and determine if seed transmission occurs in alternative hosts. The development of a predictive model based on aphid vector arrival, environmental and cultural factors prior to the growing season would be used to predict potential virus epidemics.

SECTION 1

Minimising losses caused by *Zucchini yellow mosaic virus* in vegetable cucurbit crops in tropical, sub-tropical and Mediterranean environments through cultural methods and host resistance

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(Draft of paper published in Virus Research, 2011)

Abstract

Between 2006 and 2009, 10 field experiments were done at Kununurra, Carnarvon or Medina in Western Australia (WA) which have tropical, sub-tropical and Mediterranean climates, respectively. These experiments investigated the effectiveness of cultural control measures in limiting ZYMV spread in pumpkin, and single-gene resistance in commercial cultivars of pumpkin, zucchini and cucumber. Melon aphids (*Aphis gossypii*) colonised field experiments at Kununurra; migrant green peach aphids (*Myzus persicae*) visited but did not colonise at Carnarvon and Medina. Cultural control measures that diminished ZYMV spread in pumpkin included manipulation of planting date to avoid exposing young plants to peak aphid vector populations, deploying tall non-host barriers (millet, *Pennisetum glaucum*) to protect against incoming aphid vectors and planting upwind of infection sources. Clustering of ZYMV-infected pumpkin plants was greater without a 25 m wide non-host barrier between the infection source and the pumpkin plants than when one was present, and downwind compared with upwind of an infection source. Host resistance gene *zym* was effective against ZYMV isolate Knx-1 from Kununurra in five cultivars of cucumber. In zucchini, host resistance gene *Zym* delayed spread of infection (partial resistance) in 2 of 14 cultivars but otherwise did not diminish final ZYMV incidence. Zucchini cultivars carrying *Zym* often developed severe fruit symptoms (8/14), and only the two cultivars in which spread was delayed and one that was tolerant produced sufficiently high marketable yields to be recommended when ZYMV epidemics are anticipated. In three pumpkin cultivars with *Zym*, this gene was effective against isolate Cvn-1 from Carnarvon under low inoculum pressure, but not against isolate Knx-1 under high inoculum pressure, although symptoms were milder and marketable yields greater in them than in cultivars without *Zym*. These findings allowed additional cultural control recommendations to be added to the existing Integrated Disease Management strategy for ZYMV in vegetable cucurbits in WA, but necessitated modification of its recommendations over deployment of cultivars with resistance genes.

Introduction

Crops of melon (*Cucumis melo* and *Citrullus lanatus*), cucumber (*Cucumis sativus*) pumpkin (*Cucurbita moschata* and *Cucurbita maxima*), zucchini and squash (*Cucurbita pepo*) are widely grown in Australia for domestic consumption and export markets. The area of cucurbit crops grown in tropical and sub-tropical Australia in 2007 was 7049 ha (melon), 5968 ha (pumpkin), 2438 ha (zucchini and squash) and 577 ha (cucumber) (Anon., 2008), and the regions involved are in Western Australia (WA), the Northern Territory (NT), Queensland (QLD) and northern New South Wales (NSW). Viruses cause damaging

diseases of cucurbit crops throughout horticultural cropping regions of tropical and sub-tropical Australia, greatly diminishing both yield and quality of produce, and seriously damaging industry profitability (e.g., Greber, 1969, 1979; Greber et al., 1987; Herrington, 1987; McLean et al., 1982; Coutts and Jones, 2005). The viruses that currently pose the most serious threats are two non-persistently aphid-borne potyviruses, *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV). ZYMV is the most important virus in WA and the NT, and PRSV in QLD, but the current situation in northern NSW is unclear. *Squash mosaic virus* (SqMV), *Cucumber mosaic virus* (CMV) and *Watermelon mosaic virus* (WMV) occur less frequently, causing only occasional losses (e.g., Greber, 1978; Greber et al., 1988; Herrington, 1987; Coutts and Jones, 2005). Although *Beet western yellows virus* (BWYV) occasionally infects cucurbits, at least one other unidentified virus of the *Luteoviridae* is present (Coutts and Jones, 2005). Whether the latter is *Cucurbit aphid-borne yellows virus* (CABYV) (Lecoq et al., 1992), or another member of the *Luteoviridae* that causes losses elsewhere is unknown, as is the possible occurrence of several other important cucurbit viruses not yet recorded in Australia (e.g., Zitter et al., 1996).

In 2003-04, a large-scale survey determined the incidence and distribution of viruses in cucurbit crops near Kununurra (Ord River Irrigation Area), Carnarvon (Gascoyne Horticultural Area), Broome and Perth (Perth Metropolitan Area) in WA, plus Katherine and Darwin in the NT (Coutts and Jones, 2005). Crops of melon, cucumber, pumpkin, zucchini and squash were all virus-infected; squash and zucchini were the most severely damaged. ZYMV, PRSV and SqMV occurred in all cucurbit crop types: WMV in pumpkin, zucchini and squash; and CMV and *Luteoviridae* viruses in melon, pumpkin and cucumber. ZYMV occurred commonly in 5 of 6 cucurbit growing areas, including Kununurra and Carnarvon, at final crop incidences of up to 100%. PRSV occurred in fewer crops in 4 of 6 growing areas with individual crop incidences up to 60% at Kununurra. The virus was absent from Carnarvon. Individual crop incidences of SqMV and an unknown member of the *Luteoviridae* were up to 60% and 49%, respectively, but both occurred in few crops in only 2 of 6 growing areas, including Kununurra and Carnarvon for the latter but Kununurra and Broome for SqMV. Few crops were infected with WMV, CMV or BWYV, and their within-crop incidences were always low (<8%). Subsequent studies at Kununurra rarely found SqMV (unpubl. data). Since 2005, ZYMV epidemics have decreased average cucurbit yields by >30% annually at Kununurra. In 2009 and 2010, many Kununurra (both years) and Carnarvon (2010 only) cucurbit crops were ploughed-in before harvest because the anticipated yields were too low to justify harvesting them.

Both colonising and non-cucurbit colonising aphids transmit ZYMV in the field and once the virus is introduced to a cucurbit planting its spread within the field is generally very rapid (Lisa and Lecoq, 1984; Yuan and Ullman, 1996; Desbiez and Lecoq, 1997). Melon aphids (*Aphis gossypii*) infest cucurbit crops at Kununurra and green peach aphids (*Myzus persicae*) at Carnarvon. Both species are efficient vectors of ZYMV (e.g., Desbiez and Lecoq, 1997). Which non-cucurbit colonising species are also involved in transmission is unknown as no vector transmission studies have been done with ZYMV under WA conditions. Hosts in which ZYMV can persist between cucurbit growing seasons at Kununurra and Carnarvon include volunteer cucurbit crop plants, unharvested cucurbit crops and several alternative wild cucurbitaceous hosts, but ZYMV incidences in wild cucurbits are generally small (Coutts and Jones, 2005; Coutts et al., 2010.). Similar findings are reported in other continents (Desbiez and Lecoq, 1997). ZYMV is seed-borne at low levels in zucchini and squash (*C. pepo*), and in Delicia-type butternut squash (*C.*

maxima) (Davis and Mizuki, 1986; Greber et al., 1988; Schrijnwerkers et al., 1991; Fletcher et al., 2000; Riedle-Bauer et al., 2002; Tobias and Palkovics, 2003; Tobias et al., 2008; Coutts et al., 2010), but no seed transmission has been ever been reported in Australia or elsewhere in melon, pumpkin or cucumber (e.g., Provvidenti and Robinson, 1987; Gleason and Provvidenti, 1990; Robinson et al., 1993; Desbiez and Lecoq, 1997; Riedle-Bauer et al., 2002; Glasa and Kollerova, 2007). Aphids can also acquire ZYMV from discarded infected cucurbit fruits (Lecoq et al., 2003). Thus, infected live plant hosts surviving outside the growing season, seedlings growing from infected zucchini seed lots and discarded infected fruits act as sources for spread of ZYMV to newly planted cucurbit crops.

Phytosanitary, cultural and biological control measures that help to diminish spread of ZYMV in cucurbit crops growing in open field situations include: removal of potential virus and aphid sources among weeds, removing old crops, and avoiding overlapping and side-by-side plantings (phytosanitary measures); deploying reflective or other plastic mulches to deter aphid landings and crop covers to prevent early aphid ingress (cultural measures); and using cross protection with mild ZYMV strains (biological measure) (e.g., McLean et al., 1982; Spence et al., 1996; Desbiez and Lecoq, 1997). In addition, host resistance genes against ZYMV have been described in cucumber (*zym*), watermelon, muskmelon, butternut pumpkin and *Cucurbita ecuadorensis* (all designated *Zym*). These resistances are normally found in wild accessions but some of them have been introduced into commercially available cultivars of cucumber, butternut and Jarrahdale pumpkin and zucchini (e.g., Herrington et al., 1988, 1989; Paris et al., 1988, Robinson et al., 1988; Desbiez and Lecoq, 1997; Ullah and Grumet, 2002; Brown et al., 2003; Diaz et al., 2003; Xu et al., 2004; Guner and Wehner, 2008). However, when the *Zym* gene from butternut pumpkin was introduced to zucchini through inter-specific crosses, the plants were not resistant to ZYMV but instead exhibited tolerance (i.e., systemic infection with mild symptoms) which was unstable (Desbiez et al., 2003; Lecoq et al., 2004). Paris and Cohen (2000) reported that additional pumpkin genes were required for improved expression of *Zym* in zucchini plants. Transgenic resistance to ZYMV is available commercially in squash in North America (Tricoll et al., 1995; Rowell, et al., 1999; Prendeville and Pilson, 2009). Chemical control through application of insecticides is ineffective at decreasing ZYMV spread within cucurbit crops, but frequent application of mineral oils was effective (e.g., Desbiez and Lecoq, 1997). Cultural control measures that were effective against PRSV and WMV in pumpkin and muskmelon, were use of non-host barrier crops and planting upwind of virus sources (Adlerz, 1974; Toba et al., 1977; Damicone et al., 2007), but neither measure has been evaluated against ZYMV.

In WA, the cucurbit growing season at Kununurra (tropical climate) is the cooler dry season (May-October), from autumn to spring (March-November) at Carnarvon (sub-tropical climate) and spring-autumn (October-April) at Perth (Mediterranean climate). There are 2-3 crops planted/year on the same farms at Carnarvon, and 1-2 crops/year at Kununurra and Perth, reflecting the longer growing period in the former area. The only previous research on control of ZYMV in cucurbits in WA was by McLean et al. (1982) who demonstrated in field experiments at Carnarvon that (i) reflective mulch diminished ZYMV spread (misdiagnosed as WMV) and increased yields of watermelon, and (ii) black plastic mulch also diminished spread and increased yields but to a lesser degree. This paper describes 10 field experiments done at Kununurra, Carnarvon and Perth between 2006 and 2009 that investigated the effectiveness of three cultural control measures (manipulation of planting date, non-host barriers and planting upwind) in limiting ZYMV spread in pumpkin, and of

single-gene ZYMV resistance in commercial cultivars of pumpkin, zucchini and cucumber. Also, cultivars of zucchini with single-gene resistance were inoculated with different isolates of ZYMV. Temporal and spatial dynamics of ZYMV spread were examined in some field experiments. An improved Integrated Disease Management (IDM) approach was devised for controlling ZYMV in vegetable cucurbits which was applicable to all three climatic zones.

Materials and methods

Plants, inoculations, virus isolates and antisera

Test, culture and infector plants were grown in insect-proof, air-conditioned glasshouses maintained at 18-22^{o}C. Plants of zucchini cvs Blackjack and Dunja were grown in steam-sterilised soil, sand and peat mix (1:1:1) in pots (culture plants) or jiffy pots (infector plants). For sap inoculation, infected leaves were ground in 0.1M phosphate buffer, pH 7.2, and the sap mixed with Celite before being rubbed onto the leaves of plants. All virus isolates were maintained by sap inoculation to plants of cv. Blackjack. The two ZYMV isolates used as inoculum sources in field experiments (Knx-1 and Cvn-1) came from naturally infected leaf samples: Knx-1 was from butternut pumpkin collected in 2005 at Kununurra and Cvn-1 was from the native cucurbit *Mukia maderspatana* collected in 2008 at Carnarvon (Fig. 1). Infector plants of cv. Blackjack were inoculated with infective zucchini sap containing isolates Knx-1 or Cvn-1 before transplanting outside. Four additional isolates were used to inoculate cvs Blackjack and Dunja in a glasshouse experiment: Carnarvon isolates Cvn-2 and Cvn-20 were from infected zucchini or watermelon leaves collected in 2008 and 2010, respectively, while Kununurra isolates Knx-10 and Knx-11 were from infected watermelon or rockmelon leaves in 2007 and 2008., respectively. Isolates PRSV-Qld1 and SqMV–Kun1 were from previous work (Coutts and Jones, 2005). Leaves from cultures of ZYMV isolates Knx-1 and Cvn-1, PRSV-Qld1 and SqMV–Kun1 and freeze-dried leaves containing WMV obtained from Loewe Biochemica, Germany, were used as positive controls in enzyme-linked immunosorbent assay (ELISA). Polyclonal antiserum to ZYMV was obtained from Prime Diagnostics, Netherlands and DSMZ, Germany; polyclonal antisera to PRSV, WMV and SqMV from Loewe Biochemica, Germany; and generic monoclonal antibody specific to potyviruses from Agdia Inc, USA.

Enzyme-linked immunosorbent assay

Leaves of pumpkin, zucchini and cucumber were extracted singly or in groups of 2-10 in phosphate-buffered saline (10mM potassium phosphate, 150mM sodium chloride), pH 7.4, containing 5ml/L of Tween 20 and 20g/L of polyvinyl pyrrolidone (Mol. wt, 30 000), using a mixer mill (Retsch, Germany). The sample extracts were tested for infection with individual viruses by double-antibody sandwich ELISA (Clark and Adams 1977). To test for potyviruses in general, leaf samples were extracted in 0.05M sodium carbonate buffer pH 9.6 and tested using the antigen-coated indirect ELISA protocol of Torrance and Pead (1986). Each sample was tested in duplicate wells in microtitre plates and appropriate infected and healthy leaf samples were included in paired wells as controls. The substrate used was 1.0 mg/mL of *p*-nitrophenyl phosphate in 100ml/L of diethanolamine, pH 9.8. Absorbance values (A_{405}) were measured in a microplate reader (Bio-Rad Laboratories, USA). Absorbance values regarded as positive were always at least 10 times those of

healthy sap. Virus incidence was estimated from grouped sample results using the formula of Gibbs and Gower (1960).

General details of field experiments

Department of Agriculture and Food, Western Australia (DAFWA) Research Stations at Carnarvon (24^{o} 51' S, 113^{o} 43' E), Kununurra (15^{o} 39' S, 128^{o} 42' E), and Medina (32^{o} 13' S, 115^{o} 47' E) were used for the field experiments (Fig. 1). Medina is located in southern Metropolitan Perth. Field experiments at Kununurra were flood irrigated, at Carnarvon they were drip irrigated and at Medina overhead irrigation was used. Appropriate herbicides were applied to the soil before sowing to prepare weed-free seed beds. Pumpkin, zucchini and cucumber plants were fertilised and irrigated according to standard commercial practice for each region and no insecticide was applied. Grass and broad-leafed weeds growing within plots, buffers or barriers, and around the margins of field experiments were removed by hand weeding. All leaf samples collected from each field experiment were tested by ELISA using ZYMV antiserum and generic potyvirus antibody, and the two always gave matching results.

Field experiments on cultural control

Experimental design
Experiments 1-4 were all at Kununurra in 2008 and 2009. They were planted with Jarrahdale-type pumpkin cv. WA Grey (*C. maxima*). For each experiment, details of year, cultural control measure being applied, plot area assessed, virus isolate introduced, presence or absence of a barrier, barrier area, planting dates for pumpkins and non-host barriers, days after sowing (DAS) when infector plants were introduced, and DAS when assessments were done are detailed in Table 1. All experiments consisted of blocks containing rectangular plots arranged southeast-northwest in the general direction of the prevailing wind anticipated at that time of year. All pumpkin and non-host barrier crops were machine-sown. Plots consisted of raised beds 1.8 m wide into which pumpkin seeds were sown 1 m apart along their centres. Millet (*Pennisetum glaucum*) seed was sown at 5kg/ha in four parallel rows each spaced 30 cm apart along each bed. Lablab (*Lablab purpureus*) and borlotti bean (*Phaselous vulgaris*) seeds were sown in two parallel rows spaced 10 cm apart along each bed. Introduced zucchini infector plants acted as the primary virus source in Experiments 1 and 2 in 2008, but were not required in Experiments 3 and 4 in 2009 because considerable early spread occurred from naturally occurring external sources.

Experiments 1 and 2 were planted side-by-side within the same field and were separated by a millet strip 18 m wide and 100 m long sown 4 weeks before the pumpkins (Fig. 2). Experiment 1 examined the effect of prevailing wind direction on ZYMV spread. Two rectangular plots of pumpkin 36 m wide x 48 m long were arranged end-to-end, each containing 18 raised beds. In between the two plots was a 4 m wide band within which one infector plant was transplanted into every second raised bed at 25 DAS (Fig. 2b). Experiment 2 examined the effect of non-host barriers on ZYMV spread. It consisted of three blocks separated from each other by millet strips 18 m wide x 100 m long. Each block contained a rectangular pumpkin plot, one was 36 m wide x 100 m long and two were 36 m wide x 75 m long. At the southeast end of each block there was a 1 m wide band into which infector plants were transplanted at 25 DAS, one infector plant into every second raised bed. Within two of the blocks, a 25 m long barrier zone separated the infector

primary source band from the 75 m long plot of pumpkin: in one of them this barrier zone was left fallow but a non-host barrier crop of lablab was planted within in the other (Fig. 3a). There was no barrier zone in the third block so the plot planted with pumpkin was 100 m long (Fig. 2a).

Experiment 3 resembled Experiment 2 except that it consisted of four blocks each 16 m wide, millet strips separating each block were 5.4 m wide and no infector plants were introduced, the virus entering from natural external sources. Also, in three of the blocks the 25 m long barrier zones that separated the infector primary source band from the 75 m long pumpkin plot were planted with millet, borlotti bean or left fallow, respectively. There was no barrier zone in the fourth block which was planted with pumpkin along its full 100 m length.

Experiment 4 investigated the effect of time of sowing (TOS) and presence of a non-host millet barrier on ZYMV spread and consisted of two blocks 60 m wide x 50 m long (Fig. 2c). These blocks were parallel to each other but separated from one-another by a 36 m wide area of bare earth. Each block consisted of three plots of pumpkins planted at 14-15 day intervals representing three TOS. Each plot was 16 m wide x 50 m long and contained nine raised beds. In one of the two blocks, a 6 m wide x 50 m long strip of millet was sown outside the TOS-1 plot and in between each TOS plot (Fig. 3b), but in the second block there was no separation between plots with different TOS (Fig. 3c). The millet barriers were sown 6 days before TOS-1.

Assessment of ZYMV spread

Experiments were inspected three times (Experiments 1 and 2) or four times (Experiments 3 and 4) for presence of pumpkin plants with mosaic leaf symptoms typical of ZYMV infection. In Experiments 1-3, on each occasion when characteristic symptoms were first seen in a plant, this was noted and its position was then recorded on a grid showing the position of each individual plant. In Experiment 4, the total number of plants with symptoms was counted on each assessment date. In each experiment, on each assessment date, 10-20 leaves with symptoms were collected from each pumpkin plot, placed in a separate labelled plastic bag representing each plot, stored in a cooler box, transported to the laboratory and each leaf was tested individually by ELISA for presence of ZYMV, PRSV, SqMV, WMV and potyviruses in general. ZYMV was the only virus ever detected.

Analysis of spatial pattern

In Experiments 1 and 2, infection data for individual plants were used to plot gradients of infection over increasing distance from the primary virus source. The counts for presence or absence of ZYMV infection in each set of nine adjacent plants were combined together to provide a sample unit (quadrat) value (quadrat size 3 m x 5.4 m). Spatial patterns of infected plants based on penultimate cumulative 'quadrat' counts were quantified using Spatial Analysis by Distance IndicEs (SADIE) as described by Coutts et al. (2004). For a random arrangement of the observed counts amongst the given sample units, the expected value for the index of aggregation (I_a), an index of the degree of clustering for the whole sample area, is one, while $I_a > 1$ indicates aggregation of counts into clusters (Perry et al., 1996).

The clustering indices, v, for cumulative infections were contoured using the computer program Surfer (Anon., 1997) to provide maps of spatial pattern. The contouring levels used indicate where estimated indices are half as great again as expected by chance ($v = 1.5$

for infection patches and v=-1.5 for infection gaps). The resulting maps indicate the spatial location and extent of patches and gaps of infection. Spots represent individual quadrat sample units denoting infection patches with v>0 (red) and infection gaps with v<0 (blue) (Perry et al., 1999). Small spots represent clustering indices of 0 to +/-0.99 (clustering below expectation), intermediate spots +/- 1 to +/- 1.49 (clustering slightly exceeds expectation) and large spots >1.5 or <-1.5 (clustering more than half as much again as expectation). Red lines enclosing patch clusters are contours of v=1.5 and blue lines enclosing gap clusters are contours of v=-1.5. Black lines are zero-value contours, representing boundaries between patch and gap regions where the count is close to the sample mean. The units on the contour map axes are distances in metres.

Field experiments on host resistance

General details

For each field experiment, details of year, location, crop, ZYMV isolate used, number of replications, plot size, sowing dates, DAS when infector plants were introduced, DAS when assessments were done and DAS before harvest are detailed in Table 2. Details of cucurbit type and cultivar, cucurbit virus resistance rankings used for each cultivar by its commercial seed producer, and the seed producer names are in Table 3. In the three pumpkin cultivars that carried a *Zym* gene, the gene present was originally from *C. moschata* (butternut cv. Sunset), *C. ecuadorensis* (Jarrahdale cv. Dulong) or of unspecified origin (Jarrahdale cv. Sampson). The 14 zucchini cultivars that contained *Zym* originally from *C. moschata* and five cucumber cultivars that contained *zym* from *C. sativus* were planted (Herrington et al. 1988, 1989, 1999, 2001; Paris et al., 1988; Desbiez and Lecoq, 1997; Robinson et al., 1988; Brown et al., 2003). The control cultivars planted were butternut pumpkin cv. Butternut Large and Jarrahdale pumpkin cv. WA Grey, zucchini cv. Blackjack and cucumber cv. Pronto. Plots were sown by hand in raised beds 1.8 m apart with 1 m between plants (Experiments 5-7), or 1.5 m apart with 0.5 m between plants (Experiments 8-10). There was one cultivar/plot. Except in Experiment 5 in which there was no replication and Experiment 8 which had a resolvable row-column latinised design, plots were always arranged in standard randomised block designs. Experiments 5-7 had bare-earth buffer areas surrounding each plot and Experiment 8 had an oat buffer (Fig. 3): buffer width varied between experiments (see below). For sampling, a newly emerged leaf was removed from each cucurbit plant, placed in a separate labelled plastic bag representing each plot (Experiments 5-7) or each leaf placed in a separate bag (Experiments 8-10), stored in a cooler box, transported to the laboratory and tested for ZYMV presence by ELISA. In each experiment, all leaf samples from the final assessment date were also tested for PRSV, WMV and potyviruses in general. ZYMV was the only virus ever detected.

For each cultivar, the different types of viral foliage symptoms present were recorded, and plant susceptibility and sensitivity rankings were assigned. The plant susceptibility rankings relate to the relative ease with which plants of a given genotype became infected when exposed to virus inoculation by naturally occurring aphids, while sensitivity (=symptom severity) rankings refer to the intensity of leaf symptoms after plant infection. Susceptibility rankings were based on the percentage of plants that became infected and the categories were: highly susceptible (HS), susceptible (S), moderately resistant (MR), resistant (R) and highly resistant (HR). Leaf sensitivity rankings were on a 1-5 scale: 1, symptomless infection; 2, mild mottle; 3, mottle and leaf distortion; 4, laminar bubbling, mottle and distortion; and 5, shoe-stringing, laminar bubbling, severe mottle and distortion.

In Experiments 5-9, harvested fruits were assessed for total number, individual weight and marketability. For marketability, each fruit was assigned a sensitivity ranking, and classed as being either market acceptable (no virus symptoms), unmarketable due to virus damage (obvious virus symptoms including skin blemishes and distortion), or unmarketable due to other causes (e.g. immature, split, insect damage). The sensitivity rankings were 1, absence of fruit symptoms; 2, mild skin mottle; 3, skin dimpling and mottle; 4, distortion, skin mottle, mild distortion and surface lumps; and 5, severe distortion, knobbly, reduced size. Data from each plot for area under the pathogen progress curve (AUPPC) (Experiments 7-9), angular transformed percentage ZYMV incidence and yield components were subjected to ANOVA using Genstat for Windows, release 12 (Experiments 6-9).

Experiment 5

This preliminary experiment included the three pumpkin cultivars carrying *Zym* and the two control cultivars (Table 3). Each plot consisted of two raised beds surrounded by a bare earth buffer 3.6 m wide. Individual infector plants were transplanted 5 m apart between the two raised beds. From each plot single leaves from 50 different plants were sampled at random on three occasions and tested by ELISA. At 89 DAS, plant sensitivity rankings were determined and symptom types present in infected plants recorded. At 107 DAS, a 2 x 20 m (0.004ha) area was harvested from each plot and the fruits assessed.

Experiments 6 and 7

These experiments consisted of the same five pumpkin cultivars sown in Experiment 5, but Experiment 6 also included an additional cultivar, Kent-type cv. Kens Special (*C. moschata*). Each plot consisted of three raised beds surrounded by a bare earth buffer 10 m wide (Fig. 3d). Seed was sown in the two outside raised beds. In Experiment 7, each raised bed was covered with black plastic mulch. At 22-23 DAS, two infector plants were transplanted together into the middle of the central bed of each plot. Replicates used for data collection were 6 of 6 in Experiment 6 and 3 of 5 in Experiment 7. Every plant within each of the plots of these replicates was sampled and the leaf samples tested individually by ELISA on two (Experiment 6) or four (Experiment 7) occasions. At 92 DAS (Experiment 6) and 119 DAS (Experiment 7), plant sensitivity rankings were determined, symptom types in infected plants recorded, and the complete plots harvested. Fruit assessments were as in Experiment 1.

Experiment 8

This experiment consisted of 10 commercial zucchini cultivars carrying *Zym*, six green, two yellow and two Lebanese types, and cv. Blackjack (Table 3). Each plot consisted of three raised beds 2 m long surrounded by an oat buffer 1.2 m wide (Fig. 3e). To assess ZYMV spread within plots, internal control plants were included in every plot, so each consisted of eight plants of the cultivar being tested and five additional plants of cv. Blackjack. At 13 DAS, two infector plants were transplanted into the centre of each plot. On four occasions, the plants were sampled and tested individually by ELISA. Following each test, every plant found infected was identified on a map showing all plants and such plants were not re-sampled subsequently. At 67 DAS, plant sensitivity rankings were determined and the symptom types recorded. On eight occasions, the fruits present on each plant were removed and assessed.

Experiments 9 and 10

Experiment 9 consisted of five green zucchini cultivars carrying *Zym* and cv. Blackjack, while Experiment 10 with cucumber consisted of three green-slicer and two Lebanese type

cucumber cultivars carrying *zym* and cv. Pronto (Table 3). These two experiments used single-row plots 4 m in length, and the distances between plot ends and sides were 2 m and 1.5 m, respectively (Fig. 3f). At 21 DAS (Experiment 9) and 27 DAS (Experiment 10), pairs of infector plants were transplanted into the bare earth between each test row. All plants in each test row were sampled on four occasions. In Experiment 9, the leaf samples were tested individually by ELISA. As in Experiment 8, each plant found infected was identified on a map showing all plants and such infected plants were not re-sampled. At 56 DAS, plant sensitivity rankings were determined and symptoms recorded. On eight occasions, the fruits present on each plant were removed and assessed. In Experiment 10, leaf samples from all plants within each row were tested in groups of nine by ELISA and the control plot samples were then retested individually. No fruit was harvested.

Glasshouse inoculations to zucchini

Five zucchini plants each of cv. Dunja, which was moderately resistant to aphid-borne ZYMV infection in Experiment 9, and cv. Blackjack were sap-inoculated at the two leaf stage with infective sap containing each of three ZYMV isolates from Kununurra (Knx-1, Knx-10, Knx-11) and three from Carnarvon (Cvn-1, Cvn-2, Cvn-20). In addition, five plants of each cultivar were sap-inoculated with healthy sap. Tip leaves from each plant were sampled individually 17, 24 and 32 days after inoculation and the samples tested by ELISA. Symptoms were recorded on each sampling date.

Assessment of vector aphid numbers and species

In Experiments 1 and 2 and 5-7, pumpkin or zucchini plants within each plot were observed for aphid presence on each assessment date but no counts of aphid numbers were done. In Experiments 8-10, in addition to these general observations within plots, aphids were counted on the zucchini infector plants (1 leaf/plant) at 7 (Experiment 8), 12 (Experiment 9) and 6 (Experiment 10) days after transplanting. In Experiment 3, aphids counts were done on 20 pumpkin plants (1 leaf/plant) in each plot on one occasion (28 DAS). In Experiment 4, on each assessment date aphids were counted on the oldest leaf of each of 20 pumpkin plants in all plots and plant growth stages recorded. In addition, within each millet barrier planting between pumpkin plots, aphids were counted on the top 10 cm of one shoot from each of 20 plants. Aphid identification was normally done *in situ* but some of the aphids were placed in vials containing 70% ethanol and transported to the laboratory for confirmatory identification.

Results

Cultural control field experiments

Effect of wind direction

Experiment 1

Naturally occurring winged melon aphids were present on pumpkin plants by 39 DAS and by 65 DAS this species was colonising the pumpkin plants (i.e. reproducing on them). ZYMV spread more quickly to pumpkin plants downwind than upwind of the infector plants (Fig. 4a). By 53 DAS, overall incidences of plants with typical ZYMV symptoms within each plot were 10% (upwind) and 16% (downwind). Spread then accelerated quickly and by 83 DAS incidences reached 54% (upwind) and 74% (downwind) (Table 4). In the

downwind plot, infection incidences were greatest closest to the primary infection source and declined with increasing distance (Fig. 4b). In contrast, the upwind plot incidence was unrelated to distance from primary infection source. *Ia* values for assessments at 53, 65 and 83 DAS showed that, over the entire areas of each plot, clustering of plants with symptoms was always significant downwind, but upwind this was only so for the 65 DAS assessment (Table 4). Moreover, especially at 53 and 65 DAS, in the downwind plot large patch clusters (denoted by large red spots within red contours) or gap clusters (denoted by large blue spots within blue contours) were evident close to the primary infection source or distant from it, respectively (Fig. 5). In contrast, in the upwind plot both types of clusters were smaller and their distributions much less polarised in relation to position of the primary virus source.

Effect of intervening non-host crop or fallow and time of sowing

Experiment 2

As in Experiment 1, naturally occurring winged melon aphids were present on pumpkin plants at 39 DAS, and by 65 DAS they were colonising these plants. ZYMV spread to more plants where the barrier was fallow or absent than where it was lablab (Fig. 4c). By 83 DAS the ZYMV incidences were 63% (lablab barrier), 73% (fallow barrier) and 72% (no barrier, first 75m) (Table 4). There was little evidence of virus spread between blocks, indicating that millet may be a better barrier to aphid movement than lablab. In the plots with fallow or no barriers, infection incidences were greatest closest to the primary infection source but declined with increasing distance (Fig. 4d). In contrast, in the plot with a lablab barrier, incidence was smaller closest to the infection source and declined less steeply away from the source within a 20 m wide band. *Ia* values for the assessments at 53, 65 and 83 DAS showed that, over the entire areas of each plot, clustering of plants with symptoms was always significant regardless of whether barriers were present or absent (Table 4). However, at each assessment date, the *Ia* values were smallest where the barrier was lablab (2.6-3.0) and greatest where there was no barrier (3.5-5.4, first 75m), indicating that clustering of plants with symptoms was least with the former and greatest with the latter. Also, in all three assessments, large patch clusters or gap clusters were evident close to the primary infection source or distant from it, respectively, and were most obvious in the plot without barriers and least obvious in the plot with the lablab barrier (Fig. 6).

Experiment 3

At first assessment (27 DAS), >100 melon aphids (winged, non-winged and nymphs) were counted on each of 20 pumpkin plants within each plot. At this time, no aphids were observed on 20 plants within the borlotti bean barrier, while 1-2 oat aphids (*Rhopalosiphum padi*) were counted on each of 20 millet barrier plants, both alates and nymphs being present. At 34 DAS, melon aphids occurred on all pumpkin plants examined, the majority having >20 aphids/plant (winged and non-winged, mean of 30 aphids/plant). By 41 DAS, melon aphid numbers had decreased with most pumpkin plants in each plot having <20 aphids/plant (mean of 17 aphids/plant). At 27 DAS, natural spread had already occurred within each pumpkin plot, plants with symptoms typical of ZYMV infection occurring evenly within each, and by 61 DAS all plants within each plot showed symptoms. Coincidence of a large aphid flight with the earliest stage of pumpkin growth led to a large internal melon aphid population and virus source such that each plot became totally infected by ZYMV.

Experiment 4

At first assessment, there were >100 melon aphids/pumpkin plant (winged and non-winged) in both TOS-1 plots, but plants in TOS-2 plots had very few (<1 aphid/plant) (Table 5). At second assessment of TOS-1, the number of melon aphids/plant had decreased to 30 with a barrier present and 72 without. However, the number of aphids/plant in TOS-2 plots had increased to 14 with a barrier present and 17 without. At third assessment, the number of aphids had declined in TOS-1 and increased in TOS-2 where there were still fewer with a barrier than without. By final assessment, numbers had decreased to <5/plant within both TOS-1 and TOS-2, and were also <5/plant in TOS-3. Numbers of oat aphids counted on the millet barrier plants were always <10/plant on each assessment date and included winged and non-winged aphids, with no differences in numbers between millet barriers.

At first assessment of TOS-1, 14% or 16% of plants had symptoms typical of ZYMV infection in plots without or with a barrier, respectively, and these plants with symptoms were distributed evenly throughout each plot. By final assessment, the incidences were 65% (without a barrier) or 64% (with a barrier) (Table 5, Fig. 7). In contrast, in TOS-2 more plants developed typical ZYMV symptoms in plots without than with a millet barrier, incidences reaching 32% (without a barrier) versus 11% (with a barrier). In TOS-3, on final assessment, the incidences were only 7% (without a barrier) and 4% (with a barrier). Subsequently severe powdery mildew infection in all plots prevented symptom recording. ZYMV incidence was decreased 49% by a 2-week delay in sowing from TOS-1 and TOS-2, 34% by deploying millet barriers in TOS-2, and 83% by combining both treatments.

Host resistance field experiments with pumpkin

Experiment 5

Unidentified winged aphids were found on the pumpkin plants at first assessment (55 DAS), but none at 76 or 89 DAS. At 55 DAS, all plants of cvs WA Grey and Butternut Large (controls) and cv. Sunset (with *Zym*) were infected with ZYMV (Table 6a), but no virus was detected in cvs Sampson or Dulong (with *Zym*). However, at final assessment (89 DAS), ZYMV incidences in cvs Sampson and Dulong were 80% and 40%, respectively. Cvs WA Grey and Butternut Large developed severe foliage symptoms, while symptoms in the other cultivars were moderate (Sunset) or mild (Sampson and Dulong). Plant sensitivity rankings were 4-5 for the two control cultivars and 2-3 for the others. Overall fruit yields in the Jarrahdale pumpkin cultivars were very low (3.5t/ha) in cv. WA Grey, high (26-27t/ha) in cvs Dulong and Sampson, and intermediate (14-15t/ha) in the two butternut cultivars (Butternut Large, Sunset) (Table 6b). In contrast, fruit quality (% fruit virus-affected) was severely impaired by ZYMV in the two control cultivars, unaffected in cvs Sunset and Samson and only marginally impaired in cv. Dulong (Table 6b). For the two butternut pumpkins, although their yields were similar (14-15t/ha), most cv. Butternut Large fruit were distorted and lumpy with only 3% marketable, while 92% of cv. Sunset fruit were marketable and without symptoms (Fig. 8). With Jarrahdale pumpkins, the yield of cv. WA Grey was 3.5t/ha with no marketable fruit, whereas yields of cvs Sampson and Dulong were 26-27 t/ha; 50% (Dulong) and 76% (Sampson) were marketable. Fruit sensitivity rankings were 3-5 in the control cultivars and 2 for cv. Dulong.

Experiment 6

Melon aphids were found colonising pumpkin plants on the first assessment date (49 DAS), but no aphids were seen at 77 DAS. At 49 DAS, the virus incidence in cv. Dulong was significantly smaller than in all other cultivars and in cv. Sunset were significantly smaller

than those in cv. WA Grey (Table 6a). By 77 DAS, ZYMV incidence had reached 86% in cv. Dulong and 100% in all the other cultivars. Susceptiblity rankings were MR for cv. Dulong, S for cv. Sunset and HS for the other cultivars. Plants of cvs WA Grey and Butternut Large developed severe foliage symptoms, while the other cultivars had moderate (cvs Kens Special and Sunset) or mild (cvs Sampson and Dulong) symptoms. Plant sensitivity rankings were 5 for the two control cultivars, 3 for cvs Kens Special and Sunset, and 2 for cvs Sampson and Dulong.

Overall fruit yields were significantly smaller (10-11t/ha) in two of the cultivars without *Zym* (WA Grey, Kens Special), than in any of the others (Table 6b). Overall fruit yields in cv. Sampson were significantly larger (33t/ha) than those in any other cultivar, while those in cv. Sunset were significantly larger (27t/ha) than those of control cv. Butternut Large (23t/ha), which in turn had significantly larger overall yields than those of cv. Dulong (19t/ha). Thus, in this experiment a control butternut cultivar (Butternut Large) yielded more than a Jarrahdale cultivar carrying *Zym* (Dulong). Fruit quality (% fruit virus-affected) was significantly more damaged in control cvs Butternut Large and WA Grey than in the others, including cv. Kens Special which lacks *Zym*. However, cv. Kens Special had a significantly greater % of unmarketable fruit than all the other cultivars, 45% being immature. Marketable yield was minimal in the three cultivars without *Zym*, and, due to its fruit maturing at different rates, significantly smaller in cv. Dulong than in the other two cultivars carrying *Zym.* For the two butternut pumpkins, although their yields were relatively similar (23-28t/ha), most cv. Butternut Large fruit were distorted and lumpy with only 7% marketable, while 35% of cv. Sunset fruit were marketable and without symptoms (Fig. 8). In Jarrahdale pumpkin, 88% of cv. WA Grey fruit were small and severely distorted, only 10% being marketable, 36% of cv. Sampson fruit were marketable while 46% were virus-affected, and only 17% of Dulong fruit were marketable, with 67% virus-affected. Fruit sensitivity rankings were lower than those for plant sensitivity, 3-4 in the two control cultivars and 2 in the three cultivars carrying *Zym*, and in cv. Kens Special without *Zym.*

Experiment 7

Cool minimum temperatures in August and September (average $<10^{o}$C) resulted in little aphid activity during these months, and aphids were never found alighting on or colonising pumpkin plants on any assessment date, so all virus transmission was attributed to migrant aphids. Spread of isolate Cvn-1 was slower than that of isolate Knx-1 in Experiments 5 and 6 (Table 6a, Fig. 9a). It was not detected until second assessment (76 DAS) when it occurred at low levels in control cvs Butternut Large and WA Grey, eventually rising to 32% and 93% by 119 DAS, respectively. In the cultivars carrying *Zym*, spread was minimal (Samson at 4%) or did not occur (Sunset and Dulong), contrasting with the substantial spread of isolate Knx-1 found previously in these three cultivars. AUPPC and final % incidence values were significantly greater in cv. WA Grey than in cv. Butternut Large. Susceptiblity rankings under these low inoculum pressure (late spread) conditions were HS for cv. WA Grey, S for cv. Butternut Large, R for cv. Sampson, and HR for cvs Dulong and Sunset. Cvs WA Grey and Butternut Large developed moderately severe plant symptoms, while Sampson was infected symptomlessly. Plant sensitivity rankings were lower than in Experiments 5 and 6, being 3 for the two control cultivars and 1 for cv. Sampson.

Overall fruit yields were significantly smaller in control cv. Butternut Large (18t/ha) than in all other cultivars, and in cv. Sunset (34t/ha) were significantly smaller than those in cv. Sampson (55t/ha); the values for cvs Sunset, Dulong and WA Grey (43-42t/ha) were not significantly different from each other (Table 6b). Fruit quality (% fruit virus-affected) was significantly more damaged in control cvs Butternut Large and WA Grey than in the other three cultivars, being nil in the two that remained uninfected. However, due to variation in their maturity date, cv. Dulong had a significantly higher proportion of unmarketable fruit (74%) than all the other cultivars. Marketable yields of cv. Sampson (30t/ha) were significantly greater than those in all other cultivars, while those in cv. Butternut Large (2t/ha) were significantly smaller than those in all cultivars except cv. Dulong (11t/ha). Marketable yields for cvs Sunset, Dulong and WA Grey (34-48t/ha) were not significantly different from each other. For the two butternut pumpkins, most cv. Butternut Large fruit had skin dimpling and uneven skin colour, and were of reduced size. Only 14% were marketable, while 50% of cv. Sunset fruit were marketable and all without virus symptoms (Fig. 8). With the Jarrahdale types, 59% of cv. WA Grey fruit were distorted and lumpy due to virus infection with only 26% marketable, while 52% and 25% of cvs Samson and Dulong, respectively, were marketable. Fruit sensitivity rankings with isolate Cvn-1 in control cultivars were lower (2-3) than with isolate Knx-1 in Experiments 5-6 (3-5).

Host resistance field experiments with zucchini

Experiment 8

At 20 DAS, winged green peach aphids occurred naturally on each infector plant (1-2 aphids/plant). No aphids were observed within the plots subsequently. There were no significant differences in incidences of ZYMV between any of the 11 cultivars on any of the four assessment dates (Table 7a, Fig. 9b). By final assessment at 81 DAS, incidences were 94-100% in the 10 cultivars carrying *Zym* and and 100% in cv. Blackjack, However, AUPPC values revealed significant differences in rates of ZYMV spread which was slowest in cv. Top Gun and fastest in cv. Hummer. Rate of spread in control cv. Blackjack was not significantly different from that in cv. Top Gun but was significantly slower than those in cvs Batal, Black Adder, and Hummer. All cultivars received susceptibility rankings of HS. Plant symptoms varied from very mild leaf mottle in cvs Columbia, Sungold and Top Gun to severe mosaic, leaf distortion and plant stunting in cvs Gold Coast, Black Adder, Midnight and control cv. Blackjack (Fig. 10a-d), and this was reflected in their leaf sensitivity rankings of 2-4.

Overall fruit yields in cv. Blackjack (137g/plant) were not significantly different from those in Black Adder, Bond and Gold Coast, but were significantly smaller than those in the other seven cultivars. Those in cv. Columbia (3491g/plant) were significantly greater than those in all other cultivars, and those in cv. Top Gun (2562g/plant) were significantly greater than all except cvs Sungold and Batal (Table 7b). However, these overall yield differences bore little resemblance to those for marketable yields as those of cv. Blackjack (21g/plant) were significantly exceeded only by those of cvs Batal, Columbia, Gold Coast, Sungold and Top Gun. The marketable yield of cv. Sungold (1098g/plant) was significantly greater than those of all other cultivars except cv. Columbia, and this was reflected in its % fruit virus-affected value (22%) which was significantly smaller than in any other cultivar apart from cv. Batal (41%). Fruit sensitivity rankings were 5 in control cv. Blackjack and Columbia and 3-4 in all the other cultivars except cv. Sungold which had a ranking of 2. Fruit symptoms varied from faint ringspots alone in cv. Sungold to mottle, ringspots, knobbliness and mis-shapenness in control cv. Blackjack (Fig. 10a-d). In cvs Columbia, Top Gun and

Jaguar the severity of fruit quality symptoms was unrelated to those of leaf symptoms as they developed mild leaf symptoms (sensitivity rankings of 2), but severely affected fruits (sensitivity rankings of 4-5). The two sensitivity rankings were the same in Midnight (both 4) and Sungold (both 2), or similar in Batal, Black Adder, Bond, Gold Coast, Hummer (3-4) and Blackjack (4-5).

Experiment 9

At 32 DAS naturally occurring winged green peach aphids were present (1-2 aphids/plant) on each infector plant and every zucchini plant (7 leaf stage). No aphids were ever observed subsequently. The initial rate of ZYMV spread was slowest in cvs Paydirt and Dunja and fastest in cvs Amanda and Blackjack (Fig. 9c). By first assessment (40 DAS), 65% of plants of cv. Blackjack were infected while incidences in cultivars carrying *Zym* ranged from 0% to 47% (Table 7a). Incidence values in cvs Paydirt and Dunja were significantly smaller than those in any of the other cultivars except Sintia. By 48 DAS, values were significantly smaller than those in any of the other four cultivars, and values for cv. Midnight were significantly smaller than those for cvs Amanda and Blackjack. However, by final assessment (75 DAS), there were no significant differences in incidence values between any cultivar (Fig 9c). AUPPC values for cv. Paydirt were significantly smaller than those for all other cultivars; those for cv. Dunja were significantly smaller than those for cvs Amanda, Blackjack, Midnight and Sintia, and those for cvs Midnight and Sintia were significantly smaller than those for cvs Amanda and Blackjack. Based on differences in rates of ZYMV spread, susceptibility rankings ranged from R (cv. Paydirt) and MR (cv. Dunja) to HS (cvs Amanda and Blackjack). Plants of cvs Paydirt and Dunja were symptomlessly infected (Fig. 10e), and symptoms in the other four cultivars ranged from mild mosaic and leaf distortion to severe mosaic, leaf distortion and plant stunting. Sensitivity rankings ranged from 1 (cvs Paydirt and Dunja) to 4 (cvs Amanda and Blackjack).

Overall fruit yields were significantly smaller in control cv. Blackjack than in all other cultivars except Amanda (1823-1842g/plant), but were significantly greater in cvs Paydirt and Dunja (3689-4063g/plant) than in any other cultivar (Table 7b). The marketable yields of cvs Amanda and Midnight were not significantly different from those of control cv. Blackjack but were significantly smaller than those of the other three cultivars; those of cv. Sintia were significantly smaller than those of cvs Paydirt and Dunja. The marketable yield of cv. Dunja (1983g/plant) was significantly greater than those of all other cultivars, and this was reflected in its low % fruit virus-affected value (13%). This was significantly smaller than in any other cultivar and was due to greater production of healthy early-formed fruit (Fig. 10e). Similarly, the higher marketable yield cvs Paydirt (1137g/plant) was reflected in their lower % fruit virus-affected values (45%) again due to greater production of healthy early-formed fruit. Cv. Midnight had a significant higher % fruit virus-affected value (91%) than any other cultivar apart from cv. Amanda and this was reflected in its low marketable yield (101g/plant). Fruit sensitivity rankings ranged from 3 to 4. Fruit symptoms ranged from dimpling alone in cv. Paydirt to mottle, ringspots and knobbliness in cvs Midnight and Amanda. In cvs Dunja, Midnight and Paydirt, fruit symptoms were more severe, and fruit sensitivity rankings higher, than those for plant symptoms and leaf sensitivity rankings. However, in the other three cultivars, symptom severity observations and sensitivity rankings were the same.

Although final ZYMV incidences in Experiments 8 and 9 were similar (98-100%), the overall yields for cvs Blackjack and Midnight were considerably larger in Experiment 9:

with Blackjack 137 g/plant (Experiment 8) versus 1823 g/plant (Experiment 9), and with Midnight 1511 g/plant (Experiment 8) versus 2634 g/plant (Experiment 9). The likely explanation for this is that in Experiment 8 the zucchini plants were exposed to greater inoculum pressure at an earlier growth stage as they were growing in closer proximity to the infection source which was introduced earlier (at 13 DAS instead of 21 DAS). Combining results from both experiments, severe plant symptoms developed in 4 of 14 cultivars (Amanda, Gold Coast, Black Adder and Midnight), and severe fruit symptoms in 8 of 14 cultivars (Amanda, Batal, Bond, Columbia, Hummer, Jaguar, Midnight and Top Gun).

Host resistance field experiments with cucumber

Experiment 10
Naturally occurring winged green peach aphids occurred (1-2 aphids/plant) on each infector plant at 39 DAS (ie. at 12 days after transplanting infectors), but no aphids were ever observed later. ZYMV did not infect any plants of the five cucumber cultivars carrying *zym* (Camelot, Germlin, Khassib, Lancelot and Nouran). In contrast, all control cv. Pronto plants became infected developing leaf pallor, mild leaf mosaic, plant stunting and fruit skin symptoms of mild mottle

Glasshouse inoculations to zucchini

All six ZYMV isolates caused systemic infection in all 5 plants of control cv. Blackjack. Apart from Cvn-1, which induced a severe mottle, all isolates caused mottle symptoms, but for Cvn-1 and Knx-11 these were associated with leaf deformation. In cv. Dunja, the isolates caused systemic infection in 0/5 (Knx-1, Knx-10), 1/5 (Cvn-1), 2/5 (Cvn-2, Knx-11) or 3/5 (Cvn-20) plants. Also, where infection occurred it was always associated with mild symptoms of mild systemic chlorotic blotching (Cvn-1, Cvn-2, Knx-11) or very mild mottle (Cvn-20). No infection was detected in any of the plants of either cultivar inoculated with healthy sap (all 0/5).

Discussion

Our field experiments showed that alterating cropping practices by manipulating planting date to avoid exposing young plants to peak aphid vector populations, planting upwind of virus infection sources and using millet as a tall non-host barrier around crops can all diminish ZYMV spread in pumpkin. However, such barriers were ineffective when sown late and exceptionally large aphid vector populations were active at an early stage of crop growth. Lablab as a short non-host barrier was relatively ineffective, even when aphid populations were lower. Clustering of ZYMV-infected pumpkin plants was greater in plots downwind compared with upwind of the virus infection source, and in plots without a 25 m wide non-host barrier between the infection source and the pumpkin plants than when one was present. In pumpkin cultivars, resistance gene *Zym* was effective against ZYMV isolate Cvn-1 under low ZYMV inoculum pressure. In contrast, it was ineffective against isolate Knx-1 under high inoculum pressure, although leaf and fruit symptoms were milder and their marketable yields greater than those in cultivars without *Zym*. Of the 14 zucchini cultivars carrying *Zym,* all became infected with isolate Knx-1. Eight developed severe fruit symptoms, four developed severe foliage symptoms, one had useful tolerance of leaf and fruit infection and two displayed useful partial resistance (delayed rate of spread). This lack of useful tolerance in most zucchini cultivars carrying *Zym* differs from findings

elsewhere with other ZYMV isolates, as does the high incidences of infection with isolate Knx-1 in pumpkin cultivars carrying *Zym* (Desbiez et al., 2003; Lecoq et al., 2004). In contrast, *zym* resistance in cucumber was effective against isolate Knx-1.

Within plantings of annual crops, a marked effect of proximity to infection source on the extent of spread over distance is typical for non-persistently aphid-borne viruses. This is because the majority of incoming aphids alight initially at the crop margin before moving deeper into the stand and lose any virus that they carry when they probe healthy plants (e.g., Thresh, 1974, 1976, 1983; Jones, 1993, 2004, 2005). Also, when planting crops likely to become infected by epidemics of such viruses, orientation in relation to prevailing wind direction is important as less spread by aphids occurs upwind than downwind of an external source (e.g., Hampton, 1967; Jones et al., 2005). Both of these scenarios were illustrated in our field experiments investigating cultural control methods against ZYMV in pumpkin. Incidences in the downwind plot were greatest closest to the primary infection source and declined rapidly with increasing distance, but those in the upwind plot were unrelated to primary infection source position (Experiment 1). Also, clustering of ZYMV-infected plants was greater downwind than upwind. Moreover, in the downwind plot large patch clusters or gap clusters were evident close to the primary infection source or distant from it, respectively, but in the upwind plot both types of clusters were smaller and their distributions little influenced by source position.

In our first non-host barrier experiment (Experiment 2), when fallow, lablab or no barriers were present between pumpkin plots and infection sources, incidences were greatest closest to the primary infection source in the plots with no or fallow barriers and declined steeply over distance, but incidence was much smaller closest to the infection source in the plot with a lablab barrier, declining slowly only within a 20 m wide band. Clustering of ZYMV-infected plants was greatest in the plot without a barrier and least in the plot with a lablab barrier. Moreover, large patch or gap clusters were evident close to the primary infection source or distant from it, respectively, and were most obvious where a barrier was lacking and least obvious with the lablab barrier, confirming that clustering was greatest where the barrier was absent and least where it was lablab. The explanation for these findings is that in the upwind-downwind experiment aphids tended to be blown downwind of the virus source and spread upwind was delayed. In the non-host barrier experiment aphids landing on and probing the lablab instead of flying over it are likely to have been rendered non-viruliferous before moving on to the pumpkins.

Planting upwind of the virus source decreased overall ZYMV incidence in the pumpkin plot by 20% (Experiment 1), while the lablab barrier in our first non-host barrier experiment (Experiment 2) decreased it by 11%, so neither planting upwind nor the 25 m lablab barrier were very effective as control treatments if used alone. However, the lablab barrier was more effective in decreasing ZYMV incidence within a 20 m wide band closest to the virus source than further away. The 25 m fallow barrier provided little benefit. Planting upwind is likely to be more effective when the prevailing wind is stronger and more sustained, when there is low early inoculum pressure and a taller non-host crop (which low flying aphids would be less likely to overfly without alighting on the barrier) is planted instead. Elsewhere, barrier crops of wheat (*Triticum aestivum*) and swiss chard (*Beta vulgaris*) diminished spread of PRSV and WMV in muskmelon (Toba et al., 1977), and tall barrier crops of the non-host sorghum (*Sorghum bicolor*) were effective in decreasing spread of these two viruses in pumpkin in field experiments in which borders of non-host peanut (*Arachis hypogea*), soybean (*Glycine max*) and maize (*Zea mays*) were ineffective

(Damicone et al., 2007). Tall non-host barrier crops not only act as 'cleansing barriers' by removing virus adhering to aphid stylets (as do short non-host barriers), but also act as physical barriers (Thresh, 1974, 1976, 1983; Fereres, 2000; Jones, 2004, 2005, 2006; Jones et al., 2005; Hooks and Fereres, 2006). In Experiment 2, in which the pumpkin plots were separated by 5.4 m wide strips of the tall non-host millet, there was little evidence of virus spread between blocks, indicating that millet may be a better barrier to aphid movement than lablab. Subsequently, TOS-2 in the time of sowing experiment (Experiment 4) confirmed this, as the 6 m wide millet barrier decreased ZYMV incidence by 34%. In contrast, presence of a late-sown 6 m wide millet barrier established only 6 days before planting the pumpkins made little difference within TOS-1 plots sown 2 weeks earlier when the coincidence of a large aphid flight and young pumpkin growth led to a high inoculum pressure. Similarly, in our second non-host barrier experiment (Experiment 3) planted at the same time as TOS-1 (Experiment 4), there was no difference in ZYMV spread between pumpkin plots with no barrier and plots with fallow, borlotti bean or late sown millet barriers. In the time of sowing experiment (Experiment 4), within the plots without millet barriers delaying sowing by 2 weeks decreased ZYMV incidence by 49% and combining both treatments by 83%. Thus, combining manipulation of planting date with a tall non-host barrier provided very effective control of ZYMV in pumpkin when inoculum pressure was lower. To maximise its effect, the barrier needs to be established well before planting the cucurbit crop and, preferably, planted all round the crop perimeter to guard against any alterations in wind direction.

An ideal plant barrier should be a non-host for both virus and its vector, but should encourage aphid landing, be attractive to their natural enemies and should allow sufficient residence time to allow aphid probing before take-off occurs (Hooks and Fereres, 2006). For logistical reasons we were unable to undertake detailed studies on aphid vector efficiencies, aphid landings, probing, attraction and natural enemies in our field experiments with non-host barriers at the remote Kununurra location. However, such studies would be of great interest for the future.

Based on studies elsewhere, Desbiez et al. (2003) described the single-gene resistance conferred by *Zym* and associated modifier genes as 'almost complete' in butternut pumpkin. However, in our two field experiments with pumpkins carrying *Zym*, Kununurra isolate Knx-1 readily infected butternut pumpkin cv. Sunset and Jarrahdale pumpkin cvs Dulong and Sampson, all of which carry *Zym*. However, *Zym* was still useful in such cultivars because the rate of virus spread between plants was diminished (Dulong only), infected plants were more tolerant, fruit symptoms were either absent or diminished and marketable fruit yields were greater than in cultivars without this gene. Cv. Dulong, performed less well than cvs Sampson or Sunset mainly because of its low overall fruit yields and variable fruit maturity. In contrast, in our field experiment with the Carnarvon isolate Cvn-1 in which there was lower aphid activity than in the field experiments with isolate Knx-1, Cvn-1 either did not infect plants carrying *Zym* (cvs Dulong and Sunset) or infected very few plants (cv. Sampson), and fruit symptoms were milder in cultivars without *Zym*. Thus, *Zym* was effective against isolate Cvn-1, at least under the conditions of low inoculum pressure that occurred, so it apparently behaved more like the typical isolates in pumpkins carrying *Zym* (Desbiez et al. 2003). To establish whether *Zym* is more effective against Cvn-1 than Knx-1 in pumpkin, additional studies are needed with cultivars carrying *Zym* involving (i) field experiments with Cvn-1 under high inoculation pressures comparable to those reported here with Knx-1, and (ii) glasshouse experiments with aphid and sap inoculations involving both isolates.

ZYMV resistance in commercial zucchini hybrids was described as tolerance because, although cultivars carrying *Zym* became infected, only mild symptoms developed (Desbiez et al., 2003; Lecoq et al., 2004). Evolution towards increased aggressiveness of ZYMV that was associated with a point mutation in its P3 protein occurred in such hybrids. However, although fit when infecting such hybrids, these aggressive mutants were much less fit than wild-type mutants in mixed infection of zucchini cultivars without *Zym* (Desbiez et al., 2003). In our two field experiments with zucchini, all 14 cultivars carrying *Zym* became infected when exposed to isolate Knx-1. Eight of these cultivars developed severe fruit symptoms and two of these cultivars plus two others also developed severe leaf symptoms, although a few might be described as tolerant, most could not. Indeed, cv. Blackjack sometimes outperformed cultivars carrying *Zym* in overall yield and proportion of market-acceptable fruit (e.g., cv. Black Adder). In our first field experiment with zucchini (Experiment 8), only cv. Sungold was sufficiently tolerant of fruit infection with isolate Knx-1 to be suitable for planting in regions where isolates resembling Knx-1 occur. Thus, this isolate behaves like a naturally occurring aggressive ZYMV isolate in that it often induces severe symptoms in zucchini cultivars carrying *Zym*. However, unlike the aggressive isolates of Desbiez et al. (2003), there was no evidence of it being unfit. Our second field experiment with zucchini (Experiment 9) also revealed that spread of ZYMV was delayed substantially in cvs Paydirt and Dunja which led to considerably increased marketable yields due to greater production of healthy early-formed fruit, such partial resistance to ZYMV apparently not having been reported before in zucchini carrying *Zym*. Sap inoculation of three Kununurra and three Carnarvon isolates to plants of cvs Dunja and Blackjack established infection in all plants of the latter but few of the former, which is consistent with cv. Dunja having partial resistance. The greater gross yield of cvs Paydirt and Dunja combined with their lower % fruit-affected values increased their marketable yields sufficiently for them to be suitable for planting in regions where isolates resembling Knx-1 occur. We did not determine whether isolate Knx-1 has a point mutation in its P3 protein that might explain its aggressiveness in zucchini cultivars carrying *Zym* (Desbiez et al., 2003), but sequencing of its coat protein gene and those of 24 other ZYMV isolates from Kununurra revealed that all 25 group with Singapore/Reunion Island ZYMV isolates (Coutts et al., 2010). In contrast, coat protein gene sequences of isolate Cvn-1 and 17 others from Carnarvon all grouped with isolates from eastern Australia (B. Coutts et al., unpubl.).

The question arises as to whether the behaviour of isolates Knx-1 and Cvn-1 in pumpkin cultivars carrying *Zym* is typical of all ZYMV isolates present in the isolated, remote locations from which they came. At tropical Kununurra, *Zym*-carrying cv. Sunset is widely grown and in the epidemic years of 2009 and 2010 many crops of this cultivar became ZYMV-infected at high incidences and showed obvious symptoms. Recent information on natural ZYMV epidemics in cvs Dulong and Sampson is unavailable as they are rarely grown for agronomic reasons, but Coutts and Jones (2005) reported no infection in one cv. Sunset crop at Kununurra in 2003. The high ZYMV incidences in cv. Sunset in 2009 and 2010 suggest that isolates that behave like Knx-1 are widespread. At sub-tropical Carnarvon, cv. Sunset is also grown widely, but cvs Dulong and Sampson are not. In the 2010 epidemic year, ZYMV incidences of 4-50% were found in three crops of cv. Sunset at Carnarvon, but such data is lacking for other Sunset crops. Coutts and Jones (2005) reported a <1% infection in one of two cv. Dulong crops at Carnarvon in 2003. This is insufficient information from which to draw firm conclusions but the 2010 findings in cv. Sunset indicate that (i) there may be more virulent isolates than Cvn-1 at Carnarvon, or (ii)

when inoculation pressure is exceptionally high (as in 2010) *Zym* may be overcome by isolates that resemble Cvn-1. However, this did not occur in our Experiment 7 in a year of low inoculum pressure (2008).

Melon and green peach aphids colonised our field experiments at Kununurra and Medina, respectively. In addition, oat aphids were found colonising millet non-host barriers at Kununurra in 2009. In 2010 at Kununurra, winged rice root aphid (*R. rufi-abdominalis*) and both winged and wingless corn leaf aphid (*R. maidis*) were found on millet, and both winged and wingless melon and cowpea (*Aphis craccivora*) aphids on legume weeds (B. Coutts, unpubl.). At Carnarvon, winged green peach and turnip aphids were trapped in earlier studies (McLean et al., 1975). In 2010, colonising green peach aphids were found on cucurbits, winged green peach, oat and bluegreen (*Acyrthosiphon kondoi*) aphids on beans, winged oat aphid on tomato, winged and wingless black citrus aphid (*Toxoptera citricidus*) on lemon, and winged or wingless brown sowthistle aphid (*Uroleucon sonchi)* and green peach aphid on weeds (S. Broughton, pers com.). At Medina, migrant aphid species trapped in earlier studies were green peach, melon, bluegreen, cabbage (*Brevicoryne brassicae*), honeysuckle (*Hydaphis foeniculi*) and sowthistle (*Hyperomzus lactucae*) (Jones et al., 2005, 2006). Which cucurbit colonising (melon and green peach aphids) and transient non-colonising aphid species are responsible for the ZYMV spread occurring in each of the tropical (Kununurra), sub-tropical (Carnarvon) and Mediterranean (Perth) climatic zones where our field experiments were located was not determined, but would be important to establish in future studies.

For control of cucurbit viruses in WA, Coutts and Jones (2005) recommended an integrated disease management (IDM) approach involving use of healthy seed stocks; isolation of new cucurbit plantings from older ones; removing any potential alternative virus reservoirs (weeds, volunteer cucurbit plants, old finished or abandoned crops) during and between growing seasons; roguing of plants with virus symptoms; growing virus-resistant cultivars when available; and restricting movement and handling of plants to minimise SqMV spread. Inclusion of reflective mulch was appropriate for Carnarvon but not Kununurra because of the differing irrigation practices at these two locations. Unfortunately, limited attention has been paid to most of these recommendations by many cucurbit growers. The measures that were adopted were frequent roguing of crops by squash and zucchini growers and planting cultivars of zucchini and butternut pumpkin carrying *Zym,* and cucumber carrying *zym*. Also, rigorous removal of potential alternative virus reservoirs was adopted by several growers. However, most growers continue to use repeated spraying with insecticides to decrease the aphid populations that colonise cucurbits, although some do realise that insecticides are ineffective with non-persistently aphid-borne viruses like ZYMV. Also, growers still prefer to use cheaper black plastic rather than reflective mulch. The widespread ZYMV epidemics and crop damage experienced in recent years (2009 and 2010) have highlighted the need to adopt IDM more widely and comprehensively. For ZYMV, new additions to the IDM approach arising from our earlier research (Coutts and Jones, 2005) are the three cultural control measures: planting upwind of potential virus sources, manipulation of sowing date and deployment of tall non-host barriers of millet. Despite high incidences of ZYMV in pumpkin cultivars carrying *Zym* their use is still worthwhile at Kununurra because symptoms were milder than in cultivars without *Zym* and marketable yields were sufficient to justify their use. Among the zucchini cultivars carrying *Zym,* we identified cv. Sungold as having sufficient tolerance of fruit infection to be suitable for inclusion in the IDM approach despite its high susceptibility to infection, and two cultivars with partial ZYMV resistance (cvs Dunja and Paydirt) in which delayed

spread resulted in marketable yields that were sufficiently high for inclusion. In contrast, the *zym* resistance in cucumber held up well against isolate Knx-1 in the five cultivars evaluated, so these can all be included.

Acknowledgements

We thank Eva Gajda, Stuart Vincent, Gae Plunkett and staff at Carnarvon and Kununurra Research Stations for technical assistance, Mario D'Antuono for help with experimental design and statistical analyses and Cameron Brumley for aphid identifications. Horticulture Australia Limited provided financial support.

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Fig. 1. Locations where field experiments were done (●) in Western Australia.

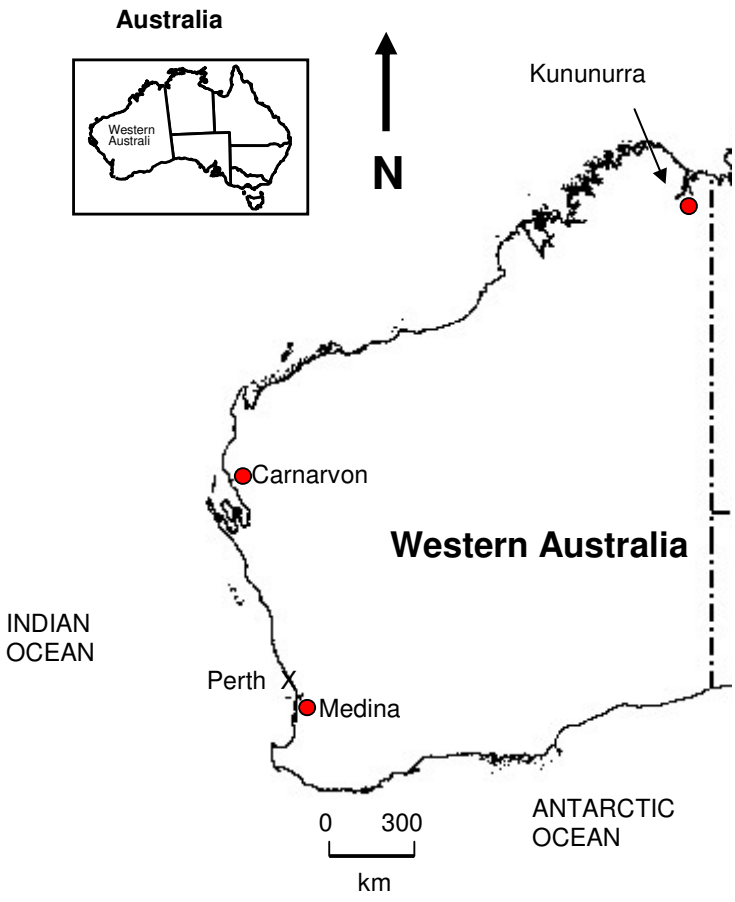


Fig. 2. Design of cultural control field experiments with Jarrahdale pumpkin cv. WA Grey in Kununurra in 2008 and 2009. a) Experiment 2, three pumpkin blocks with (i) no barrier, (ii) fallow barrier and (iii) non-host lablab barrier between pumpkin planting and ZYMV source; dotted line in (i) indicates top 25 m not used in statistical analyses. b) Experiment 1, two pumpkin plots arranged end-to-end with ZYMV source between them. c) Experiment 4; two blocks each with three pumpkin plots each sown 2 weeks apart, i) with, or ii) without millet barrier strips separating the plots. Grey shaded area indicates millet barrier strip. Prevailing wind blew from bottom to top (Experiments 1 and 2) and from left to right (Experiment 4).

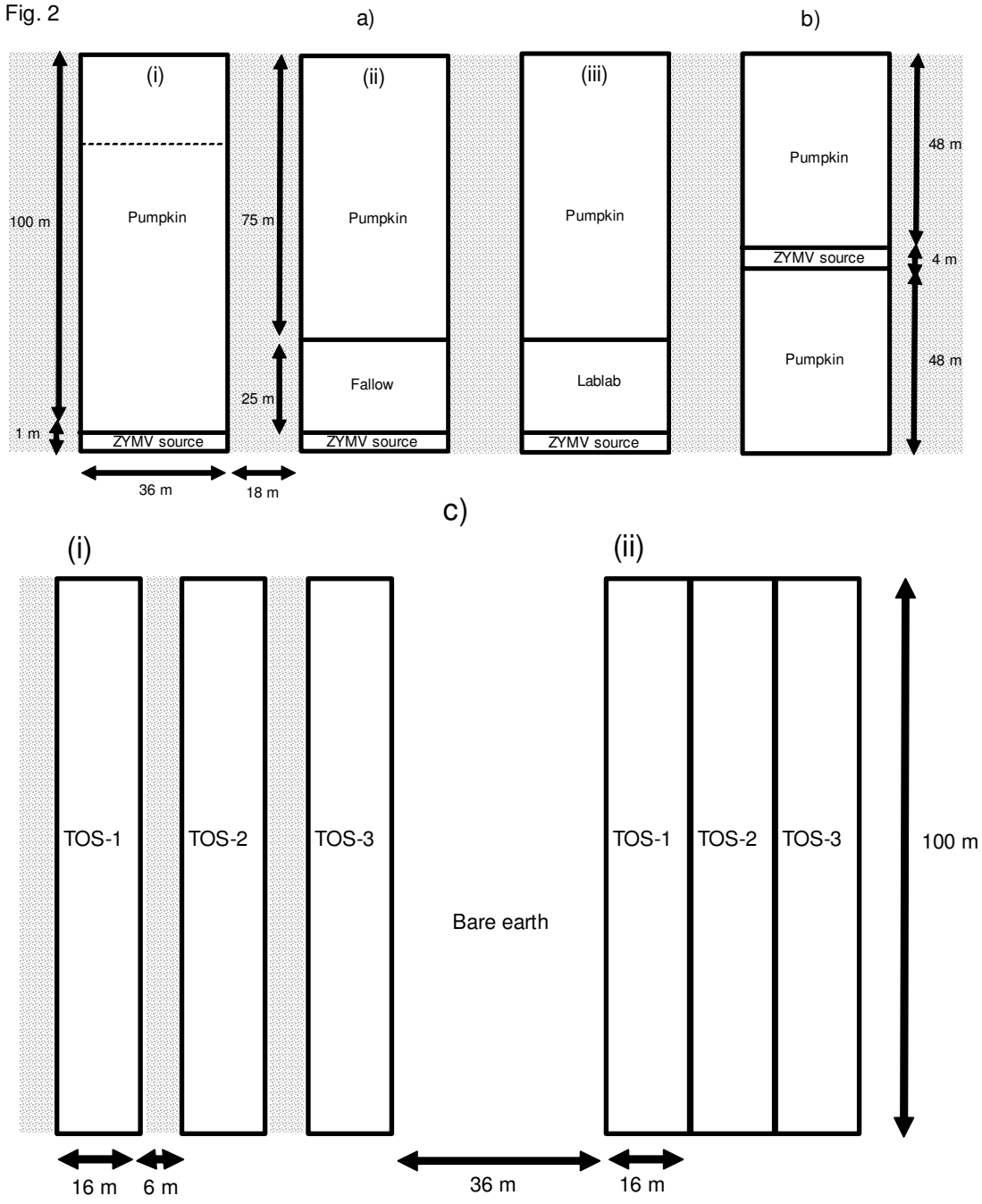


Fig. 3. a) Experiment 2 with pumpkin cv. WA Grey, 25m non-host barrier (lablab) in foreground, pumpkin plot behind with millet strips sown along both sides. b) Experiment 4 with pumpkin cv. WA Grey, first time of sowing pumpkin plot with millet barrier strip on both sides. c) Experiment 4, plots without any barrier separating them, first (left) and second (right) times of sowing. d) Experiment 6 with pumpkin cultivars, plot with three raised beds consisting of pumpkin plants in outer beds and zucchini infector plant focus (two transplants) in central bed; plot surrounded by 10 m bare earth buffer. e) Experiment 8 with zucchini cultivars, plot consisting of three raised beds of plants, infector plant focus (two transplants) in central bed, and surrounding oat buffer. f) Experiment 9 with zucchini cultivars, single row plots along raised beds and infector plant foci (single transplants) in bare earth bed between each plot. Arrows in d) – f) indicate positions of ZYMV infector plants.

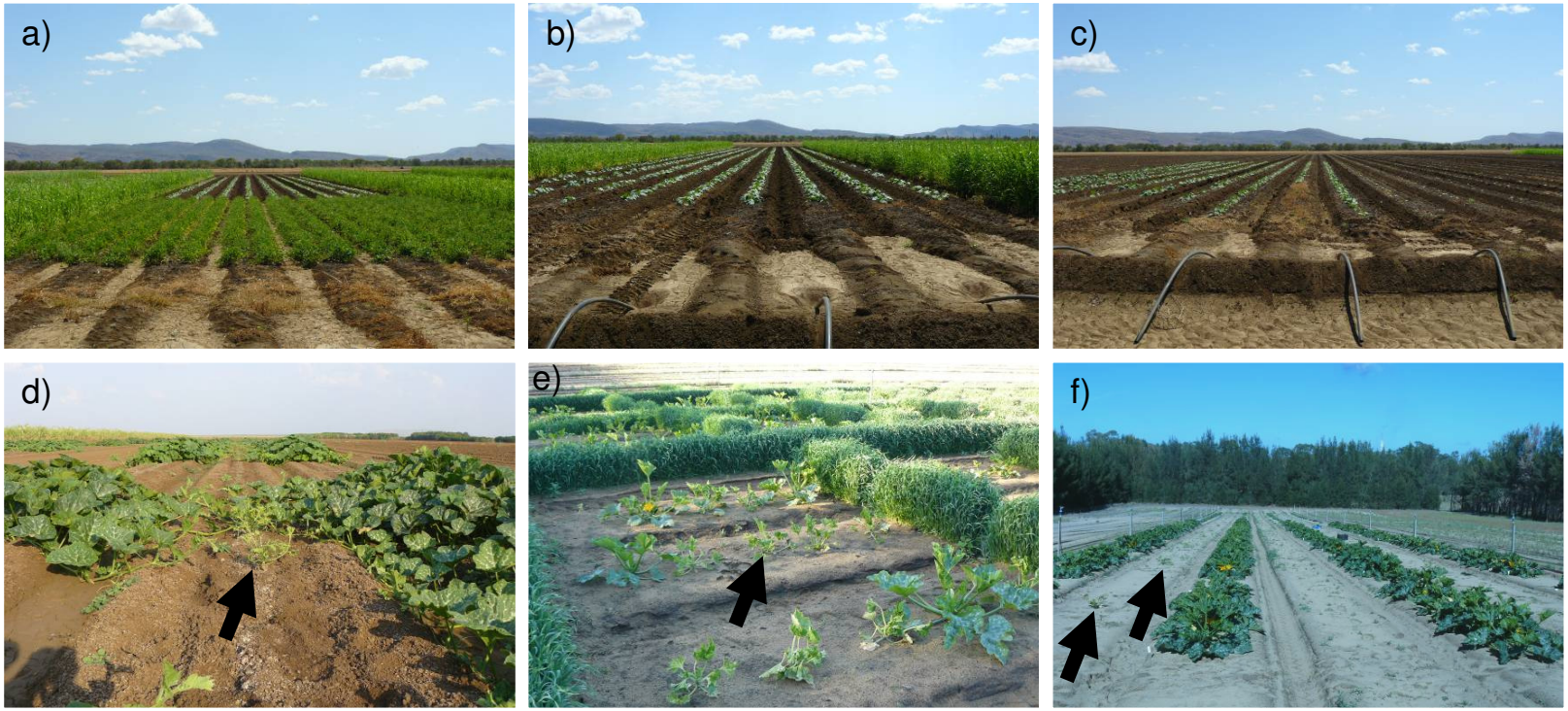
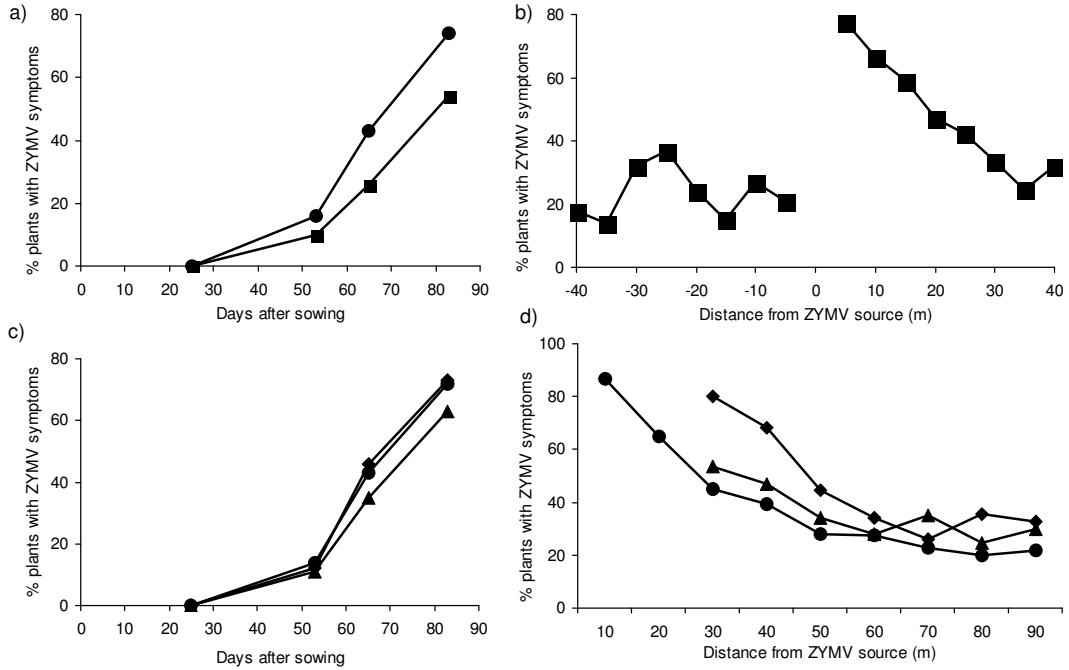


Fig. 4. Pathogen progress curves for pumpkin cv. WA Grey plants infected with ZYMV. a) Experiment 1 comparing planting upwind (■) with downwind (●), and c) Experiment 2 comparing separation from the ZYMV source by a 25 m wide fallow (♦) or non-host lablab (▲) barrier, or without any barrier (first 75 m only) (●). Gradients of ZYMV infection in pumpkin cv. WA Grey plants away from the introduced virus source at 83 days after sowing in b) Experiment 1, upwind (left) or downwind (right), and d) Experiment 2, with a 25 m wide fallow (♦) or non-host lablab (▲) barrier, or without any barrier (●).

Fig. 4



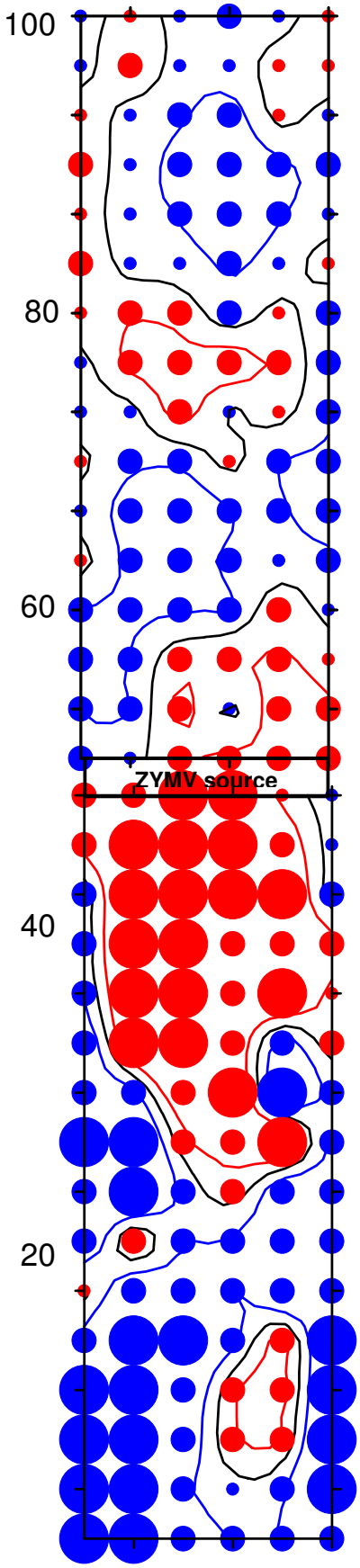


Fig. 5. Map of clustering indices for cumulative numbers of pumpkin cv. WA Grey plants infected with ZYMV in Experiment 1 at 65 days after sowing; upwind plot above and downwind plot below. Axes show distance in metres. Spots represent units denoting infection patches with v>0 (red) and infection gaps v<0 (blue). Small spots represent clustering indices of 0 to +/-0.99 (clustering below expectation), intermediate sized spots +/-1 to +/-1.49 (clustering slightly exceeds expectation) and large spots >1.5 or <-1.5 (clustering more that half as much again as expectation). Red lines enclosing patch clusters are contours of v=+1.5 and blue lines enclosing gap clusters are of v=-1.5. Black lines are zero-value contours, representing boundaries between patch and gap regions where the count is close to the overall sample mean.

Fig. 6

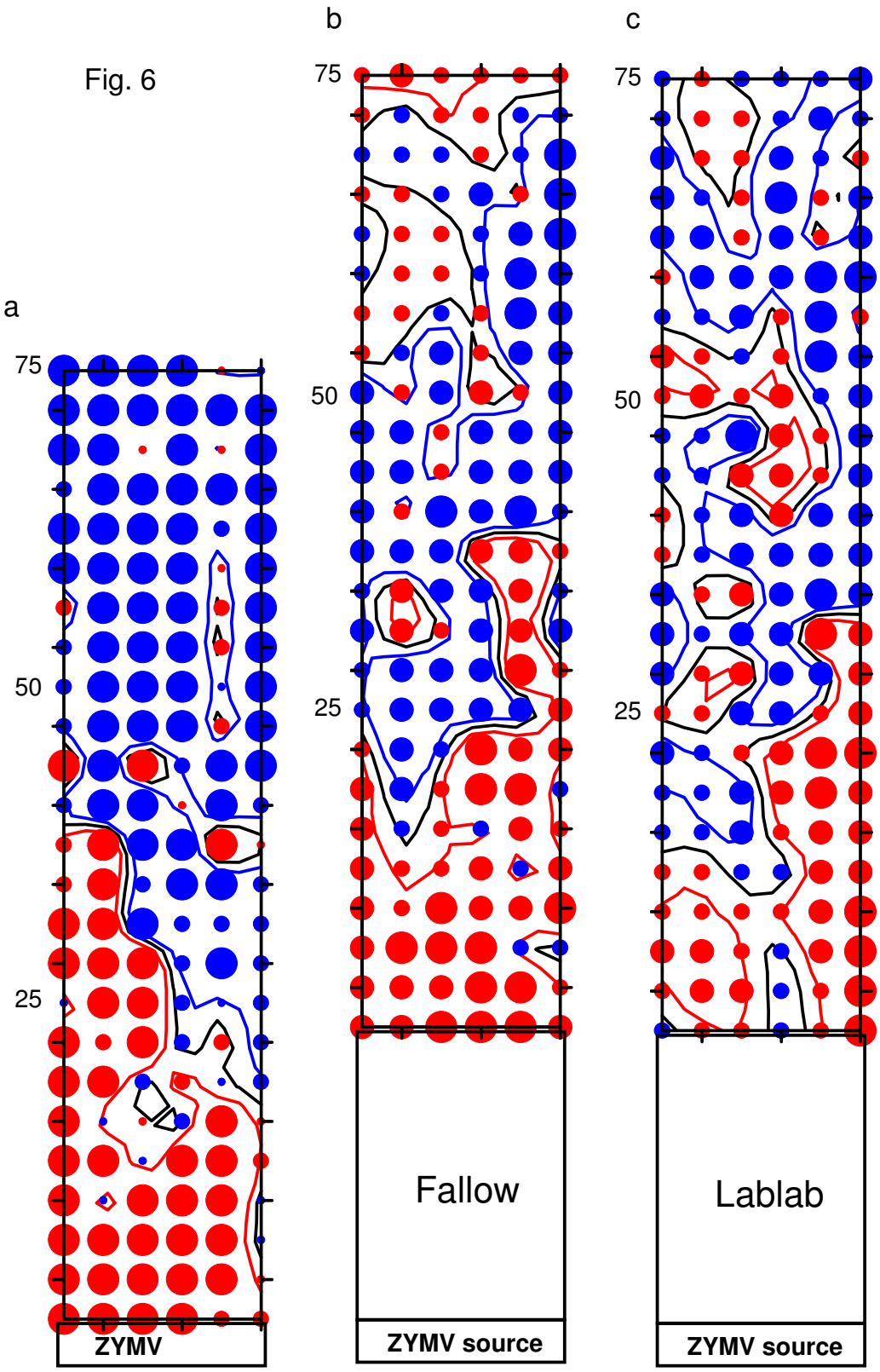


Fig. 6. Map of clustering indices for cumulative numbers of pumpkin cv. WA Grey plants infected with ZYMV in Experiment 2 at 65 days after sowing. a) No barrier (first 75 m), b) fallow barrier and c) non-host barrier of lablab. Symbols, contours and axes are as for Fig. 5.

Fig. 7. Pathogen progress curves for pumpkin cv. WA Grey plants infected with ZYMV in Experiment 4: time of sowing 1 with 18m wide tall non-host millet barrier (□) or without any barrier (■), and time of sowing 2 with 18m tall non-host millet barrier (○) and without any barrier (●).

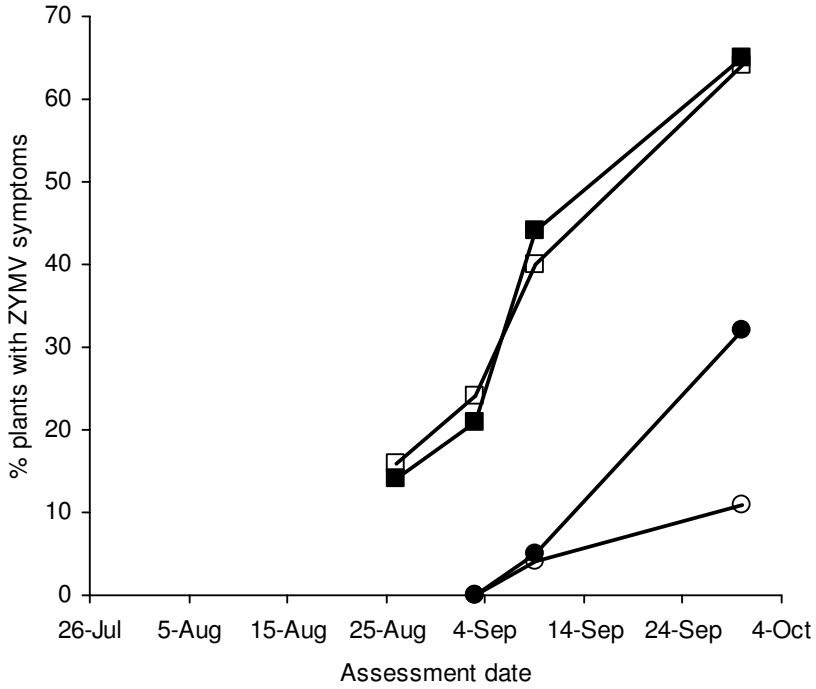


Fig. 8. Symptoms in five ZYMV-infected pumpkin cultivars from Experiment 6 with ZYMV isolate Knx-1: butternut pumpkin cvs Butternut Large and Sunset, and Jarrahdale cvs WA Grey, Sampson and Dulong. Cultivars carrying *Zym* are Sunset, Sampson and Dulong. a) cv. Sampson, mild leaf mottle (above) and faint fruit skin dimpling (below); b) cv. Dulong, mild leaf mottle (above) and faint fruit distortion with skin dimpling (below); c) cv. WA Grey, severe leaf mottle (above) and distorted, knobbly fruit (below); d) Butternut Large, severe leaf mottle (left) and distorted fruit with lumpiness and uneven colour (right); and e) cv. Sunset, leaf mottle (left) and mild fruit skin dimpling (right).

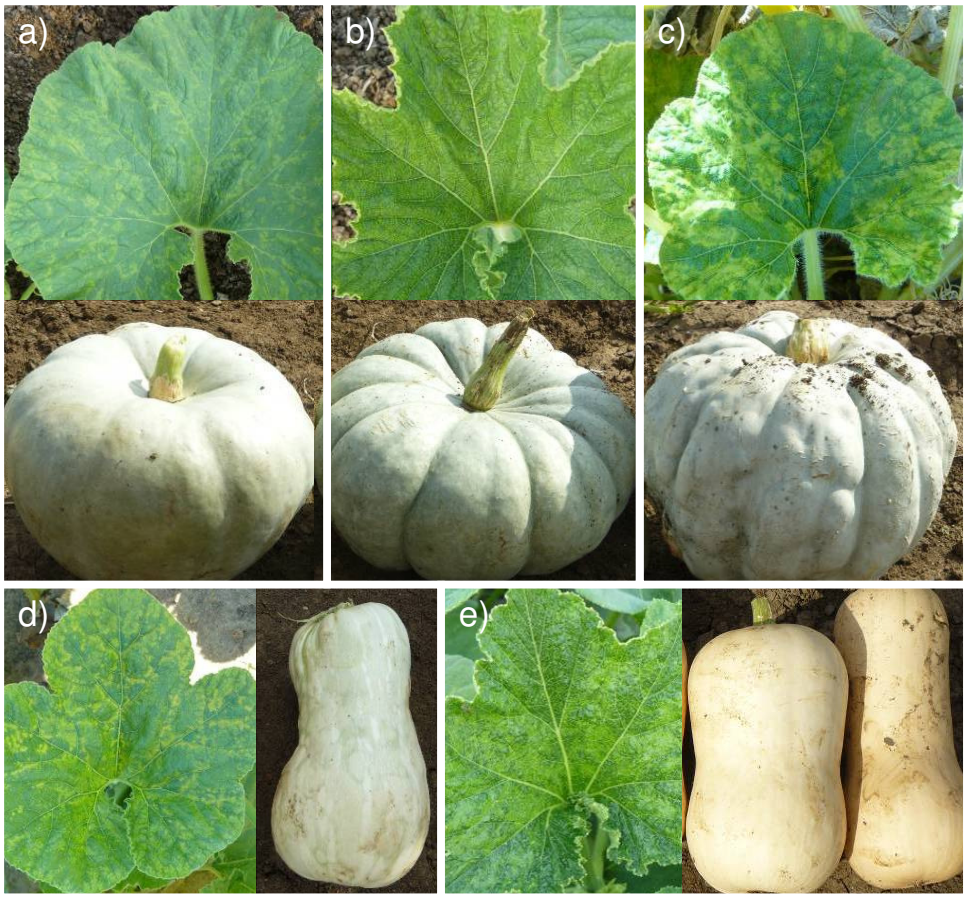


Fig. 9. Pathogen progress curves for cucurbit plants infected with ZYMV. a) Experiment 7 at Carnarvon in 2008 with butternut pumpkin cvs Butternut large (●) and Sunset (♦), and Jarrahdale pumpkin cvs WA Grey (▲), Sampson (■) and Dulong (**X**); cultivars carrying *Zym* are Sunset, Sampson and Dulong. b) Experiment 8 at Medina in 2007 with 11 zucchini cultivars without (cv. Blackjack) or with *Zym* (other 10 cultivars: Blackjack ■, Batal ♦, Black Adder ◊, Bond ●, Columbia □, Gold Coast Δ, Hummer *, Jaguar +, Midnight ▲, Sungold **X**, Top Gun ○). c) Experiment 9 at Medina in 2008 with six zucchini cultivars without (cv. Blackjack) or with *Zym* (other 5 cultivars: Blackjack ■, Amanda *, Dunja ♦, Midnight ▲, Paydirt ●, Sintia ○).

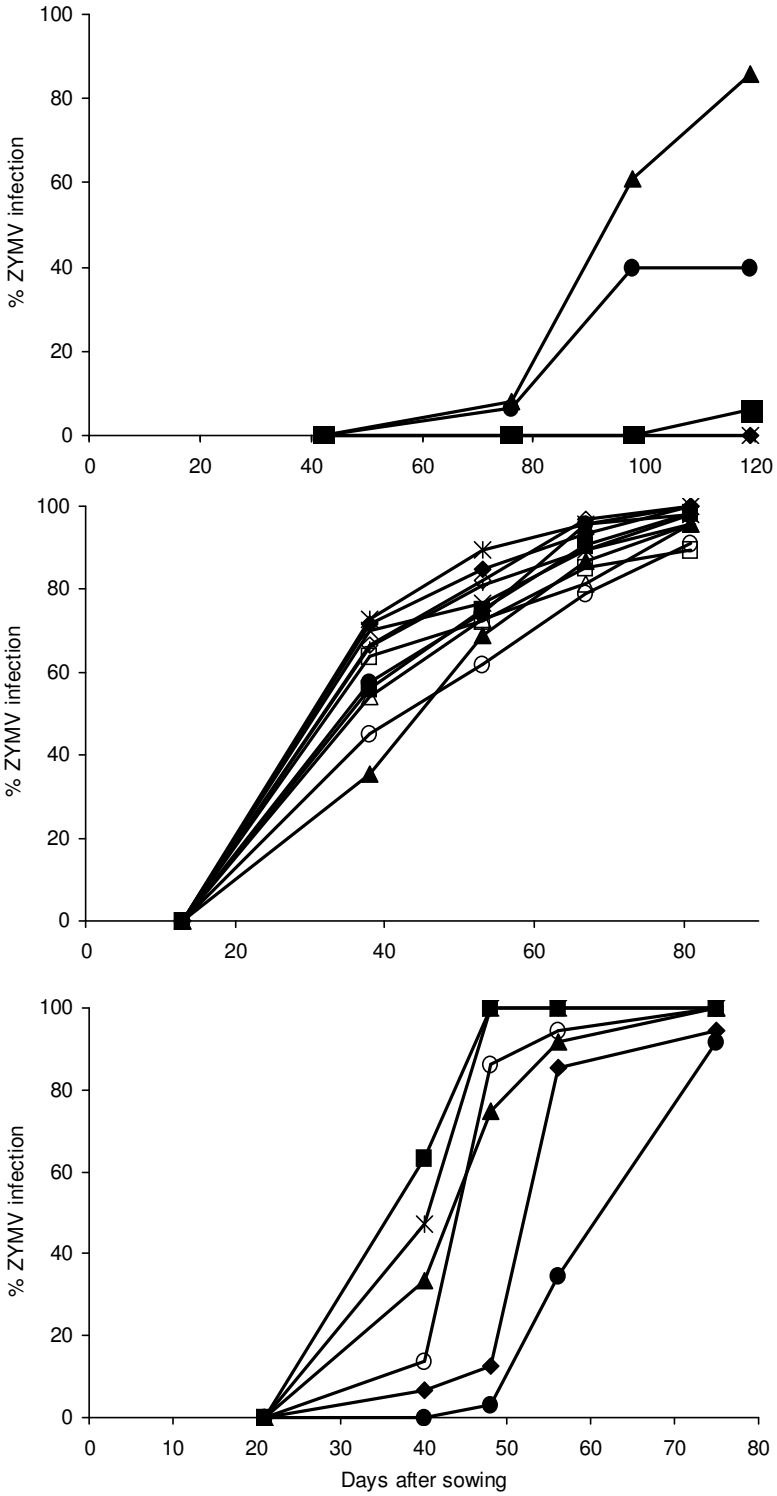


Fig. 10. Symptoms in infected zucchini cultivars without (cv. Blackjack) or with *Zym* (all other cultivars) from Experiments 7 and 8 with ZYMV isolate Knx-1. a) Cv. Blackjack, severe leaf mottle and leaf distortion (left), and fruit with mottle, distortion and knobbliness (right). b) Cv. Columbia, leaf mottle (left), severe fruit distortion and knobbliness (right). c) Cv. Gold Coast, severe leaf mottle and distortion (left), fruit with mottle and distortion (right). d) Cv. Sungold with mild leaf mottle (left) and faint rings on fruit (right). e) Cv. Dunja, symptomless leaf infection (left) and early formed fruit with no symptoms (right).

Table 1.
General details of the four field experiments on cultural control with Jarrahdale pumpkin cv. WA Grey at Kununurra.

Expt	Year	Cultural control	Plot size (m)	ZYMV source introduced (isolate)	Barrier present (type)[a]	Barrier area (m)[b]	Barrier planting date	Crop planting date	Infector plants introduced (DAS)[b]	Assessments (DAS)[b]
1	2008	Upwind/downwind	36 x 100	Yes (Knx-1)	No	-	-	18 July	25	53, 65, 83
2 (i)	2008	Barrier	36 x 100	Yes (Knx-1)	No	-	-	18 July	25	53, 65, 83
2 (ii)	2008	Barrier	36 x 75	Yes (Knx-1)	Yes (Fallow)	36 x 25	-	18 July	25	53, 65, 83
2 (iii)	2008	Barrier	36 x 75	Yes (Knx-1)	Yes (Lablab)	36 x 25	18 July	18 July	25	53, 65, 83
3 (i)	2009	Barrier	16 x 100	No	No	-	-	30 July	-	27, 34. 41, 61
3 (ii)	2009	Barrier	16 x 75	No	Yes (Fallow)	16 x 25	-	30 July	-	27, 34. 41, 61
3 (iii)	2009	Barrier	16 x 75	No	Yes (Borlotti bean)	16 x 25	6 July	30 July	-	27, 34. 41, 61
3 (iv)	2009	Barrier	16 x 75	No	Yes (Millet)	16 x 25	24 June	30 July	-	27, 34. 41, 61
4 (i)	2009	Time of sowing with barrier	16 x 50	No	Yes (Millet)	6 x 50	24 July	30 July, 13 Aug, 27 Aug	-	[c]26 Aug, 3 Sept, 9 Sept, 29 Sept
4 (ii)	2009	Time of sowing without barrier	16 x 50	No	No	-	-	30 July, 13 Aug, 27 Aug	-	26 Aug, 3 Sept, 9 Sept, 29 Sept

[a] Lablab (*Lablab purpureus*); Millet (*Pennisetum glacum*); Borlotti bean (*Phaselous vulgaris*).
[b] m, metres; DAS, days after sowing
[c] DAS not given as these differed because of staggered sowing dates.

Table 2.
General details of the six field experiments on host resistance.

Expt	Year	Location	Crop	ZYMV isolate introduced	No. of replications	Plot size (m)[a]	Planting date	Infector plants introduced (DAS) [a]	Assessments (DAS) [a]	Harvests (DAS) [a]
5	2006	Kununurra	Pumpkin	Knx-1	1	30 x 3.6	2 August	0	55, 76, 89	107
6	2007	Kununurra	Pumpkin	Knx-1	6	5 x 3.6	7 August	22	49, 77	92
7	2008	Carnarvon	Pumpkin	Cvn-1	5	5 x 3.6	6 August	23	42, 76, 98, 119	119
8	2007	Medina	Zucchini	Knx-1	6	2 x 4.5	28 September	13	38, 53, 67, 81	61, 63, 66, 68, 70, 74, 77, 81
9	2008	Medina	Zucchini	Knx-1	4	4 x 1.5	23 October	21	40, 48, 56, 75	48, 50, 53, 56, 60, 64, 71, 77
10	2008	Medina	Cucumber	Knx-1	4	4 x 1.5	17 October	27	46, 54, 62, 81	Not harvested

[a] m, metres; DAS, days after sowing.

Table 3.
Details of cucurbit cultivars used in the six field experiments on host resistance.

Cultivar	Type	Resistance present[a]	Seed producer	Expt.
Pumpkin – *Cucurbita maxima*				
Dulong	Jarrahdale	Z, P,W	South Pacific Seeds	5, 6, 7
Sampson	Jarrahdale	Z, P,W	Terranova Seeds	5, 6, 7
WA Grey	Jarrahdale	NR	Terranova Seeds	5, 6, 7
Pumpkin – *Cucurbita moschata*				
Butternut large	Butternut	NR	Terranova Seeds	5, 6, 7
Kens special	Kent	NR	South Pacific Seeds	6
Sunset	Butternut	Z, P	South Pacific Seeds	5, 6, 7
Zucchini – *Cucurbita pepo*				
Amanda	Green	Z	Clause	9
Batal	Lebanese	Z	Lefroy Valley	8
Blackjack	Green	NR	Yates	8, 9
Black Adder	Green	Z, W	Terranova Seeds	8
Bond	Green	Z, P	Fairbank's Selected Seeds	8
Columbia	Lebanese	Z, W	South Pacific Seeds	8
Dunja	Green	Z, P, W	Enza Zaden	9
Gold coast	Yellow	Z	Syngenta	8
Hummer	Green	Z, P, W	South Pacific Seeds	8
Jaguar	Green	Z, W	Lefroy Valley	8
Midnight	Green	Z, W	Syngenta	8, 9
Paydirt	Green	Z, W	Syngenta	9
Sintia	Green	Z, W	Clause	9
Sungold	Yellow	Z, W	Terranova Seeds	8
Top Gun	Green	Z, P, W	Charlcon Seeds	8
Cucumber – *Cucumis sativus*				
Camelot	Slicer	Z, P, W, C	Terranova Seeds	10
Gremlin	Slicer	Z, W, P	South Pacific Seeds	10
Khassib	Lebanese	Z, P, W, C	Rijk Swan	10
Lancelot	Slicer	Z, P, W, C	Terranova Seed	10
Nouran	Lebanese	Z	Rijk Swan	10
Pronto	Slicer	NR	Yates	10

[a] Resistance present according to seed producer. Z, ZYMV; W, WMV; P, PRSV; C, CMV; NR, no resistance.

Table 4.
Analyses of spatial spread data for Jarrahdale pumpkin cv. WA Grey plants infected with *Zucchini yellow mosaic virus* in Experiments 1 and 2.

Experiment[a]	Assessment (DAS)[b]	Total no. of plants assessed	Cumulative no. of plants with symptoms (%)	*Ia*[c]	Significance (P)
1, Upwind	53	789	77 (10)	1.18	n.s.
	65		209 (26)	1.43	<0.05
	83		426 (54)	1.17	n.s.
1, Downwind	53	748	118 (16)	3.15	<0.05
	65		323 (43)	3.56	<0.05
	83		554 (74)	2.32	<0.05
2 (i)[d]	53	1186	171 (14)	4.76	<0.05
	65		509 (43)	5.35	<0.05
	83		859 (72)	3.47	<0.05
2 (ii)	53	1223	146 (12)	2.85	<0.05
	65		563 (46)	3.87	<0.05
	83		894 (73)	3.74	<0.05
2 (iii)	53	1176	128 (11)	2.80	<0.05
	65		415 (35)	2.96	<0.05
	83		744 (63)	2.59	<0.05

[a] (i), no barrier; (ii), fallow barrier; (iii), lablab barrier.
[b] DAS, days after sowing.
[c] *Ia*, Spatial Analysis by Distance IndicEs Overall Mean Index of Aggregation for cumulative numbers of infected plants, where *Ia* = 1 indicates randomly arranged infected plants and *Ia* >1 indicates clustering of affected plants.
[d] first 75m of pumpkin plot used for spatial analysis.

Table 5.
Incidence of *Zucchini yellow mosaic virus* and average melon aphid numbers in three time of sowing plots of Jarrahdale pumpkin cv. WA Grey with and without millet barriers in Experiment 4.

Time of sowing plot	Millet barrier present	Total no. of plants	% of plants with ZYMV symptoms				Average no. of aphids on 20 pumpkin plants[a]			
			26 Aug	3 Sept	9 Sept	30 Sept	26 Aug	3 Sept	9 Sept	30 Sept
1	Yes	405	16	24	40	64	100	30	17	3
	No	400	14	21	44	65	100	72	14	3
2	Yes	418		0	4	11	<1	14	17	4
	No	340		0	5	32	<1	17	26	5
3	Yes	385			0	4				4
	No	434			0	7				5

[a] Aphids counted on the oldest leaf of each plant. Counts included both winged and non-winged aphids.

Table 6.
Zucchini yellow mosaic virus (ZYMV) incidence, yield data, susceptibility and sensitivity rankings and predominant symptoms in pumpkin cultivars in Experiments 5-7.

A. ZYMV incidence, susceptibility and leaf sensitivity rankings and predominant plant symptoms.

Pumpkin cultivar	ZYMV incidence (%)[a]				AUPPC[b]	Susceptibility ranking[c]	Sensitivity Ranking[d]	Predominant symptoms[e]
Experiment 5[f]	55 DAS	76 DAS	89 DAS					
Butternut Large	100	100	100			HS	4	sm, ld, st
Sunset	100	100	100			HS	3	m, ld
WA Grey	100	100	100			HS	5	sm, ld, st
Dulong	0	30	40			MR	2	mm
Sampson	0	80	80			HS	2	mm
Experiment 6	49 DAS	77 DAS						
Butternut Large	90 (77.4) bc	100				HS	5	sm, lb, ld, st
Sunset	83 (66.3) b	100				S	3	m, mld
WA Grey	97 (84.3) c	100				HS	5	sm, lb, ld, st
Dulong	60 (51.0) a	86				MR	2	mm
Sampson	97 (85.8) c	100				HS	2	mm
Kens Special	92 (76.6) bc	100				HS	3	m, ld
P	<0.001	ns						
d.f.	25							
l.s.d.[g]	13.39							
Experiment 7[h]	42 DAS	76 DAS	98 DAS	119 DAS	AUPPC			
Butternut Large	0	6 (14.5) b	41 (39.6) b	32 (34.6) b	1397 b	S	3	m, ld, st
Sunset	0	0 (0.6) a	0 (0.6) a	0 (0.6) a	0 a	HR	-	-
WA Grey	0	5 (13.7) b	61 (51.6) c	93 (75.3) c	2446 c	HS	3	m, ld, st
Dulong	0	0 (0.6) a	0 (0.6) a	0 (0.6) a	0 a	HR	-	-
Sampson	0	0 (0.6) a	0 (0.6) a	4 (11.8) a	63 a	R	1	ns
P		0.014	<0.001	<0.001	<0.001			
d.f.		8	8	8	8			
l.s.d.		9.73	5.85	21.13	550.5			

[a] All percentage incidence data were angular transformed before analysis, values in parentheses. Values followed by the same letter are not significantly different at P<0.05.
[b] AUPPC, area under the pathogen progress curve.
[c] Susceptiblity ranking: HS, highly susceptible; S, susceptible; MR, moderately resistant; R, resistant.
[d] Sensitivity ranking (1-5): 1, symptomless infection to 5, extremely severe symptoms.
[e] Symptom codes: m, mottle; sm, severe mottle; vmm, very mild mottle; lb, leaf bubbling; ld, leaf distortion; mld, mild leaf distortion; st, plant stunting.
[f] Data based on 1 replicate only, no statistics done on virus incidence.
[g] l.s.d., least significant difference.
[h] Data based on 3 replicates only.

B. Yield data, fruit sensitivity rankings and predominant fruit symptoms.

Pumpkin cultivar	Total yield (t/ha)	Marketable yield (t/ha)[a]	Virus affected yield (t/ha)[a]	Unmarketable yield (t/ha) - other causes[a]	% fruit marketable	% fruit virus affected	% fruit unmarketable - other causes	Marketable fruit wt (kg/fruit)	Sensitivity ranking[b]	Predominant symptoms[c]
Experiment 5[d]										
Butternut Large	15.1	0.5	11.7	2.9	3	77	20	0.9	3	d, l
Sunset	14.6	13.9	0	0.7	92	0	8	1.1	-	-
WA Grey	3.6	0	2.2	1.4	0	50	50	0	5	sd, k, rs
Dulong	27.1	15.6	5.6	5.9	50	17	33	3.6	2	md
Sampson	26.7	23.6	0	2.9	76	0	24	5.9	-	-
Experiment 6										
Butternut Large	22.7 c[e]	1.8 ab	19.4 d	1.5 ab	7 ab	84 c	9 ab	1.1 a	3	d, l, rs, sc
Sunset	27.6 d	10.1 c	15.1 c	2.4 b	35 d	53 ab	12 a	1.2 a	2	dp
WA Grey	10.5 a	1.5 ab	8.8 ab	0.2 a	10 bc	88 c	2 a	3.9 b	4	sd, k, rs
Dulong	18.9 b	3.4 b	13.1 c	2.4 b	17 c	67 b	16 b	3.5 b	2	md, dp
Sampson	33.1 e	15.3 d	12.6 bc	5.1 c	36 d	46 a	18 b	5.5 c	2	dp
Kens Special	9.8 a	0.2 a	4.5 a	5.0 c	1 a	54 ab	45 c	1.1 a	2	dp, sc
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001		
d.f.	20	20	20	20	20	20	20	20		
l.s.d.[f]	3.37	3.06	4.01	1.87	8.8	15.3	11.5	1.17		
Experiment 7[g]										
Butternut large	18.3 a	2.1 a	10.1 b	6.1 a	14 a	52 b	34 ab	1.0 a	2	dp, sc, rs
Sunset	34.1 b	16.0 b	0 a	18.0 b	50 c	0 a	49 b	1.3 a	-	-
WA Grey	48.2 bc	12.4 b	28.3 c	7.6 a	26 a	59 b	14 a	3.9 b	3	d, l
Dulong	41.8 bc	11.4 ab	0 a	30.4 c	25 a	0 a	74 c	4.0 b	-	-
Sampson	55.3 c	30.4 c	4.0 ab	20.7 b	52 c	7 a	41 b	5.5 c	2	dp
P	0.005	0.002	<0.001	0.001	0.005	<0.001	0.002	<0.001		
d.f.	8	8	8	8	8	8	8	8		
l.s.d.	15.57	9.58	8.67	9.09	18.0	19.8	21.3	0.56		

[a] Fruit assessments: marketable, no visible virus symptoms; virus symptom-affected, noticeable virus symptoms including skin dimpling, lumpy/knobbly and distortion; unmarketable, fruit immature, split or insect damaged.
[b] Sensitivity ranking (1-5): 1, symptomless infection to 5, extremely severe symptoms.
[c] Fruit symptoms codes: d, distortion; md, mild distortion; sd, severe distortion; l, lumpy; k, knobbly; dp, skin dimpling; sc, uneven skin colouring; rs, reduced size; ns, no symptoms
[d] Data based on 1 replicate only, no statistics done on virus incidence.
[e] Values followed by the same letter are not significantly different at P<0.05.
[f] l.s.d., least significant difference.
[g] Data based on 3 replicates only.

Table 7.
Zucchini yellow mosaic virus (ZYMV) incidence, yield data, susceptibility and sensitivity rankings and predominant symptoms in zucchini cultivars in Experiments 7 and 8.

A. ZYMV incidence, susceptibility and leaf sensitivity rankings and predominant plant symptoms.

Zucchini cultivar	ZYMV incidence (%)[a]				AUPPC[b]	Susceptibility ranking[c]	Sensitivity Ranking[d]	Predominant symptoms[e]
Experiment 8	38 DAS	53 DAS	67 DAS	81 DAS				
Batal	74	92	97	100	3687 cd	HS	3	m, ld, st
Blackjack	73	86	99	100	3136 ab	HS	4	sm, ld, st
Black Adder	57	57	92	100	3687 cd	HS	4	sm, ld, st
Bond	65	81	98	99	3440 bcd	HS	3	m, ld, st
Columbia	65	76	90	94	3258 abc	HS	2	m, mld
Gold coast	54	80	86	98	3182 ab	HS	4	sm, ld, st
Hummer	77	94	98	100	3788 d	HS	3	m, ld, st
Jaguar	67	88	94	98	3482 bcd	HS	2	m, mld
Midnight	40	78	93	98	3128 ab	HS	4	sm, ld, st
Sungold	76	81	96	99	3494 bcd	HS	2	mm
Top Gun	40	62	83	96	2891 a	HS	2	mm
P	ns	ns	ns	ns	0.013			
d.f.					50			
l.s.d.[f]					499.8			
Experiment 9	40 DAS	48 DAS	56 DAS	75 DAS				
Amanda	47 (43.4) cd	100 (89.4) c	100 (89.4) c	100	3714 d	HS	4	sm, ld, st
Blackjack	65 (53.5) d	100 (89.4) c	100 (89.4) c	100	3921 d	HS	4	sm, ld, st
Dunja	4 (10.8) a	10 (18) a	92 (73.4) b	98	2238 b	MR	1	ns
Midnight	27 (31.4) bc	85 (67.2) b	96 (77.8) bc	100	3221 c	S	2	mm, mld
Paydirt	0 (0.2) a	1 (5.3) a	34 (35.4) a	97	1365 a	R	1	ns
Sintia	8 (15.9) ab	92 (73.9) bc	97 (80.0) bc	100	3094 c	MR	3	m, ld
P	<0.001	<0.001	<0.001	ns	<0.001			
d.f.	15	15	15		15			
l.s.d.	19.06	19.10	14.48		461.4			

[a] All percentage incidence data were angular transformed before analysis, values in parentheses. Values followed by the same letter are not significantly different at P<0.05.
[b] AUPPC, area under the pathogen progress curve. Values followed by the same letter are not significantly different at P<0.05.
[c] Susceptiblity ranking: HS, highly susceptible; S, susceptible; MR, moderately resistant; R, resistant.
[d] Sensitivity ranking (1-5): 1, symptomless infection to 5, extremely severe symptoms.
[e] Symptom codes: m, mottle; sm, severe mottle; vmm, very mild mottle; ld, leaf distortion; mld, mild leaf distortion; st, plant stunting.
[f] l.s.d., least significant difference.

B. Yield data, fruit sensitivity rankings and predominant fruit symptoms.

Zucchini cultivar	Total yield (g/plant)	Marketable yield (g/plant) [a]	Virus symptom affected yield (g/plant) [a]	Unmarketable yield (g/plant) [a]	Total no. fruit/plant	% fruit marketable /plant	% fruit virus affected /plant	% fruit unmarketable/ plant	Sensitivity ranking [b]	Predominant symptoms [c]
Experiment 8										
Batal	1811 de[d]	682 cd	749 bc	380 bcd	4.0 d	28 ab	41 ab	31 c	4	m, k
Blackjack	137 a	21 a	93 a	23 a	2.8 cd	22 ab	59 bc	19 b	5	m, k ,ms, r
Black Adder	462 ab	95 ab	308 ab	59 a	1.3 a	22 ab	66 c	12 ab	3	m
Bond	802 abc	216 ab	378 ab	208 abc	1.9 ab	24 ab	58 bc	18 b	4	m, k
Columbia	3490 f	833 de	2203 e	453 cd	6.8 e	23 ab	61 bc	16 b	5	m, k, r
Gold Coast	929 abc	454 bcd	406 ab	69 a	3.3 d	38 b	57 bc	5 b	3	m, r
Hummer	1126 bcd	226 ab	697 bc	202 abc	3.0 cd	15 a	74 bc	11 ab	4	m, k, r
Jaguar	1453 cd	219 ab	954 c	281 abcd	4.1 d	14 a	75 c	11 ab	4	m, k, r
Midnight	1511 cd	371 abc	781 bc	358 bcd	3.9 d	22 ab	61 bc	16 b	4	m, k
Sungold	1808 de	1098 e	565 abc	145 ab	6.9 e	57 c	22 a	21 bc	2	r
Top Gun	2526 e	470 bcd	1537 d	519 d	6.2 e	20 a	61 bc	19 b	4	m, d, r
P	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	0.002	0.004		
l.s.d. [e]	822.9	380.3	494.8	270.3	1.28	17.3	23.1	11.1		
d.f.	50	50	50	50	50	50	50	50		
Experiment 9										
Amanda	1842 a	224 a	1489 b	129 a	10.1 abc	8 a	85 de	6 a	4	m, r, k
Blackjack	1823 a	229 a	1406 b	203 a	9.4 ab	9 a	82 d	9 a	4	m, k
Dunja [f]	3689 c	1983 d	318 a	1388 c	10.9 bc	61 d	13 a	26 b	3	d, r
Midnight	2634 b	101 a	2380 d	153 a	9.0 a	3 a	91 e	5 a	4	m, r, k
Paydirt [f]	4063 c	1137 c	1865 c	1061 b	11.2 c	34 c	45 b	21 b	3	d
Sintia	2780 b	659 b	1885 c	236 a	12.9 d	24 b	63 c	13 a	3	d, r
P	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	<0.001		
l.s.d.	484.6	290.7	372.5	303.0	1.71	7.8	7.4	8.2		
d.f.	15	15	15	15	15	15	15	15		

[a] Fruit assessments: marketable, no visible virus symptoms; virus symptom-affected, noticeable virus symptoms including skin dimpling, lumpy/knobbly and distortion; unmarketable, fruit immature, split or insect damaged.
[b] Sensitivity ranking (1-5): 1, symptomless infection to 5, extremely severe symptoms.
[c] Fruit symptoms codes: d, skin dimpling; k, knobbly; m, skin mottle; ms, misshapen; r, ringspots.
[d] Values followed by the same letter are not significantly different at $P<0.05$.
[e] l.s.d., least significant difference.
[f] Early fruit formed had no symptoms, fruit sensitivity ranking and predominant fruit symptoms refer to late formed fruit.

SECTION 2

Zucchini yellow mosaic virus: biological properties, detection procedures and comparison of coat protein gene sequences.

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(Draft of paper submitted for publication in Archives of Virology, 2011)

Abstract

Between 2006 and 2010, 5324 samples from at least 34 weed, two cultivated legume and 11 native species were collected from three cucurbit growing areas in tropical or sub-tropical Western Australia. Two new alternative hosts of ZYMV were identified, the Australian native cucurbit *Cucumis maderaspatanus,* and the naturalised legume species *Rhyncosia minima*. Seed transmission of ZYMV (0.7%) was found in seedlings grown from seed collected from ZYMV-infected fruit of zucchini (*Cucurbita pepo*) but not of pumpkin (*C. maxima* and *C. moschata*). Leaf samples from symptomatic cucurbit plants collected from fields in five cucurbit growing areas in four Australian states were tested and the coat protein (CP) gene of the ZYMV isolates found sequenced. When 42 complete CP nucleotide (nt) sequences from the new isolates were compared to those of 101 other complete CP nt sequences from five other continents, phylogenetic analysis of the 143 ZYMV sequences revealed three distinct groups (A, B and C), and four subgroups within A (I-IV). The new Australian sequences fitted within groups A and B, and within A into subgroups I and II. They grouped according to their collection location. The 16 new sequences from one isolated location in tropical northern Western Australia were in group B with 85.6-89.1% nt identity to sequences from Singapore, Reunion Island and Vietnam. In contrast, the three sequences from the Northern Territory fitted into A-II with 94.6-99.0% nt identities with isolates from United States, Iran, China and Japan. The 23 new sequences from the central west coast and two east coast locations all fitted into A-I, with 95.9-98.9% nt identities to sequences from Europe and Japan. These findings suggest (i) at least three separate ZYMV introductions into Australia and (ii) there are few changes to local isolate CP sequences following their establishment in remote growing areas. In pumpkin, isolates from A-I, A-II and B overcame (*C. moschata*) or partially overcame (*C. maxima*) the *Zym* gene for ZYMV resistance, and most isolates induced chlorotic symptoms in inoculated leaves of *Chenopodium quinoa*, but an isolate from A-II caused symptomless infection in it. One of three commercial ZYMV-specific antibodies did not detect all Australian isolates reliably by ELISA. A multiplex real-time PCR using dual-labelled probes was developed which distinguished between Australian ZYMV isolates belonging to phylogenetic groups A-I, A-II and B.

Introduction

Zucchini yellow mosaic virus (ZYMV; family *Potyviridae*, genus *Potyvirus*) is transmitted non-persistently by a number of colonising and non-colonising aphid species [31]. It is one of more than 30 viruses infecting cucurbit crops and occurs worldwide. Infected cucurbit plants develop a range of foliage symptoms including mosaic, leaf deformation and blistering and plant stunting. Fruits formed on infected plants develop knobbly areas and are malformed and discoloured. Early season infection of cucurbit crops cause up to 100% yield losses and up to 95% losses in marketable fruit [e.g., 2, 4, 10, 14, 16]. Although ZYMV epidemics are common in many cucurbit growing areas of the world, very few naturally occurring alternative hosts have been found, their occurrence is often sporadic and ZYMV incidence in them often low even when inoculum pressure is very high. They include volunteer cucurbit crop plants, wild cucurbits [e.g., 1, 9, 13, 38], non-cucurbitacous weed species from more than eight families (e.g., 1, 14, 36, 38, 40, 46] and some ornamental plants [6, 7]. Seed transmission occurs occasionally at low levels in squash and zucchini (*Cucurbita pepo*) and Delica type butternut squash (*Cucurbita maxima*) [12, 16, 25, 40, 42, 45, 50, 51], but has not been reported in melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita moschata*), or cucumber (*Cucumis sativus*) [e.g., 14, 19, 21, 39, 40, 41].

Differences in biological properties between ZYMV isolates (e.g. symptom expression, host range, aphid transmission) reflect changes in the nucleotide (nt) sequences in its coat protein (CP), helper component-protease (HC-Pro) or P3 protein genes [17]. Serological differences found using monoclonal antibodies raised against ZYMV isolates from different geographical locations indicated up to 15 distinct serotypes [14]. In initial studies with 47 partial CP nt ZYMV sequences, Desbiez et al. [15] found isolates clustered into two groups, A consisting of three sub-groups that included isolates from Europe, North America and Eastern Asia, and B, which was highly divergent from A, was limited to an isolate from Reunion Islands. Subsequently, Zhao et al [54] compared 39 complete ZYMV CP nt sequences and suggested three groups (I-III), I worldwide, II containing isolates only from Asia and III containing isolates only from China. When Simmons et al. [44] compared 55 complete CP nt sequences, they suggested combining I with II, but their analyses omitted new sequences from Iran and Vietnam. Ha et al. [26] analysed 61 complete nt sequences and suggested three main clusters, I was worldwide, II comprising Reunion Island, Singapore and Vietnam isolates, and III consisting of Vietnam and China isolates. When Bananej et al. [3] compared 208 partial sequences (231 nt) two main groups (A and B) were suggested, A was a worldwide group with three sub-groups within it and B comprised isolates from China, Reunion Island, Singapore and Vietnam. Thus, these phylogenetic analyses all revealed two or three major groups.

In Australia, ZYMV was first detected in 1984, but symptomatic cucurbit plants were recorded in Western Australia in 1973 [5, 24, 25, 35]. Subsequently, it became widespread in cucurbit growing areas in tropical Kununurra (Western Australia, WA), Ayr (Queensland, Qld) and Darwin (Northern Territory, NT), subtropical Carnarvon (WA), and temperate Swan Hill (Victoria, Vic). These growing areas have seasonal breaks in cucurbit production of up to 4 months but, despite this break, crops often become infected with individual crop incidences and yield losses of up to 100% [9,

10. 25, 30, 34]. Aphid transmission of ZYMV often involves *Aphis gossypii* (melon aphid) and *Myzus persicae* (green peach aphid) [10, 25]. Seed transmission of ZYMV was found in squash (*C. pepo*), but not in 'Jarrahdale' pumpkin (*C. maxima*) or rockmelon (*C. melo*) [24, 25]. Alternative hosts found to be ZYMV-infected were wild prickly paddy melon (*Cucumis myriocarpus*), wild Afghan melon and volunteer watermelon (both *Citrullus lanatus*) [3, 9, 35]. Australian-bred pumpkin cultivars (*C. moschata* and *C. maxima*) with the *Zym* gene for ZYMV resistance are grown [9, 25, 27, 28, 29], and control involves an integrated virus disease management approach [9, 10, 34]. Currently, no full length CP nt sequences are available for Australian ZYMV isolates.

The aims of this study were to (i) identify additional alternative hosts of ZYMV, (ii) provide additional data on ZYMV seed transmission, (iii) compare the complete CP nt sequences of 42 Australian ZYMV isolates with those of 101 others from five different continents available in GenBank, (iv) determine if there are biological differences in host responses to infection with ZYMV isolates from different locations, and (v) develop a multiplex real-time PCR assay to accurately detect and distinguish between different Australian isolates.

Materials and Methods

Plants, inoculations, virus isolates and antisera

Culture and test plants were grown in insect-proof, air-conditioned glasshouses maintained at 18-22^{o}C. Plants of 'butternut' pumpkin (*C. moschata*), 'Jarrahdale' pumpkin and zucchini, and virus indicator hosts were grown in steam-sterilised soil, sand and peat mix (1:1:1) in pots. For sap inoculation, infected leaves were ground in 0.1M phosphate buffer, pH 7.2, and the sap mixed with celite before being rubbed onto leaves. All isolates were maintained by sap inoculation to zucchini cv. Blackjack.

In 2005-2009, ZYMV isolates from leaf samples with symptoms (mottle, blistering, leaf distortion) were collected from naturally infected zucchini, 'Jarrahdale' and 'butternut' pumpkin, watermelon, rockmelon and cucumber crop plants growing in five of the major Australian cucurbit growing areas: Carnarvon (WA), Kununurra (WA), Darwin (NT), Ayr (Qld) and Swan Hill (Vic) (Fig. 1). In addition, three isolates were from freeze-dried cucurbit leaves collected at Carnarvon in 2001 and 2003, and another two from Kununurra in 2001 [9]. ZYMV isolates Knx-1 and Cvn-1, and *Papaya ringspot virus* (PRSV) isolate Qld1 were from previous work [9, 10] and freeze-dried leaves containing *Watermelon mosaic virus* (WMV) obtained from Loewe Biochemica, Germany were used as positive controls in enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) assays. Polyclonal antisera to ZYMV were obtained from Prime Diagnostics, Netherlands, DSMZ, Germany and Loewe Biochemica, Germany; polyclonal antisera to PRSV and WMV from Loewe Biochemica, Germany; and generic monoclonal antibody specific to potyviruses from Agdia Inc, USA.

Enzyme-linked immunosorbent assay

Leaves were extracted singly or in groups of 2-10 in phosphate-buffered saline (PBST) (10mM potassium phosphate, 150mM sodium chloride), pH 7.4, containing 5ml/L of Tween 20 and 20g/L of polyvinyl pyrrolidone, using a mixer mill (Retsch, Germany). Sample extracts were tested with individual viruses by double-antibody

sandwich ELISA [8]. To test for potyviruses in general, leaf samples were extracted in 0.05M sodium carbonate buffer pH 9.6 and tested using the antigen-coated indirect ELISA protocol of Torrance and Pead [52]. Each sample was tested in duplicate wells in microtitre plates, and appropriate infected and healthy leaf samples were included in paired wells as controls. The substrate used was 1.0 mg/mL of *p*-nitrophenyl phosphate in 100ml/L of diethanolamine, pH 9.8. Absorbance values (A_{405}) were measured in a microplate reader (Bio-Rad Laboratories, USA). Absorbance values regarded as positive were always at least 10 times those of healthy sap. Virus incidence was estimated from grouped sample results using the formula of Gibbs and Gower [18].

Alternative host survey

Between 2006-2010, leaf and shoot samples were collected from naturalised weed, cultivated legume and native species growing in cucurbit growing regions in Western Australia: Carnarvon, Broome, and Kununurra (Fig. 1). Native plant samples were collected under licences from Western Australian Department of Environment and Conservation (SW011694 and SW010823). Collections were along roadside verges, fence lines and irrigation channels, within and along the edges of ZYMV-infected cucurbit crops, and in native bushland near cucurbit crops. Leaves with and without potential viral symptoms were sampled from different plants at these sites. Extracts from samples with symptoms were tested individually or samples without symptoms were tested in groups of 2-10 by ELISA. Initially, all samples were tested for presence of potyviruses in general. Samples that tested potyvirus positive were then retested for presence of ZYMV, PRSV and WMV. Samples that tested positive for ZYMV were then tested by RT-PCR using ZYMV-specific primers and sequenced.

Seed transmission

Seed of 'butternut' pumpkin cv. Butternut Large and 'Jarrahdale' pumpkin cv. WA Grey was obtained from mature fruits infected with ZYMV isolate Knx-1 grown in field experiments at Kununurra (2007 and 2008) [10], or small plots at South Perth (2007) (Fig. 1). Seed of zucchini cv. Blackjack from fruit infected with ZYMV-isolate Cvn-1 was from a field experiment at Carnarvon (2008) [10]. Seeds were germinated and the first true leaf from each seedling sampled and tested for ZYMV in groups of 10 by ELISA. Individual infected seedlings were then identified by retesting single plant samples by ELISA. The ZYMV positive seedlings were then tested by RT-PCR using ZYMV specific primers and sequenced.

Inoculations to hosts

Four ZYMV isolates (Cvn-1, Knx-1, Nt-3 and Vic-1) from different locations (Table 1) were sap inoculated onto plants of *Chenopodium quinoa*, *C. amaranticolor* (5 plants/isolate) and zucchini cv. Blackjack (2 plants/isolate). Symptoms were recorded and samples from inoculated and tip leaves tested for ZYMV by ELISA 3 and 4 weeks after inoculation.

Five plants each of pumpkin cultivars, 'Jarrahdale' pumpkin cvs Dulong and Sampson, and 'butternut' pumpkin cv. Sunset (with *Zym* gene for ZYMV resistance), and ZYMV susceptible 'Jarrahdale' pumpkin cv. WA Grey and 'butternut' pumpkin cv. Butternut Large (without *Zym*) were sap inoculated at the two leaf stage with infective sap containing three ZYMV isolates each from Kununurra (Knx-1, Knx-10, Knx-11) and Carnarvon (Cvn-1, Cvn-2, Cvn-20) collected from different hosts in

different years (Table 1). In addition, five plants of each cultivar were sap inoculated with healthy sap. Tip and inoculated leaves from each plant were sampled individually and symptoms recorded 12, 19, 26 and 32 days after inoculation and the samples tested by ELISA.

Effectiveness of antisera

Four isolates (Cvn-1, Knx- 1, Nt-3, and Vic-1) were sap inoculated to two plants each of zucchini cv. Blackjack. Two plants were left uninoculated as negative controls. Four weeks after inoculation, tip leaves were sampled, and extracted in PBST (1:20) and the sap extract for each isolate tested by ELISA using polyclonal ZYMV-specific antiserum obtained from three commercial manufacturers: DSMZ, Loewe Biochemica, and Prime Diagnostics. Absorbance values (A_{405}) were measured 30, 60, 90 and 120 minutes after substrating. In addition, plants infected with each of the four isolates were tested using ZYMV ImmunoStrips (Agdia, Elkartm IN) according to the manufacturer's instructions.

DNA extraction, RT- PCR, sequencing and sequence analysis

Samples found infected with ZYMV by ELISA were ground in liquid nitrogen and total RNA extracted with either RNeasy Plant Miniprep (Qiagen, Australia) or Spectrum Plant Total RNA (Sigma-Aldrich, USA) kits. The complete CP nt sequence was amplified by RT-PCR using ZYMV-specific primers ZY2 (5'GCT CCA TAA TAG CTG AGA CAG C-3') and ZY3 (5'TAG GCT TGC AAA CGC AGT CTA ATC-3') [49]. RT-PCR was done according to manufacturers instructions using either ImPromII (Promega, Australia) and *Taq* (Fisher Biotech, Australia) or a Qiagen OneStep RT-PCR kit (Qiagen, Australia). The PCR conditions were: 50ºC for 30 min, followed by 95ºC for 15 min, and then 30 cycles of 94ºC for 30 s, 65ºC for 30 s, 72ºC for 30 s, and a final extension step of 72ºC for 10 min. The expected product of 1186 bp for ZYMV obtained was purified using a PCR purification kit (Qiagen, Australia). The resulting product was then sequenced directly using an Applied Biosystems/Hitachi 3730 DNA Analyzer with BigDye terminator V3.1 chemistry. Complete (837 bp) and partial CP gene sequences were submitted to GenBank (Table 2).

The 42 new complete ZYMV CP nt sequences consisted of (i) 31 isolates from ZYMV-infected cucurbit crop plants from Kununurra (14), Carnarvon, (9), NT (3), Qld (4) and Vic (1); (ii) seven isolates from Carnarvon from alternative hosts *Cucumis maderaspatanus* (5) and wild Afghan melon (*C. lanatus*) (2); and (iii) four isolates from freeze-dried cucurbit crop plant leaves collected from Carnarvon (2) and Kununurra (2) (Table 1). In addition to these 42 new sequences, 101 full length ZYMV CP sequences from 26 countries in five other continents were retrieved from GenBank (Supplementary Table 1). All 143 complete CP nt sequences were aligned and analysed using Molecular Evolutionary Genetics Analysis (MEGA) program version 4.1 with Clustal W [47]. Direct pairwise comparisons were used to establish percentage identities and evolutionary distances. One sequence each of *Bean common mosaic virus* (BCMV), *Soybean mosaic virus* (SMV) and WMV were retrieved from GenBank (Supplementary Table 1) to act as outgroups. Phylogenetic tree creation and analysis used the neighbour-joining method with 1000 bootstrap resamplings to assess the robustness of the lineages in the trees. The maximum composite likelihood model and the Poisson correction methods were used to compute evolutionary distances for nucleotides and amino acids, respectively. Further analyses were done

including six new partial ZYMV CP nt sequences, five from infected cucurbit crop plants from Kununurra (4) and NT (1), and one from the naturalised weed *Rhynchosia minima* from Kununurra (1). When the partial sequences were included with 143 full length sequences for analysis, the alignment was trimmed to the length of the shortest sequence. Prior to this study, the only ZYMV sequences from Australia were two partial sequences from Qld (S81377 and S81381) [49], but as they contained only 130 nt of the CP we excluded them from this analysis.

dN/dS ratios

Selection pressures on the ZYMV CP were calculated using all 143 complete amino acid sequences using the Li-Wi-Luo method in MEGA 4.1 [48, 47]. The mean number of non-silent substitutions (*dN*) and silent substitutions (*dS*), and *dN/dS* ratios were determined [37]. The *dN/dS* ratios for the nt sequences of complete CP genes were estimated for all isolates in each phylogenetic group. Negative selection was indicated by a ratio of <1, neutral selection by a ratio of =1, and positive selection by a ratio of >1.

Multiplex real-time RT-PCR

Three sets of primer and probe sequences were designed using RealTimeDesign (Biosearch Technologies, CA, USA), one each for ZYMV isolates sequences Vic-1, Nt-1 and Knx-1 (Table 2) representing the three phylogenetic groups Australian isolates fitted into (see below) (Fig. 1). Specificities of primers and probes were confirmed by aligning them with the other 42 new sequences using MEGA 4.1 and a BLAST search in the GenBank database. The probes were dual-labelled with a black hole quencher (BHQ) and fluorescent reporter dye. Primers and probes were synthesized by Biosearch Technologies (CA, USA) (Table 2). The assay was performed in a Corbett Rotor-Gene 6000 Rotary Analyzer (Sydney, Australia) using a Qiagen Rotor-Gene Multiplex RT-PCR kit according to manufacturer's instructions with template adjusted to 0.5 µl/reaction. Cycling conditions were: one step of reverse transcription at 50°C for 15min, followed by an activation step at 95°C for 5 min, and then 40 cycles of 95°C for 15s and 60°C for 15s. Each assay was performed in duplicate. Two negative controls were always included: (i) extraction from healthy zucchini leaf material and (ii) non-template nuclease free water.

To generate standard curves, leaf samples infected with isolates Knx-1, Vic-1 or Nt-1 were extracted and a 50µl standard RT-PCR done using primers ZY2 and ZY3. The expected product (1186bp) obtained by gel electrophoresis was purified using a Qiaquick PCR purification kit (Qiagen, Australia) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA). Serial dilutions were done in nuclease free water to achieve final concentrations from 0.5 to 0.004 ng/µl. To validate the assay, two 'blind' studies were done on separate occasions, one with 12 and the other with 17 samples. One sample (Cvn-20) from watermelon collected in 2010 from Carnarvon was also included. Samples contained leaves infected with ZYMV isolates with sequences from the three phylogenetic groups, or from healthy plants. The status of the leaves within each sample was unknown by the experimenter. Sample extracts of isolates Knx-1, Vic-1 or Nt-1 were used as positive controls and healthy zucchini leaves were used as a negative control. Threshold cycle (Ct) values above 35 were considered negative.

Results

Alternative host survey

At 87 sites, 5324 individual samples were collected belonging to at least 34 naturalised weed, two cultivated legume and 11 native plant species. The numbers of samples and species collected from each location were: (a) Kununurra (2006-2009), 50 sites, 3456 samples from 38 species; (b) Carnarvon (2008 and 2010), 26 sites, 1732 samples from 33 species; (c) Broome (2008), 11 sites 136 samples from 4 species. ZYMV was detected in <1% of samples belonging to three species from Kununurra in 2007 (i), or Carnarvon in 2008 and 2010 (ii and iii): (i) a plant of the naturalised legume weed *Rhynchosia minima* with mild mottle and leaf distortion growing at the edge of a ZYMV-infected cucurbit crop (0.2% incidence); (ii) plants of the native cucurbit *Cucumis maderaspatanus* (family, Cucurbitaceae) with mild leaf mottle and leaf distortion from six sites, growing along fence lines adjacent to cucurbit crops (70% incidence); and (iii) plants of the naturalised weed Afghan melon with very mild mottle and leaf pallor from two road verge sites (25% incidence). Five isolates from *C. maderaspatanus*, two from Afghan melon and one from *R. minima* were sequenced (Table 1). In addition, infection with other potyviruses was detected in symptomatic leaf samples of (i) the cultivated legume species (number of plants tested positive in parentheses) *Phaselous vulgaris* (common bean) borlotti type (15) and *Glycine max* (soybean) cvs Bunya and Oakie (12) from commercial crops from Kununurra, and (ii) the naturalised weed species *Clitorea ternatea* (butterfly pea) (8), *Macroptilium atropurpureum* (siratro) (140), *R. minima* (19) and *Vigna trilobata* (wild mung bean) (45) (family, Fabaceae), and *Passiflora foetida* (stinking passion flower) (155) (family Passifloraceae), from Kununurra, Broome and Carnarvon. However, no ZYMV, PRSV and WMV were detected in these samples [11].

No ZYMV or other potyviruses were detected in symptomless plants of the following naturalised weed species (number of plants tested in parentheses): *Mesembryanthemum crystallinum* (16), *Trianthema portulacastrum* (63) (family, Aizoaceae); *Amaranthus* spp. (54) (family, Amaranthaceae); *Calatropis procera* (10) (family, Asclepiadaceae); *Lactuca serriola* (9), *Sonchus oleraceus* (182), *Tridax procumbens* (166) (family Asteraceae); *Brassica sisymbrium* (44), *Raphanus raphanistrum* (8) (family, Brassicaeae); *Chenopodium* spp. (5) (family Chenopodiaceae); *Ipomoea* spp. (125), *Merremia dissecta* (1) (family, Convoluvlaceae); *Citrullus latanus* (volunteer watermelon) (47) (family Cucurbitaceae); *Euphorbia* spp. (51) (family Euphorbiaceae); *Clitorea ternatea* (87), *Glycine* spp. (33), *Macroptilium atropurpureum* (10), *Macroptilium lathyoides* (208), *Medicago* spp. (42), *R. minima* (461), *Swainsona* spp. (10), *Vicia benghalensis* (64), *Vigna trilobata* (336), unidentified legume weeds (130) (family, Fabaceae); *Hyptis suaveolens* (22) (family, Lamiaceae); *Abuliton oxycarpum* (48), *Malva* spp. (210), *Melochia pyramidata* (96) (family, Malvaceae); *Boerharvia* spp. (243) (family, Nyctaginaceae); *Passiflora foetida* (312) (family, Passifloraceae); *Emex* spp. (11) (family, Polygonaceae); *Portulaca* spp. (252) (family, Portulaceae); *Physalis* spp. (129), *Solanum* spp. (168) (Solanaceae); *Tribulus terrestris* (31) (family, Zygophyllaceae); and unidentified other weed species (222). Neither was it detected in the following native species (number of plants tested in parentheses): *Gomphrena* spp. (10) (family, Amaranthaceae); *Atriplex semilunaris* (43) *Rhagodia ereameae* (24) (family, Chenopodiaceae); *Cleome viscose* (73) (family, Cleomaceae); *Convolvulus* spp. (31), *Jacquemontia* spp. (40), *Operculina brownii* (506), (family

Convolvulaceae); *C. maderaspatanus* (12) (family, Cucurbitaceae); *Adriana* spp. (8) (family Euphorbiaceae); *Crotalaria cumminghamii* (9) (family, Fabaceae); *Abelmoschus ficulneus* (71), *Hibiscus panduriformis* (86), (family, Malvaceae).

Seed transmission
Three out of 430 zucchini seedlings were found ZYMV-infected when tested by ELISA and RT-PCR, representing a seed transmission rate of 0.7%. The infected seedlings showed mild leaf mottle and plant stunting. No seed transmission was detected in a total of 9560 pumpkin seedlings tested.

Effectiveness of antisera
When leaves from zucchini plants infected with isolates Cvn-1, Vic-1, Nt-3 and Knx-1 were tested by ELISA, absorbance values were always lowest with DSMZ and highest with Prime antisera, and those for Knx-1 were always the lowest, regardless of antiserum used. For example, after 60 min, mean absorbance values for Knx-1 were 0.124, 0.449 and 0.878 for DSMZ, Loewe and Prime antisera respectively. The corresponding values for Cvn-1 were 0.629, 0.930 and 0.995; for Vic-1 were 0.628, 0.867 and 1.034; and for Nt-3 were 0.535, 0.847 and 1.045. The negative control values were 0.021-0.029. When leaves from the four isolates were tested by ImmunoStrips, Cvn-1, Vic-1 and Nt-3 reacted strongly and the positive test line was visible in <5min, while the reaction to Knx-1 was weaker and the test line developed in >5 min.

Inoculation to hosts
Regardless of ZYMV-isolate used, ZYMV was always detected in inoculated leaves of *C. quinoa* and *C. amaranticolor*, but no systemic infection was found over a 4 week period after inoculation. Isolates Cvn-1, Knx-1 and Vic-1 induced local chlorotic blotches and small local chlorotic spots in inoculated leaves of *C. quinoa* and *C. amaranticolor,* respectively. By contrast, isolate Nt-3 caused symptomless infection in inoculated leaves of *C. quinoa*, and only induced very few chlorotic spots with red halos in *C. amaranticolor.* This was repeated three times always giving the same result. Regardless of ZYMV-isolate used, 2 weeks after inoculation of zucchini plants no symptoms developed on inoculated leaves, but all plants became infected systemically developing symptoms of leaf mottle and distortion that became severe after 3 weeks.

All six isolates caused symptomless infection in inoculated leaves of pumpkin cvs WA Grey, Butternut Large and Sunset. Inoculated leaves developed few small chlorotic spots or blotches (cv. Dulong) or small chlorotic spots (cv. Sampson) and ZYMV was detected in them (Table 3). All six isolates caused systemic infection in all plants of cvs Butternut Large and WA Grey (without *Zym*), and cv. Sunset (with *Zym*), and symptoms were always severe (Table 3). In contrast, cv. Dulong (with *Zym*) only developed systemic infection in all plants with 1/6 isolates and cv. Sampson (with *Zym*) with 4/6 isolates. Also, where systemic infection occurred in cv. Dulong and Sampson, it was always associated with mild symptoms. No ZYMV was detected in inoculated or tip leaves of any plants of any cultivar inoculated with healthy sap.

Sequence analysis

When the 143 full length ZYMV CP nt sequences were analysed, they clustered into three groups (A-C), with bootstrap support of 100% for A, 85% for B and 100% for C (Fig. 1). Group A comprised four subgroups (I-IV) with bootstrap support of 99%, 51%, 98% and 99%, respectively. Overall, the % nt identities within each group and subgroup were >88.9% (A), >95.9% (A-I), >93.2% (A-II), >91.8% (A-III), >95.3% (A-IV), >85.6% (B) and >85.8% (C). The new isolates from Carnarvon, Vic and Qld were in A-I which also contained isolates from Europe and Asia. Isolates from NT were all in A-II, which also contained isolates from North America, Europe and Asia. Isolates from Kununurra were in B and this group contained only four others, from Vietnam (1), Singapore (2) or Reunion Island (1). When the six new partial sequences were included with the 143 others, the resulting Neighbour-Joining tree (346 nt) (Supplementary Fig. 1) gave the same groups and subgroups as those obtained for full length sequences, but there was indication of possible further subdivision of subgroup A-II into four with bootstrap support of >76%. The five partial sequences from Kununurra (Knx-17-21) grouped together with the complete Kununurra sequences in B, while one isolate (Nt-4) from NT grouped with the complete NT sequences in A-II.

When the 42 new ZYMV CP nt sequences were compared with each other, their nt identities were >85.3% and when aligned with the 101 ZYMV sequences retrieved from GenBank, their nt identities were >78.5%. When the new sequences from within each location were compared separately, those from Kununurra (16) had >98.7% nt identity, NT (3) had 100% nt identity, those from Carnarvon (18) had >98.9% nt identities, and those from Qld (4) had >98.4% nt identity. When the 23 sequences representing Australian isolates in A-I (Carnarvon, Qld, Vic) were combined they had >96.9% nt identity. When Carnarvon isolates were compared with Vic and Qld isolates there was 97.0% and >96.9% nt identity, respectively, while Qld and Vic isolates had 99.5% nt identity. When the Australian isolates from the three different groups were compared to each other the nt identities were, >85.3% for B (Kununurra) and A-I (Qld, Vic and Carnarvon), 85.4% for B and A-II (NT), and >93.5% for A-I and A-II.

When the complete CP nt sequences of the five ZYMV isolates from *C. maderaspatanus* (Table 1) were compared with each other, they had nt identities of >99.0%, and when compared with other isolates from Carnarvon they had >98.9% nt identities. The partial sequence from *R. minima* (Knx-17) clustered in group B with the other Kununurra isolates. When the three sequences from seed-infected zucchini seedlings (Cvn-3, Cvn-4, Cvn-5) were compared to each other and to Carnarvon isolate Cvn-1, there was 100% nt identity between Cvn-4, Cvn-5 and Cvn-1, while Cvn-3 had 99.5% nt identity with Cvn-1, Cvn-4 and Cvn-5 (difference of 4 nucleotides or 2 amino acids).

Selection pressure (dN/dS ratios)

Using all 143 complete CP amino acid sequences, the mean *dN/dS* ratios for each ZYMV group or subgroup were A-I, 0.085; A-II, 0.0592; A-III, 0.0949; A-IV, 0.787; A, 0.044; B, 0.0373 and C, 0.555. Most CP nucleotide substitutions within groups were silent, with mean *dN/dS* ratios <1 indicating negative selection pressure on the CP gene, and suggesting selection for amino acid conservation.

Multiplex real-time RT-PCR

The multiplex assay differentiated between representative sequences from A-I (Vic-1 and Cvn-1), A-II (Nt-3) and B (Knx-1). Standard curves produced had R^2 values of 0.99, 0.98 and 0.99 for the A-I, A-II and B group specific reactions, respectively (Fig. 3). Ct values of 35 or less were considered to be positive. The assay was validated on 'blind' samples and correctly identified the phylogenetic group each infected sample belonged to, with one exception (Cvn-2). For Cvn-2, although all controls were valid, and the shape of the fluorescence curve was similar to other ZYMV-infected samples, it began fluorescing at cycle 16 compared with cycles 5-10 for other samples also the curve did not reach the threshold. Sample Cvn-20, which was not sequenced, was identified as resembling others from A-I.

Discussion

This study of ZYMV infecting cucurbit crops growing in tropical and subtropical Australia showed that it is seed transmissible in zucchini, two new alternative hosts were identified, resistance gene (*Zym*) was overcome or partially overcome in pumpkin and there are genetic differences between isolates collected from different locations. Cucurbit growing areas in Australia are often remote and widely separated and when 42 new ZYMV CP gene nt sequences of isolates from four locations were compared with 101 others from five other continents, there were three major groupings (A, B, and C) and four subgroups (I-IV) within the largest of them (A). The Australian isolates were in groups A-1, A-11 and B with new isolates in A-II and B being restricted to one location each. Selection pressure within each group and subgroup was estimated to be negative with *dN/dS* ratios of <1 which suggest genetic stability within each location examined. Isolates from group B were not detected reliably by commercially available ZYMV antisera in ELISA, so a multiplex real-time RT-PCR assay was devised that not only detected the three Australian groups reliably but also distinguished between them.

Our study confirmed seed transmission of ZYMV isolate Cvn-1 at low rates (0.7%) in zucchini. However, despite exhaustive testing of pumpkin seeds from infected fruit (>9000) and lack of any seed transmission, we are unable to conclude that ZYMV is never seed transmitted at very low levels in the cultivars of the pumpkin species (*C. maxima* or *C. moschata*) used, or the possibility that the Knx-1 isolate used is not seed-borne. Testing of pumpkin seeds from fruit infected with the Cvn-1 isolate and zucchini seeds from fruit infected with Knx-1 might help resolve this. Simmons et al. [45] recently reported low rates (1.6%) of seed transmission in *C. pepo* subsp. *texana* (wild gourd), with infected seedlings being symptomless. In this study, we used ELISA to test all seedlings and infected seedlings were symptomatic.

This study again demonstrated the rarity of wild alternative hosts of ZYMV (see Introduction), extensive surveys in areas with high ZYMV inoculum and aphid vector pressure (Kununurra and Carnarvon) finding <1% of samples infected which were limited to just three species. Two of these species are new ZYMV host records, *C. maderaspatanus* and *R. minima*. *C. maderaspatanus* is an annual twining cucurbit native to regions of Africa, Asia and Australasia [43]. When ZYMV sequences from naturally infected cucurbit crop plants (zucchini, cucumber, watermelon, squash) growing in Carnarvon were compared with five from *C. maderaspatanus* also from Carnarvon, they had >98.9% nt identities. *R. minima* is a leguminous weed

naturalised in Australasia, and also found in Africa, Asia, North and South America [53]. The partial sequence obtained from it grouped with ZYMV isolates collected from cucurbit crop plants originating from Kununurra. Afghan melon was also found ZYMV infected in this study which was reported previously [9]. The isolate obtained from it grouped with others collected from cucurbit crops at Carnarvon. *C. maderaspatanus* and Afghan melon can act as virus reservoirs for ZYMV spread to cucurbit crops as they are commonly found growing nearby. *R. minima* is also found in close proximity to cucurbit crops but it was rarely found infected. It would be interesting to collect seed from ZYMV-infected *C. maderaspatanus* and Afghan melon fruit to determine if ZYMV is seed-borne in them.

As mentioned in the Introduction, previous ZYMV studies used complete or partial sequences to distinguish phylogenetic groups. Our findings with a larger number of sequences (143) which included our 42 new ones from Australia, support the division of ZYMV isolates into three main groups as proposed by Ha et al. [26] which they named I-III. However, since the group names A and B suggested by Desbiez et al. [15] have precedence, we use them here. In addition, we call the third group C. Groups A, B and C correspond to groups I (worldwide), II (Vietnam, Reunion and Singapore) and III (Vietnam and China) of Ha et al. [26], and our group C corresponds to group III of Simmons et al. [44]. Within group A, we suggest division into four subgroups (A-I - A-IV). Desbiez et al. [15] originally suggested 3 clusters within group A, one of which corresponds to A-I, two combined into subgroup A-II, and two new subgroups (A-III and A-IV). Group A-111 includes an isolate from China AJ307036 even though its genetic divergence reduces bootstrap support for this subgroup to 45%. When this sequence is excluded, the bootstrap support for A-III is 98% suggesting this isolate represents a monotypic group and therefore further subdivisions within group A which will only be confirmed when other isolates resembling it are sequenced. Similarly, bootstrap support for subgroup A-II is only 51%, but there is indication this subgroup could be further subdivided into four (>76% bootstrap support). Sequencing of the complete CP of more ZYMV isolates could help clarify if more subgroups are needed to accurately describe the variation within the worldwide population of ZYMV.

A study of 36 French ZYMV isolates divided them into five 'haplotypes' each having one or more nucleotide differences from others within the same molecular grouping [33]. These 'haplotypes' fit within three of our subgroups, A-II (3), A-III (1) and A-IV (1). When we assessed our isolates on this basis, no correlation was evident between collection year, original location, host or 'haplotype'.

The new Australian isolates cluster into A-I, A-II or B. Isolates from Kununurra fitted into B which previously only contained four isolates from Reunion Island, Singapore, and Vietnam. NT isolates grouped into A-II with isolates from Asia, North America, and Africa. Carnarvon, Vic and Qld isolates grouped into A-1 with Asian and European isolates. These groupings suggests at least three separate introductions of ZYMV to Australia, one each to Kununurra and NT, and one to either Carnarvon, Vic, or Qld. We include the east coast (Qld to Vic) as one growing area as it is a continuously populated zone, whereas Carnarvon, Kununurra and NT are remote and isolated. The rarity of introductions may be the reason ZYMV has not been found in the remote Broome cucurbit growing area [9]. How the virus first entered Australia is difficult to determine but there are several possible pathways. Prior to adoption of

stringent quarantine procedures infected cucurbit material such as whole plants or fruits may have entered from elsewhere, providing the initial virus source [32]. Alternatively, imported cucurbit seed might have provided the initial source despite the low levels of seed transmission. We calculated an overall *dN/dS* ratio of <1 for 143 CP sequences, similarly Simmons et al. [44] previously reported mean *dN/dS* ratio of 0.108 for 55 ZYMV CP sequences, and suggested in situ evolution of ZYMV within several countries and human activity has played a central role in ZYMV dispersal. Thus, once an isolate becomes established at a location a lack of positive selection occurring amongst the population means there is little change unless a new variant is introduced as occurred in France [33]. For example, 16 isolates from Kununurra collected in 2001-2009 from different cucurbit crop types showed little variation (>1.3%). Likewise, the five isolates from the alternative host *C. maderaspatanus* were very similar to the 11 cucurbit crop isolates collected at Carnarvon in 2008-2010.

We attempted to determine if there were differences in host responses to inoculation with ZYMV isolates from different phylogenetic groups. When four isolates representing groups A-I, A-II and B were inoculated to *C. quinoa*, Cvn-1, Vic-1 (A-I) and Knx-1 (B) all induced obvious symptoms on inoculated leaves, while Nt-3 (A-II) did not. Thus, it may be possible to differentiate A-II isolates by their reactions in *C. quinoa*. When six ZYMV isolates from A-I and B were inoculated to five pumpkin cultivars all infected *C. moschata* cultivars with or without *Zym* and *C. maxima* without *Zym* systemically. However, although inoculated leaves became infected, systemic movement did not occur with some isolates in some plants of the two *C. maxima* cultivars with *Zym*, indicating partial resistance to systemic movement in *C. moschata* resulting from presence of ZYMV. The isolates used originated from Carnarvon and Kununurra but were collected in different years and from different hosts. Thus, isolate (Cvn-1) from an alternative host was infectious on cucurbits, and regardless of host or year isolates from these two locations overcame the *Zym* gene in *C. moschata* and partially overcame it in *C. maxima*. In our previous studies [10], the *Zym* gene in pumpkin (*C. moschata* and *C. maxima*) was partially effective against Cvn-1 under low ZYMV inoculum pressure in the field, but ineffective against Knx-1 under high inoculum pressure. Also, under high inoculum pressure in natural epidemics *Zym* was overcome in *C. moschata* cv. Sunset at both locations. When zucchini cultivars with and without *Zym* were sap inoculated with the same six isolates, systemic infection occurred and there was little difference in systemic leaf symptoms.

ELISA testing is useful when many samples need to be tested in a short time frame at low cost so determining the most appropriate antibody to use is important, underestimation of virus incidence being possible when weak reactions occur. When using ELISA to test for ZYMV in leaf samples, we found ZYMV specific polyclonal antibodies sourced from Prime Diagnostics consistently reacted strongly with all isolates but antibodies from DSMZ did not. Possibly the DSMZ antiserum was not raised against isolates that included a representative from group B. Alternatively, weak reactions with DSMZ antibodies may indicate that group B is a different serotype. When testing cucurbit crop samples collected from various WA locations we found Loewe antibodies did not detect ZYMV reliably (data not shown). The ImmunoStrips (Agdia) reacted with all four isolates tested, but were weakest when testing the isolate representing group B. Generic potyvirus antibody (Agdia) was

equally effective in detecting all isolates from all locations, but did not distinguish ZYMV from other potyviruses.

Our multiplex real-time RT-PCR assay distinguished ZYMV isolates from the three phylogenetic groups (A-I, A-II, B) to which Australian isolates belonged reliably, except with isolate Cvn-2 (A-II) which fluoresced around cycle 16. When the probe sequence was compared with the other sequences from A-I, a single base pair difference was found in the middle of the probe which could be rectified by adjusting the sequence of the probe accordingly. Although this assay is unlikely to replace ELISA as a diagnostic tool,it does have the advantages of increased sensitivity, convenience and significant time savings (1 vs 7 days) over conventional RT-PCR assays and sequencing required to identify which phylogenetic group a ZYMV isolate belongs to.

Acknowledgements

We thank Denis Persley, Brendan Rodoni and Barry Conde of supplying isolates from Qld, Vic and NT, respectively. We also thank Eva Gajda for assisting with ELISA testing of samples. This research was funded by Horticulture Australia Ltd project VG06022.

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Table 1. *Zucchini yellow mosaic virus* isolates with coat protein sequences from this study: isolate codes, hosts, years collected, original locations and GenBank accession numbers

Isolate[a]	Host[b]	Common name, cultivar	Year collected	Geographical origin[c]	Accession number
Cvn-1	*Cucumis maderaspatanus*[b]	-	2008	Carnarvon, WA	JF792448
Cvn-2	*Cucurbita pepo*	Zucchini	2008	Carnarvon, WA	JF792449
Cvn-3	*C. pepo*	Zucchini	2008	Carnarvon, WA	JF792450
Cvn-4	*C. pepo*	Zucchini	2008	Carnarvon, WA	JF792451
Cvn-5	*C. pepo*	Zucchini	2008	Carnarvon, WA	JF792452
Cvn-6	*Cucumis sativus*	Cucumber	2009	Carnarvon, WA	JF792453
Cvn-7	*C. pepo*	Squash	2010	Carnarvon, WA	JF795791
Cvn-8	*C. maderaspatanus*[b]	-	2010	Carnarvon, WA	JF795792
Cvn-9	*Citrullus lanatus*	Watermelon	2010	Carnarvon, WA	JF795793
Cvn-10	*C. pepo*	Zucchini	2010	Carnarvon, WA	JF795794
Cvn-11	*C. maderaspatanus*[b]	-	2010	Carnarvon, WA	JF795795
Cvn-12	*C. maderaspatanus*[b]	-	2010	Carnarvon, WA	JF795796
Cvn-13	*C. maderaspatanus*[b]	-	2010	Carnarvon, WA	JF795797
Cvn-14	*C. lanatus*[b]	Afghan melon	2010	Carnarvon, WA	JF795798
Cvn-15	*C. lanatus*[b]	Afghan melon	2010	Carnarvon, WA	JF795799
Cvn-16	*C. pepo*	Zucchini	2001	Carnarvon, WA	JF795800
Cvn-17	*C. sativus*	Cucumber	2003	Carnarvon, WA	JF792361
Cvn-18	Unknown cucurbit	-	2003	Carnarvon, WA	JF792362
Knx-1	*Cucurbita moschata*	Butternut pumpkin	2005	Kununurra, WA	JF792363
Knx-2	*C. moschata*	Kent pumpkin	2005	Kununurra, WA	JF792364
Knx-3	*Cucumis melo*	Honeydew melon	2005	Kununurra, WA	JF792365
Knx-4	*Cucurbit maxima*	Jarrahdale pumpkin cv. WA Grey	2006	Kununurra, WA	JF792366
Knx-5	*C. moschata*	Butternut pumpkin cv. Sunset	2006	Kununurra, WA	JF792367
Knx-6	*C. melo*	Rockmelon	2007	Kununurra, WA	JF792368
Knx-7	*C. maxima*	Jarrahdale pumpkin	2007	Kununurra, WA	JF792369
Knx-8	*C. moschata*	Butternut pumpkin	2007	Kununurra, WA	JF792370
Knx-9	*C. pepo*	Zucchini cv. Gold coast	2007	Kununurra, WA	JF792371
Knx-10	*C. lanatus*	Watermelon	2007	Kununurra, WA	JF792372
Knx-11	*C. maxima*	Jarrahdale pumpkin	2008	Kununurra, WA	JF792373
Knx-12	*Cucurbita sp.*	Pumpkin	2009	Kununurra, WA	JF792374
Knx-13	*C. pepo*	Zucchini	2005	Kununurra, WA	JF797207
Knx-14	*Cucurbita sp.*	Pumpkin	2001	Kununurra, WA	JF797208
Knx-15	*C. sativus*	Cucumber	2001	Kununurra, WA	JF797209
Knx-16	*Cucurbita sp.*	Pumpkin	2006	Kununurra, WA	JF797210

Knx-17[a]	*Rhynchosia minima*[b]	-	2007	Kununurra, WA	JF797212
Knx-18[a]	*C. pepo*	Zucchini cv. Houdini	2006	Kununurra, WA	JF797213
Knx-19[a]	*C. maxima*	Jarrahdale pumpkin	2006	Kununurra, WA	JF797214
Knx-20[a]	*C. maxima*	Jarrahdale pumpkin	2007	Kununurra, WA	JF797215
Knx-21[a]	*Cucurbita sp.*	Pumpkin	2009	Kununurra, WA	JF797216
Nt-1	*C. sativus*	Cucumber	2008	Darwin, NT	JF792440
Nt-2	*C. melo*	Rockmelon	2008	Darwin, NT	JF792441
Nt-3	*C. melo*	Rockmelon	2008	Darwin, NT	JF792442
Nt-4[a]	*C. melo*	Rockmelon	2008	Darwin, NT	JF797211
Qld-1	*C. moschata*	Butternut pumpkin	2008	Ayr, Qld	JF792444
Qld-2	*C. maxima*	Jarrahdale pumpkin	2008	Ayr, Qld	JF792445
Qld-3	*C. moschata*	cv. WA Grey Kent pumpkin	2008	Ayr, Qld	JF792446
Qld-4	*Cucurbita sp.*	Pumpkin	2008	Ayr, Qld	JF792447
Vic-1	unknown cucurbit	-	2008	Vic	JF792443

[a], denotes partial sequence only. [b], denotes alternative host. [c], WA, Western Australia; NT, Northern Territory; Qld, Queensland; Vic, Victoria.

Table 2. Multiplex real-time PCR primers and probes for detection of *Zucchini yellow mosaic virus* (ZYMV) isolates representing three phylogenetic groups found in Australia

Phylogenetic group	Isolate	Type of primer/probe	5'-3' sequence	Probe Label
B	Knx-1	Forward	GCTGCGACAAATAATGCATCAC	
		Reverse	GTGCCTCTGCGTTTCTCATC	
		Probe	TTCTCAGATGCAGCGGAGGC	CAL Fluor Orange 560-BHQ1
A-I	Vic-1	Forward	CAGGCACTCAGCCAACTGT	
		Reverse	GAGCCGGAGCCTGTAACATC	
		Probe	ACGCTGGAGCCACAAAGAAAGACAA	CAL Fluor Red 610-BHQ2
A-II	Nt-1	Forward	CATGCCGAGGTATGGTTTGCTT	
		Reverse	GCGGGCTCTTTCAGGAGTT	
		Probe	AAACCTACGGGATAGGAGTTTAGCACGA	Quasar 670-BHQ2

Table 3. Responses of pumpkin cultivars with and without *Zym* to inoculation with six *Zucchini yellow mosaic virus* (ZYMV) isolates.

Pumpkin cultivar	*Zym* gene	Predominant symptoms[a]		Number of plants infected systemically[b]					
		Inoculated leaves	Tip leaves	ZYMV isolate					
Cucurbita moschata				Knx-1	Knx-10	Knx-11	Cvn-1	Cvn-2	Cvn-20
Butternut large	No	si	sm, ld, st	5	5	5	5	5	5
Sunset	Yes	si	sm, ld, st	5	5	5	5	5	5
Cucurbita maxima									
WA Grey	No	si	sm, ld, st	5	5	5	5	5	5
Dulong	Yes	lcs, lcb	scs, st	3	3	1	4	1	5
Sampson	Yes	lcs	mm, scs, st	5	2	5	5	5	4

[a], Symptom descriptions: si, symptomless infection; lcs, local chlorotic spots; lcb, local chlorotic blotches; mm, mild mottle; scs, systemic chlorotic spots or blotches; ld, leaf distortion; sm, severe mottle; st, plant stunting. ZYMV was detected in inoculated leaves of all plants including those in which no systemic infection was found.

[b], Five plants of each pumpkin cultivar inoculated with each ZYMV isolate. Samples from tip leaves of all plants were tested for ZYMV infection by ELISA 32 days after inoculation.

Fig. 1. Locations in Australia where *Zucchini yellow mosaic virus* isolates, alternative host survey samples and fruit samples were collected.

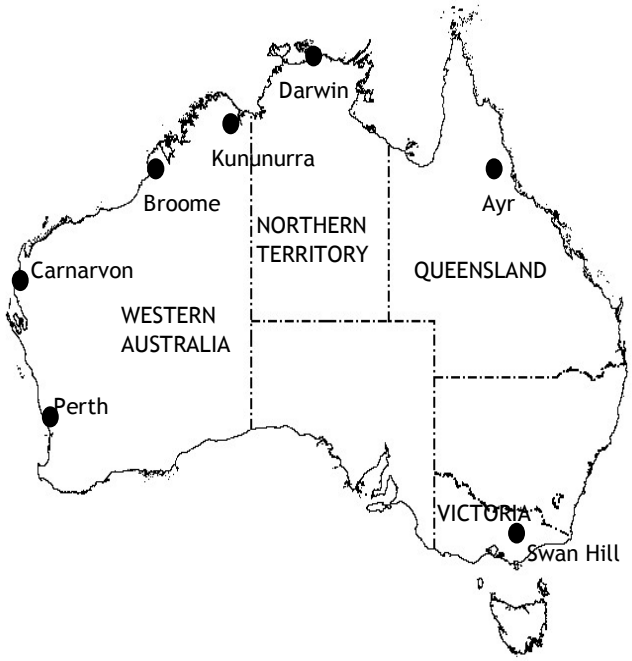


Fig. 2. Neighbour-joining relationship phylogram obtained from alignment of complete coat protein nucleotide sequences of 42 new Australian *Zucchini yellow mosaic virus* (ZYMV) isolates and 101 ZYMV sequences from GenBank. Three groups (A-C) and within A four subgroups (I-IV) are shown. The tree was generated using the ClustralW and MEGA 4.1 programmes set to default parameters. Tree branches were bootstrapped with 1,000 replications. Numbers at nodes indicate bootstrap scores of >45%. The scale bar represents a genetic distance of 0.05 for horizontal branch lengths. New sequences are without GenBank codes, in bold and highlighted, but other sequences show their GenBank codes and countries of origin. For isolate designation see Table 1 (new isolates) and Supplementary Table 1 (other isolates). The tree was rooted with *Bean common mosaic virus* (BCMV, EU761198), *Watermelon mosaic virus* (WMV, L22907) and *Soybean mosaic virus* (SMV, U25673) sequences.

Fig. 2

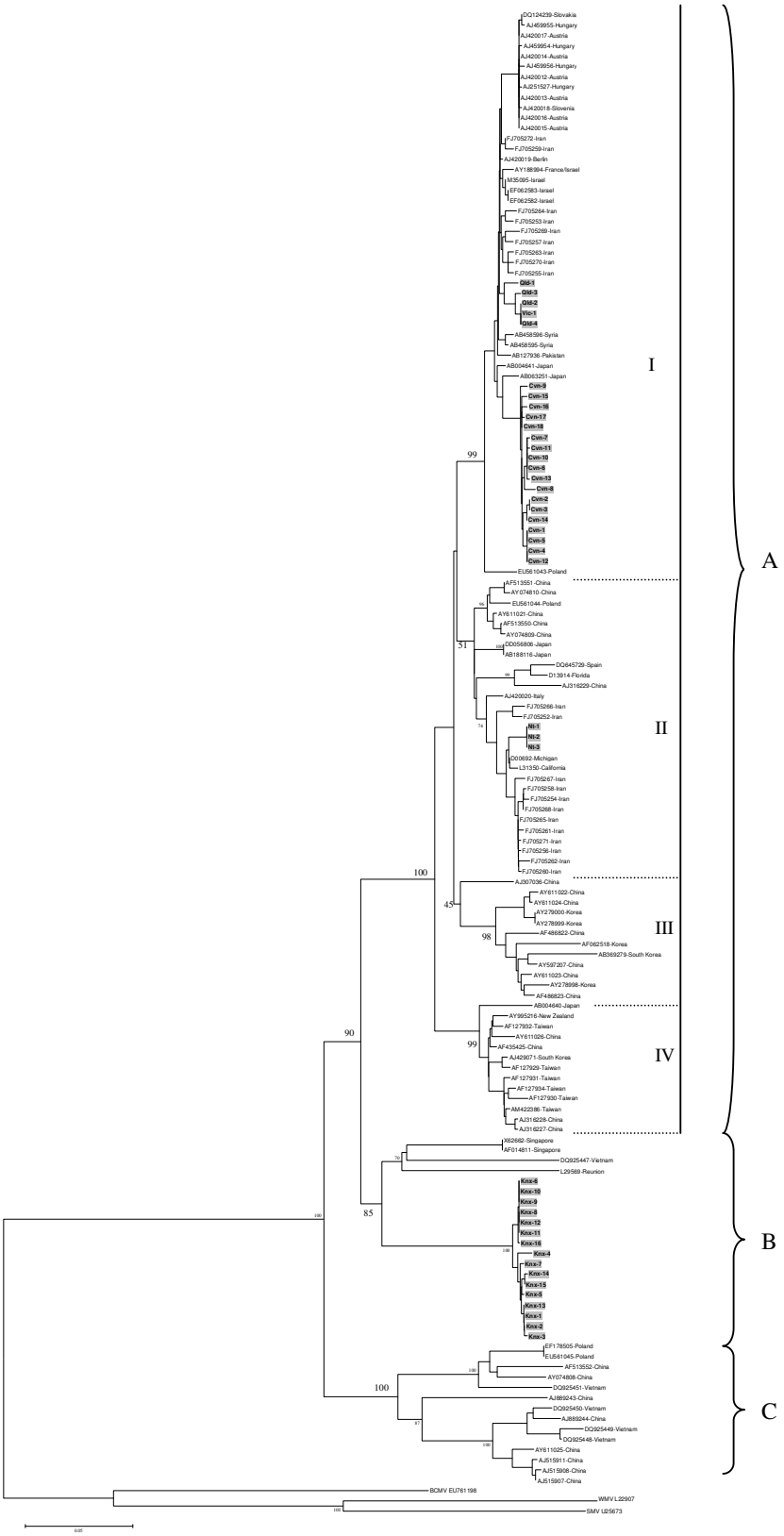
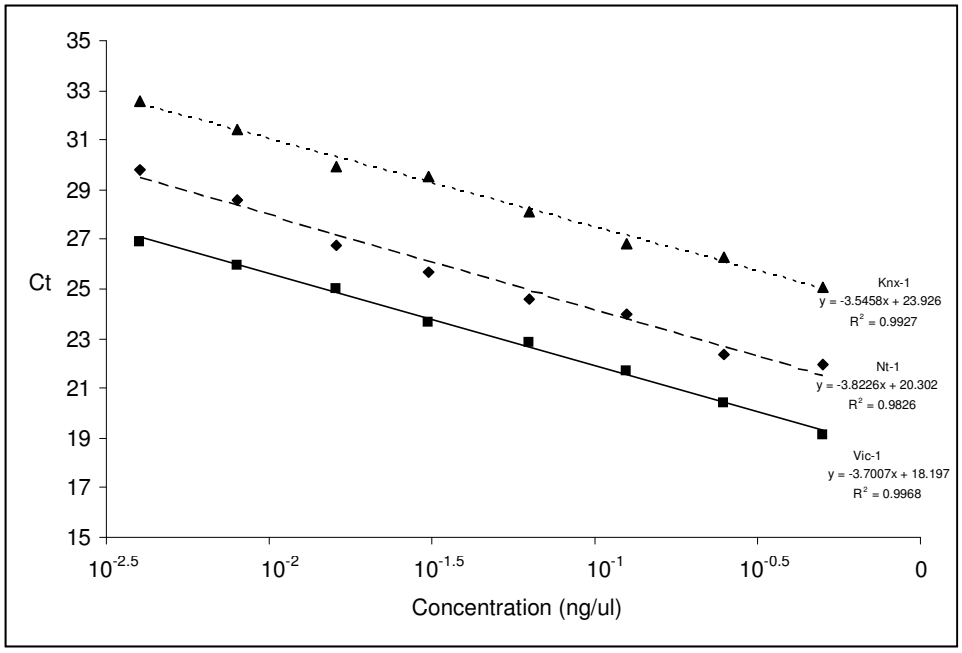

DQ124239-Slovakia
AJ459955-Hungary
AJ420017-Austria
AJ459954-Hungary
AJ420014-Austria
AJ459956-Hungary
AJ420012-Austria
AJ251527-Hungary
AJ420013-Austria
AJ420016-Austria
AJ420015-Austria
FJ705272-Iran
FJ705259-Iran
AJ420019-Berlin
AY188994-France/Israel
M35095-Israel
EF062583-Israel
EF062582-Israel
FJ705264-Iran
FJ705253-Iran
FJ705269-Iran
FJ705257-Iran
FJ705263-Iran
FJ705270-Iran
FJ705255-Iran
Qld-1
Qld-3
Qld-2
Vic-1
Qld-4
AB458596-Syria
AB458595-Syria
AB127936-Pakistan
AB004641-Japan
AB063251-Japan
Cvn-9
Cvn-15
Cvn-16
Cvn-17
Cvn-18
Cvn-7
Cvn-11
Cvn-10
Cvn-6
Cvn-13
Cvn-8
Cvn-2
Cvn-3
Cvn-14
Cvn-1
Cvn-5
Cvn-4
Cvn-12
EU561043-Poland
AF513551-China
AY074810-China
EU561044-Poland
AY611021-China
AF513550-China
AY074809-China
DQ056806-Japan
AB188116-Japan
DQ645739-Spain
D13914-Florida
AJ316229-China
AJ420020-Italy
FJ705266-Iran
FJ705252-Iran
Mc-1
Mc-2
Mc-3
DQ09592-Michigan
L31130-California
FJ705267-Iran
FJ705258-Iran
FJ705254-Iran
FJ705268-Iran
FJ705265-Iran
FJ705261-Iran
FJ705271-Iran
FJ705256-Iran
FJ705262-Iran
FJ705260-Iran
AJ307036-China
AY611022-China
AY611024-China
AY279000-Korea
AY278999-Korea
AF486822-China
AF062518-Korea
AB369279-South Korea
AY597207-China
AY611023-China
AY278998-Korea
AF486823-China
AB004640-Japan
AY995216-New Zealand
AF127932-Taiwan
AY611026-China
AF435426-China
AJ429071-South Korea
AF127929-Taiwan
AF127931-Taiwan
AF127934-Taiwan
AF127930-Taiwan
AM422586-Taiwan
AJ316228-China
AJ316227-China
X62960-Singapore
AF014811-Singapore
DQ925447-Vietnam
L29569-Reunion
Kna-6
Kna-10
Kna-9
Kna-8
Kna-12
Kna-11
Kna-16
Kna-4
Kna-7
Kna-14
Kna-15
Kna-5
Kna-13
Kna-1
Kna-2
Kna-3
EF178505-Poland
EU561045-Poland
AF513552-China
AY074808-China
DQ925451-Vietnam
AJ889243-China
DQ925450-Vietnam
AJ889244-China
DQ925449-Vietnam
DQ925448-Vietnam
AY611025-China
AJ515911-China
AJ515908-China
AJ515907-China
BCMV EU761198
WMV L22907
SMV U25673
I
II
III
IV
A
B
C
99
51
100
45
98
90
99
85
100

Fig. 3. *Cucumis maderaspatanus* infected with *Zucchini yellow mosaic virus* growing at Carnarvon, Western Australia: a) leaf with symptoms of mild mottle, b) infected plant growing along a fence-line.

Fig. 4. Standard curves from serial dilutions of purified PCR products of Australian *Zucchini yellow mosaic virus* isolates Knx-1, Nt-1, and Vic-1 representing phylogenetic groups B, A-II and A-I, respectively. Symbol codes: (▲) Knx-1; (♦) Nt-1; (■) Vic-1.



Supplementary Table 1. List of *Zucchini yellow mosaic virus* isolates with coat protein sequences from GenBank.

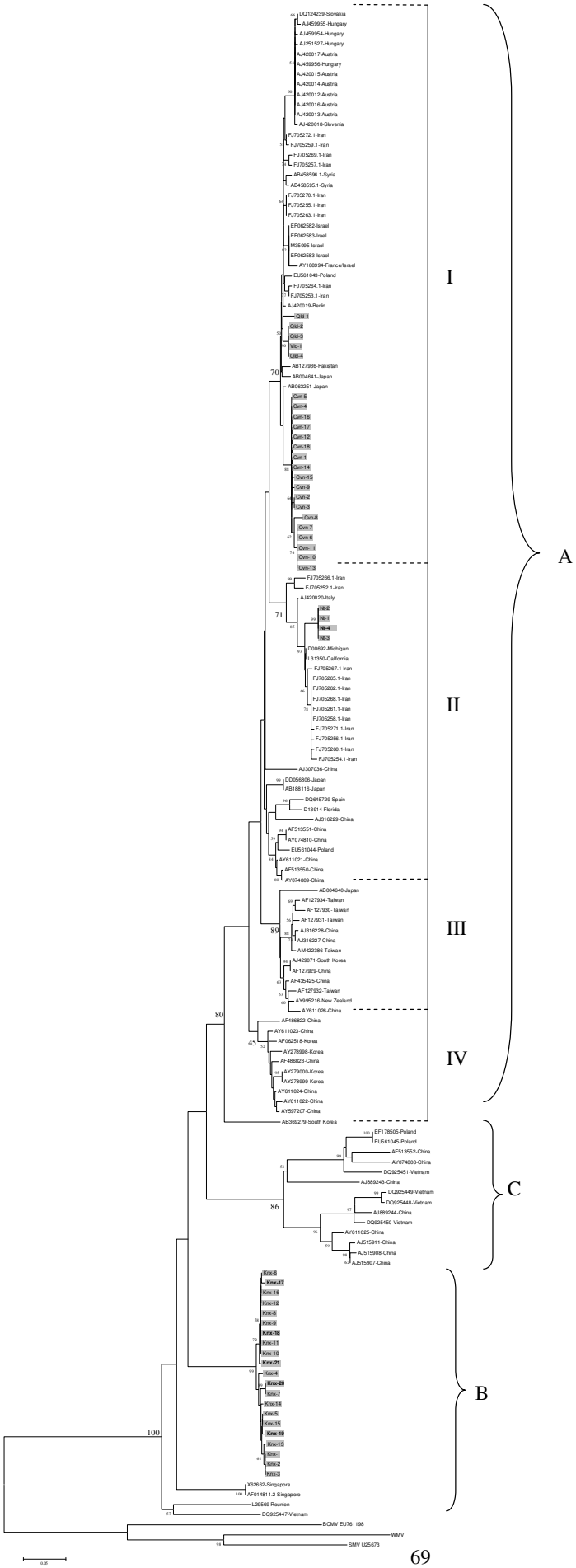
Virus	Geographical Origin	Accession number	Isolate
ZYMV	Michigan	D00692	-
ZYMV	South Korea	AB369279	-
ZYMV	Taiwan	AM422386	begonia
ZYMV	China	AY611023	CH99/193
ZYMV	China	AY611022	CH99/87
ZYMV	China	AJ889243	LG1
ZYMV	Poland	EF178505	zug
ZYMV	Israel	EF062582	NAT
ZYMV	Slovakia	DQ124239	kuchyna
ZYMV	Spain	DQ645729	zymv c-16
ZYMV	Korea	AY279000	KR-PS
ZYMV	Korea	AY278999	KR-PE
ZYMV	Japan	DD056806	-
ZYMV	Japan	AB188116	2002
ZYMV	Italy	AJ420020	Italy 1
ZYMV	Berlin	AJ420019	Berlin 1
ZYMV	Slovenia	AJ420018	Slovenia 1
ZYMV	Austria	AJ420016	Austria 11
ZYMV	New Zealand	AY995216	-
ZYMV	Pakistan	AB127936	pak
ZYMV	California	L31350	-
ZYMV	Reunion	L29569	-
ZYMV	Poland	EU561045	zug
ZYMV	Poland	EU561044	zuy
ZYMV	Poland	EU561043	cu
ZYMV	Vietnam	DQ925451	ZYMV-VN/Bh1
ZYMV	Vietnam	DQ925450	ZYMV-VN/Cm2
ZYMV	Vietnam	DQ925449	ZYMV-VN/Cs1
ZYMV	Vietnam	DQ925448	ZYMV-VN/Cm1
ZYMV	Vietnam	DQ925447	ZYMV-VN/Cm3
ZYMV	China	AY611021	CH99/116
ZYMV	China	AJ889244	TY
ZYMV	Israel	EF062583	AG
ZYMV	Korea	AY278998	KR-PA
ZYMV	Austria	AJ420017	Austria 12
ZYMV	Austria	AJ420015	Austria 10
ZYMV	Austria	AJ420014	Austria 6
ZYMV	Austria	AJ420013	Austria 5
ZYMV	Austria	AJ420012	Austria 2
ZYMV	Hungary	AJ459956	H272-8
ZYMV	Hungary	AJ459955	H272-5
ZYMV	Hungary	AJ459954	H266-2
ZYMV	Hungary	AJ251527	10
ZYMV	China	AJ316229	WG
ZYMV	China	AJ316228	SG
ZYMV	China	AJ307036	CU
ZYMV	China	AJ515911	WM
ZYMV	South Korea	AJ429071	-
ZYMV	China	AY597207	Hefei
ZYMV	China	AY611026	HN-01
ZYMV	China	AY611025	BJ-03
ZYMV	China	AY611024	Ch99/246
ZYMV	France/Israel	AY188994	-
ZYMV	China	AJ515908	MM

ZYMV	China	AJ515907	SXS
ZYMV	China	AJ316227	p
ZYMV	Florida	D13914	-
ZYMV	China	AF513552	shandong
ZYMV	China	AF513551	ningbo
ZYMV	China	AF513550	shangyu
ZYMV	Japan	AB063251	M39
ZYMV	China	AF486823	hainan
ZYMV	China	AF486822	dongyang
ZYMV	China	AY074810	ningbo
ZYMV	China	AY074808	shanxi
ZYMV	China	AY074809	Beijing
ZYMV	Taiwan	AF127929	TW-TN3
ZYMV	China	AF435425	Hangzhou
ZYMV	Korea	AF062518	cu
ZYMV	Taiwan	AF127934	TW-PT5
ZYMV	Taiwan	AF127932	TW-TNML1
ZYMV	Taiwan	AF127931	TW-TC1
ZYMV	Taiwan	AF127930	TW-CY2
ZYMV	Japan	AB004641	-
ZYMV	Japan	AB004640	-
ZYMV	Israel	M35095	-
ZYMV	Singapore	AF014811	-
ZYMV	Singapore	X62662	-
ZYMV	Syria	AB458596	SYZY-3
ZYMV	Syria	AB458595	SYZY-1
ZYMV	Iran	FJ705272	Azr.Mak.W
ZYMV	Iran	FJ705271	Yaz.Yaz.C
ZYMV	Iran	FJ705270	the.Kar.S
ZYMV	Iran	FJ705269	Sis.Zah.C
ZYMV	Iran	FJ705268	Sis.Zab.W
ZYMV	Iran	FJ705267	Kho.Mash.S
ZYMV	Iran	FJ705266	Ker.Ker.S
ZYMV	Iran	FJ705265	Ker.Jir.W
ZYMV	Iran	FJ705264	Ker.Baf.S
ZYMV	Iran	FJ705263	Hor.Min.S
ZYMV	Iran	FJ705262	Hor.Haj.W
ZYMV	Iran	FJ705261	Ham.Mal.W
ZYMV	Iran	FJ705260	Ham.Aas.C
ZYMV	Iran	FJ705259	Gil.Ras.C
ZYMV	Iran	FJ705258	Far.Mar.M
ZYMV	Iran	FJ705257	Esf.Esf.C
ZYMV	Iran	FJ705256	Bos.Bos.M
ZYMV	Iran	FJ705255	Bor.Bor.S
ZYMV	Iran	FJ705254	Azr.Tab.S
ZYMV	Iran	FJ705253	Azr.Sha.C
ZYMV	Iran	FJ705252	Aza.Mah.W
ZYMV [a]	Australia	S81377	AU-A3
ZYMV [a]	Australia	S81381	AU-G4
BCMV	Australia	EU761198	MS1
WMV	Tonga	L22907	-
SMV	China	U25673	-

[a], denotes partial sequence only.

Supplementary Fig. 1. Neighbour-joining relationship phylogram obtained from alignment of 42 complete and 8 partial coat protein nucleotide sequences of new Australian *Zucchini yellow mosaic virus* (ZYMV) isolates and 101 ZYMV sequences from GenBank. Three groups (A-C) and within A four subgroups (I-IV) are shown. The tree was generated using the ClustralW and MEGA 4.1 programmes set to default parameters. Tree branches were bootstrapped with 1,000 replications. Numbers at nodes indicate bootstrap scores of >50%. The scale bar represents a genetic distance of 0.05 for horizontal branch lengths. New sequences are without GenBank codes, in bold and highlighted, but other sequences show their GenBank codes and countries of origin. For isolate designation see Table 1 (new isolates) and Supplementary Table 1 (other isolates). The tree was rooted with *Bean common mosaic virus* (BCMV, EU761198), *Watermelon mosaic virus* (WMV, L22907) and *Soybean mosaic virus* (SMV, U25673) sequences.

Supplementary Fig. 1



SECTION 3

Zucchini yellow mosaic virus: contact transmission, stability on surfaces, and inactivation with disinfectants

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(Draft of paper submitted for publication in Plant Disease, 2011)

Abstract

In glasshouse experiments, *Zucchini yellow mosaic virus* (ZYMV) was transmitted from infected to healthy zucchini (*Cucurbita pepo*) plants by direct contact when leaves were rubbed against each other, crushed or trampled, and, to a lesser extent, on blades contaminated by infective sap. When infective sap from zucchini plants infected with three ZYMV isolates was kept at room temperature for up to 6 h, it still infected healthy plants readily. Also, when infective sap was applied to seven surfaces (cotton, plastic, leather, metal, tyre, rubber soled footwear and skin) and left for up to 24 h before the contaminated surface was wiped onto healthy zucchini plants, ZYMV remained infective for 24 h on plastic, and up to 6 h on the other six surfaces. The effectiveness of nine disinfectants at inactivating ZYMV was evaluated by adding them to infective sap which was then inoculated to zucchini plants. None became infected when nonfat dried milk (20% w/v), bleach (42g/l sodium hypochlorite, diluted 1:4), household disinfectant (1.5% w/v benzalkonium chloride, diluted 1:20) or Farmcleanse® (diluted 1:10) were used, but infective sap without disinfectant readily infected them. When ZYMV-infected pumpkin leaves were trampled by footwear, and then used to trample healthy plants, all plants became infected but when contaminated footwear was dipped in a footbath containing bleach (42g/l sodium hypochlorite, diluted 1:4) before trampling, none became infected. This study demonstrates ZYMV can be transmitted by contact and highlights the need for on-farm hygiene practices (de-contaminating tools, machinery, clothing, etc.) to be included in integrated disease management strategies for ZYMV in cucurbit crops.

Introduction

Zucchini yellow mosaic virus (ZYMV; family *Potyviridae*, genus *Potyvirus*) is one of the most economically important viruses of cucurbit crops worldwide. When cucurbit crops become infected early by ZYMV, losses in yield and marketable fruit can be up to 100% (e.g., 3, 10, 13, 15). ZYMV was first isolated in Italy in 1973 and described in 1981 (27). Subsequently, it spread to more than 50 countries across five continents (13), and epidemics of ZYMV within cucurbit crops now occur worldwide (e.g., 14, 17, 46, 28). ZYMV is non-persistently transmitted by a number of cucurbit colonising and non-colonising aphid species (22), is seed-borne at low levels in some cucurbit species (e.g., 11, 15, 38, 40, 44), and has a limited number of alternative hosts which can act as infection reservoirs outside the cucurbit growing period (e.g., 9, 11, 13, 34, 42). Many studies have investigated the molecular properties of ZYMV (e.g., 1, 11, 13, 14, 50). Control measures have also been studied and integrated disease management approaches devised (e.g. 10, 13, 32), but controlling spread of the virus successfully in cucurbit crops has still proven difficult. This difficulty and the spread of ZYMV within cucurbit crops when aphids are apparently absent, suggests there may be other methods by which ZYMV is transmitted which are not being addressed by current control measures.

Plant viruses are transmitted by vegetative propagation, vectors, seed and contact. Readily contact-transmitted viruses typically have stable virus particles that reach very high concentrations within the epidermal cells of infected plants (e.g., 2, 30). Also, for successful contact transmission the host plant needs to be easily wounded during abrasion such that the leaf epidermis is slightly damaged enabling virus particles to penetrate its cuticle and cells were it can replicate (30). Under natural conditions in the field sublethal wounding of plant cells readily occurs readily during normal leaf abrasion, cultivation practices and other types of foliar contact including with animals and man (16). Most examples of such viruses are in the genera *Carlavirus* (e.g. *Potato virus S,* PVS)*, Sobemovirus* (eg. *Rice yellow mottle virus,* RYMV; *Subterranean clover mottle virus,* SCMoV), *Tobamovirus* (e.g. *Hibiscus latent Fort Pierce virus*, HLFPV; *Odontoglossum ringspot virus*, ORSV; *Tobacco mosaic virus,* TMV) and *Potexvirus* (e.g. *Cymbidium mosaic virus,* CYMV; *Pepino mosaic virus,* PeMV*; Potato virus X*, PVX; *White clover mosaic virus*, WCMV). These viruses spread directly from infected to healthy plants when leaves rub together under the influence of wind. They also spread indirectly (i) when infective sap contaminating clothes, hands, cutting/pruning tools, equipment and machinery comes into contact with healthy plant material, or (ii) animals graze and trample infected plants before moving to healthy plants (e.g., 6, 19, 21, 23, 31, 36, 37, 41, 45). Viruses in the family *Potyviridae* differ in that their particles are less stable and occur at lower concentration in plant cells. However, there are several reports that they spread to a limited extent by contact: *Wheat streak mosaic virus* spread in wheat when leaves of infected plants brushed against healthy plants (4, 39); *Potato virus Y* spread from infected to healthy glasshouse grown tomato plants on contaminated tools during pruning (48); and *Bean yellow mosaic virus* was to be transmitted between gladiolus plants on cutting tools (5). There is some anecdotal evidence that ZYMV spreads within cucurbit crops by contact. Fletcher et al. (15) suggested spread was by machinery because plants with disease symptoms were prevalent along machinery tracks. Similarly, Riedle-Bauer (35) suggested that wounds created during mechanical weed control operations allow plant-to-plant spread of ZYMV and that its particles may also be carried by vertebrates, such as rabbits. However, there is no experimental evidence to support these suggestions.

Traditional measures of virus stability and concentration *in vitro* including thermal inactivation point, dilution end point and longevity *in vitro* provide an indication of virus stability and therefore ability to be contact transmitted (2). *Tobamovirus* species have thermal inactivation points of 80-95^{o}C, their sap remains infective at room temperature for decades and they have dilution end-points of 10^{-5}-10^{-6} (18). Similarly, *Potexvirus* species have thermal inactivation points of 60-80^{o}C, longevities *in vitro* of weeks to months and dilution end-points of 10^{-5}-10^{-6} (24). In contrast, ZYMV has a thermal inactivation point of 55^{o}C, sap remains infective up to 3 days and the dilution end-point is 10^{-4} (26). This might be interpreted as indicating contact transmission of ZYMV is unlikely.

PVX survived and remained infective up to 3 h on metal and rubber surfaces and up to 6 h on cotton fabric, while PVS remained infective on these surfaces for up to 25 h (16, 45, 49). TMV also survived for several weeks on various glasshouse surfaces and clothing (6). For viruses spread on contaminated cutting tools, clothing or machinery, disinfectants can be used to inactivate and control virus spread between plants. Their effectiveness and usefulness depends on the type of disinfectant, its concentration and incubation time, as well as considerations such as availability, cost and potential toxicity to humans and plants. Trisodium phosphate (10% for 30 s) inactivated TMV and PVX, and was safe for equipment and hands (7). For CyMV and ORSV, NaOH (1%) was effective at inactivating both viruses without causing phytotoxic damage (19).

Seven disinfectants were effective for eliminating PepMV from five common glasshouse surfaces (33). Treating contaminated cutting tools with 10% sodium hypochlorite or 20% nonfat dry milk were effective at preventing HLFPV infection during plant propagation and pruning (23). Use of 20% nonfat dry milk plus 0.1% Tween 20 or household bleach (0.6% sodium hypochlorite, 1:10 dilution) on contaminated cutting tools completely eliminated TMV transmission to petunias (25).

In this study, mechanical transmission refers only to experimental inoculation with infective sap containing an abrasive (e.g. carborundum or diatomaceous earth), while contact transmission refers to inoculation with infective material so that minute wounding occurs in the absence of an abrasive without causing visible damage. Wound-mediated transmission refers to wounding causing visible damage such as cuts or bruises (30). This paper describes a series of glasshouse experiments undertaken with ZYMV to determine (i) if it can be transmitted to healthy plants by leaf rubbing, via crushing and trampling, or on contaminated blades, (ii) its stability in infective sap over time and on different surfaces, and (iii) the effectiveness of nine disinfectants in inactivating it in infective sap.

Materials and Methods

Virus isolates, plants, inoculations and antiserum

Test, culture, inoculum source plants and all experiments were kept in insect-proof, air-conditioned glasshouses maintained at 18-22^{o}C. Plants of zucchini (*Cucurbita pepo*) cv. Blackjack and Jarrahdale type pumpkin (*Cucurbita maxima*) cv. WA Grey were grown in pots in steam-sterilised soil, sand and peat mix (1:1:1). Zucchini plants were used in all experiments except experiments 4 and 7 that used pumpkin plants. ZYMV isolates Knx-1, Cvn-1, Nt-1 and Vic-1 were from previous studies (10, 11). They were maintained in zucchini plants by sap inoculation and were the sources of ZYMV inoculum for glasshouse experiments. For sap inoculation to maintain cultures, ZYMV-infected leaves were ground in 0.1M phosphate buffer, pH 7.2, and the sap mixed with celite before being rubbed onto the leaves of plants. Leaf samples from the isolate Knx-1 culture were used as the positive control in enzyme-linked immunosorbent assay (ELISA). The polyclonal antiserum to ZYMV used was from Prime Diagnostics, Netherlands.

Enzyme-linked immunosorbent assay

To test for ZYMV infection, leaf samples were extracted singly in phosphate buffered saline (10mM potassium phosphate, 150mM sodium chloride), pH 7.4, containing 5ml/L of Tween 20 and 20g/liter of polyvinyl pyrrolidone, using a mixer mill (Retsch, Germany). Sample extracts were tested for ZYMV by double antibody sandwich ELISA (8). Each sample was tested in duplicate wells in microtiter plates and appropriate infected and healthy leaf samples included in paired wells as controls. The substrate used was 0.6mg/mL of *p*-nitrophenyl phosphate in 100ml/L of diethanolamine, pH 9.8. Absorbance values (A_{405}) were measured in a microplate reader (Bio-Rad Laboratories, USA). Positive absorbance values were always at least 10 times those of healthy sap.

Contact-mediated transmission

Experiments 1a and 1b investigated whether ZYMV could spread from infected to healthy plants by leaf-to-leaf contact. A leaf from a zucchini plant infected with one of isolates Knx-1, Cvn-1, Vic-1 (experiments 1a and 1b) or Nt-1 (experiment 1b only) was gently rubbed gently onto the leaf surface of a healthy zucchini plant such that no visual damage occurred. For each isolate, the leaves of six (experiment 1a) or ten (experiment 1b) plants with two leaves each were rubbed with an infected leaf. The same numbers

of plants were rubbed with healthy zucchini leaves as controls. A single tip leaf was sampled from each plant at 21 (experiment 1a) and 20 (experiment 1b) days after rubbing and tested individually by ELISA.

Wound-mediated transmission

Experiments 2a and 2b simulated virus transmission on contaminated tools, such as knives used to harvest cucurbit fruit. A large scalpel blade was used to cut through a leaf petiole (experiment 2a) or small fruit (experiment 2b) of a zucchini plant infected with ZYMV isolate Knx-1. This blade was then used to cut off the leaf petioles of a zucchini test plant with six leaves. Scalpel blades were changed between each test plant. The numbers of petioles or fruits cut off were 1, 3 or 5 per infected plant followed by the same number of petiole cuts on the test plant. Five plants were used for each type of cutting combination. There were also two healthy control plants for each cutting treatment in which leaf petioles or fruits were cut from healthy plants before cutting the control plants. In addition, five plants were included as a positive control in which leaf-to-leaf rubbing occurred as used in experiment 1. A single leaf tip was sampled from each plant and tested individually by ELISA at 39 (experiment 3a) or 34 (experiments 3b) days after cutting.

Experiments 3a-d simulated virus transmission by leaf crushing such as when leaves are damaged by machinery tyres. A rubber mallet with parafilm secured with an elastic band to cover its head (5cm diameter) was used to tap zucchini leaves infected with isolates Knx-1 (experiments 3a and 3b) or Cvn-1 (experiments 3c and 3d). This rubber mallet was then used to tap a leaf of a healthy zucchini test plant (Figure 1a and b). The number of taps was 1, 3 or 5 on an infected plant leaf followed by the same number of taps on a test plant leaf. The parafilm was replaced between each test plant. There was a negative control in which leaves of healthy plants were tapped in the same way before tapping the leaf of a healthy test plant. Also, a positive control was included in which a leaf-to-leaf rubbing occurred as in experiment 1. Five plants were used for each experimental treatment. A single tip leaf was sampled from each test plant at 34, 24, 21 or 37 days after mallet inoculation in experiments 3a, 3b, 3c or 3d respectively, and tested individually by ELISA.

Experiment 4 simulated virus transmission by trampling caused by movement of humans through a cucurbit crop. Pumpkin plants were grown in large pots and placed so that their leaves grew over the glasshouse floor. Four groups, each with nine pots (2 plants/pot) were arranged so that at least 5 m separated each group. The experimental treatments were: (i) pumpkin leaves infected with Knx-1 trampled 10 times with rubber-soled shoes and then test plants trampled; (ii) as for (i) but healthy leaves trampled initially; (iii) infected leaves run over five times by a tyre of a filled wheelbarrow and then test plants run over; and (iv) plants not trampled or run over (control). Eighteen plants were used for each experimental treatment which was applied 55 days after sowing. A single tip leaf was sampled from each test plant 21 days after trampling and tested individually by ELISA.

Stability

In experiments 5a-e, to establish the stability of infectivity, sap was extracted from zucchini leaves infected with ZYMV isolate Knx-1 (experiments 5a and 5b), Cvn-1 (experiment 5c), Nt-1 (experiment 5d) or Vic-1 (experiment 5e) using a leaf press. Sub-samples of 1.5 ml each were placed into individual porcelain bowls (experiments 5a, 5d and 5e) or 2 ml sub-samples into individual holes in a 12-hole metal muffin baking tray (experiments 5b and 5c). In experiment 5a, the infective sap was left at room temperature for 5 min, 30 min, 1, 3, 6, 24 and 48 h before 1.5 ml of distilled water was added to resuspend it. In experiments 5b and 5c the time periods at room temperature

were 5 min, 30 min, 1, 2, 3 and 6 h after which 2 ml of distilled water was added to resuspend the sap. In experiments 5d and 5e, the periods at room temperature were as for experiment 5b, but with an additional 24 hr period. In experiments 5a-e, after each time period once the sap was resuspended it was immediately inoculated without buffer or abrasive onto leaves of five zucchini plants (two leaves/plant). Five plants were left uninoculated as controls. A single tip leaf was sampled from each plant 21 and 27 (experiment 5a), 14 and 22 (experiment 5b-e) days after inoculation and tested individually by ELISA.

In experiment 6, to establish the stability of infectivity on different surfaces, sap was extracted from zucchini leaves infected with ZYMV isolate Knx-1 using a leaf press and placed on each test surface. The test surfaces were: cotton fabric (worn and washed), rubber shoe sole (worn), tyre (inner rubber tube), leather (worn boot), metal (steel), plastic (black horticultural plastic mulch) and skin (human). For each surface, 0.5 ml of infective sap was applied to a 2 x 2 cm area. The applied sap was left at room temperature for 5 min, 30 min, 1, 6 and 24 h after application, except with skin which was left for 5 min, 30 min and 1 h. There were five replications of each surface for each time period. For inoculation, the surface contaminated with infective sap was wiped directly onto the surface of zucchini leaves (two leaves/plant). For controls, five plants were left uninoculated and five plants were inoculated (without buffer or abrasive) with the fresh sap extract. A single tip leaf was sampled from each test plant 15, 21 and 27 days after inoculation and tested individually by ELISA.

Disinfectants

In experiment 7, to investigate inactivation by different disinfectants, sap was extracted from zucchini leaves infected with ZYMV isolate Knx-1 using a leaf press and 1 ml of extract was diluted with 1 ml of each of the following: nonfat dried milk (20% w/v); nonfat dried milk (20% w/v) + Tween 20 (0.1%); Tween 20 (0.1%); household bleach (sodium hypochlorite 42g/l, 4% available chlorine, diluted 1:20 or 1:4); Menno-Florades® (9% benzoic acid, diluted 3%); antibacterial dishwashing concentrate with tinosan® HP100 (2-hydroxy-4,4-dichloro-diphenyl ether, diluted 0.4%); household disinfectant (benzalkonium chloride 1.5% w/w, diluted 1:20); Virkon® (potassium peroxymonosulphate 50% w/w, dilution 0.5% or 1%); Farmcleanse® (alkylbenzene sulfonic acid 1-10%, 1:10 dilution); hand sanitizer (62% ethanol, undiluted); inoculation buffer; and distilled water. All disinfectants were diluted in distilled water and used at the manufacturer's recommended rate. The mixture of infective sap and disinfectant was then used to inoculate zucchini plants without buffer or abrasive, following which inoculated leaves were rinsed promptly with tap water. For controls, plants were inoculated with undiluted sap without buffer or abrasive, and other plants left uninoculated. Five plants, (two leaves/plant) were used for each experimental treatment (disinfectants and controls). A single tip leaf was sampled from each test plant 14, 19 and 27 days after inoculation and tested individually by ELISA.

To study inactivation efficacies of different disinfectants, in experiments 8a and 8b, a rubber mallet with parafilm covering its head was used to tap a ZYMV isolate Knx-1 infected zucchini leaf five times, a disinfectant was applied to the parafilm and then a leaf of a healthy test plant was tapped five times. The parafilm was changed between each test plant. With each disinfectant or control experimental treatment, five plants were used (three leaves/plant). All disinfectants were diluted in distilled water and used at the manufacturer's recommended rate. Each disinfectant was applied as an aerosol spray to the parafilm on the mallet (3 sprays only), except for the Isowipes where the wipe was used directly on the parafilm. For controls, no disinfectant was applied after tapping an infected or healthy leaf, or plants were left undamaged. In experiment 8a, the experimental disinfectant treatments were: denatured ethanol (70%); Farmcleanse

(diluted 1:10); Virkon® (0.5%); household bleach (diluted 1:20 and 1:4); household disinfectant; antibacterial dishwashing concentrate with tinosan HP100 (0.4%); Menno-Florades (3%); Cerama Klen (alkaline salts, 34g/kg available chlorine, diluted 2.5%); Isowipe bacterial wipes (70% v/v isopropyl alcohol impregnated wipe); and distilled water. A single tip leaf was sampled from each test plant 21, 34 and 42 days after bashing and tested individually by ELISA. Experiment 8b was a repeat of experiment 8a, except that household disinfectant, dishwashing concentrate, distilled water and undamaged control treatments were omitted. A single tip leaf was sampled from each test plant 23, 30 and 37 days after inoculation and tested individually by ELISA.

Experiment 9, was similar to experiment 4, except that each experimental treatment consisted of six plants (one plant/pot). Treatments were applied 67 days after sowing and were: (i) pumpkin leaves infected with isolate Knx-1, or (ii) healthy leaf material trampled on 10 times with rubber-soled shoes, then test plants trampled; (iii) infected leaf material trampled 10 times with rubber-soled shoes, then shoes soaked for 30 s in a foot bath containing household bleach (diluted 1:20), then test plants trampled; and (iv) plants not trampled (Figure 1c and d). A single tip leaf was sampled from each test plant 49 days after trampling and tested individually by ELISA.

In experiment 10 to establish if disinfectants caused damage to metal cutting tools, carbon steel surgical scalpel blades were dipped into each of the following: bleach (1:4 dilution), Farmcleanse, nonfat dried milk, household disinfectant, inoculation buffer, undiluted fresh sap and distilled water for 30 s and then left to air-dry. In addition, blades were dipped into bleach (1:4 dilution) for 30 s followed by distilled water for 30 s. For each dipping solution three blades were used. The blades were assessed visually for area of corrosion after 1 h and 24 h.

Results

Contact transmission

In experiments 1a and 1b, the number of plants that became infected by rubbing with leaves infected with isolates Knx-1, Cvn-1, Vic-1 and Nt-1 were 16/16, 15/16, 15/16 and 8/10, respectively (Table 1). Symptoms on infected plants were leaf mottle and distortion. In experiments 2a and 2b, 0/10, 3/10 and 5/10 plants became infected following cutting 1, 3 or 5 times with a scalpel blade contaminated with infective sap, respectively. Infected plants developed mild mottle. None of the control plants ever became infected in any of experiments 1-2.

In experiments 3a-d, heavily crushed leaves (5 taps) had extensive damage often dying and falling off, whereas lightly (1 tap) or moderately (3 taps) crushed leaves had mild damage and often recovered. In experiments 3a and 3b, when healthy leaves were crushed with a rubber mallet contaminated with isolate Knx-1, 1/10, 6/10 and 9/10 plants became infected when lightly (1 tap), moderately (3 taps) and heavily (5 taps) crushed, respectively (Table 1). In experiments 3c and 3d, when healthy leaves were crushed with a mallet contaminated with isolate Cvn-1, the corresponding figures were 4/10, 3/10 and 6/10 plants. Infected plants developed leaf mottle and distortion. All plants became infected when infected leaves were rubbed onto healthy plants, and none of the healthy control plants ever became infected.

In experiment 4, when a wheelbarrow was used to crush isolate Knx-1 infected leaves and then healthy pumpkin leaves, damage was extensive and many of the growing tips and leaves died, only 1/18 plants becoming infected. When plants were trampled with footwear 9/18 plants became infected, despite leaf damage consisting of bruising and

tearing. Leaf mottle, leaf distortion and plant stunting developed on infected plants. None of the healthy control plants became infected.

Stability

In experiments 5a-c, sap containing isolates Knx-1 and Cvn-1 remained infective for up to 6 hr after extraction with 3-5/5 plants becoming infected on each occasion, but none became infected after 24 or 48 h periods (Table 2). In experiment 5d, with sap containing isolate Nt-1, 1/5 plants became infected 5 min after extraction, but 0/5 plants became infected after 30 min – 24 h time periods. In experiment 5e, with isolate Vic-1, sap remained infective up to 3 hrs, with 1-4/5 plants becoming infected after each time period, but none became infected after 6 or 24 h. None of the control plants became infected.

In experiment 6, on all surfaces except skin, sap remained infective up to 6 hr after extraction, with 1-5/5 plants becoming infected (Table 3). After 24 h, sap remained infective on plastic but not on leather or metal. With skin, 1/5 plants became infected after 5 min but none after 30 or 60 min. For the controls, all plants became infected when freshly extracted infected sap was used, but none became infected when healthy plants were uninoculated.

Disinfectants

When disinfectants were added to infective sap prior to inoculation (experiment 7), 0/5 plants became infected with nonfat dried milk (with and without Tween 20), bleach (1:4), household disinfectant or Farmcleanse; 1/5 plants with Virkon (0.5% and 1%) and bleach (1:20); 2/5 plants with hand sanitizer, dishwashing liquid or Menno; and 4/5 plants with inoculation buffer, Tween 20 or distilled water (Table 4). When infective sap was used without any disinfectant, 5/5 plants became infected. Infected plants developed symptoms of mottle on tip leaves. None of the healthy control plants became infected. No phytotoxic damage was caused to zucchini plants by any of the disinfectants.

In experiments 8a and 8b, when plants were crushed with a mallet contaminated with infective sap and no disinfectant was applied, 8/10 became infected (Table 4). None became infected when the contaminated mallet was treated with Farmcleanse, Virkon, household bleach (2 dilutions), household disinfectant, or Menno. Some plants became infected when Cerama Klen, dishwashing concentrate, ethanol, Isowipes or distilled water were used. Crushed leaves had visible bruising and lacerations, but remained attached. Infected plants developed symptoms of mottle on their tip leaves. None of the healthy control plants became infected. No phytotoxic reactions were caused by any of the disinfectants.

In experiment 9, when ZYMV-infected leaves were trampled on prior to healthy plants, 5/6 plants became infected, but none became infected when a footbath containing bleach (1:20) was used to wash footwear before trampling healthy leaves. Infected pumpkin plants developed mild mottle, distortion and stunting. None of the control plants became infected.

In experiment 10, blades dipped in bleach developed surface corrosion within 5 min and 25-50% of their surface area was corroded after 1 h (Table 4). No corrosion developed on blades dipped in bleach followed by a distilled water rinse, Farmcleanse, inoculation buffer or water. Fresh sap, nonfat dried milk and household disinfectant caused corrosion to <25% of the blade surface after 1 h.

Discussion

This study shows that ZYMV can spread from infected to healthy cucurbit plants by leaf contact (rubbing and wounding), and on blades contaminated with infective sap. This knowledge provides important new information on the epidemiology of ZYMV as previously the only known methods of natural plant-to-plant transmission were *via* aphid vectors and, occasionally, cucurbit seed. ZYMV epidemics often develop very rapidly and contact transmission between plants is likely to contribute to this. We also found that ZYMV remains infective in dried sap at room temperature for up to 6 h and after drying on different types of surface for up to 24 h. Fortunately, however, it was inactivated by several disinfectants. Unlike other studies on the stability of infectivity of contact transmitted viruses, we did not apply any abrasive (e.g. carborundum) during inoculation of test plants or dilute the sap with phosphate inoculation buffer or water during extraction or swabbing. ZYMV infectivity would have survived for longer if such procedures had been followed but we were attempting to reproduce scenarios likely to occur in cucurbit production systems.

Cucurbit plants have leaf surfaces covered with long, thick, sharp hairs (47) that easily cause fine scratches and wounds when leaves rub together, so contact transmission can occur when healthy and infected plants intertwine (e.g. in pumpkin and melon crops) or leaves rub together through wind movement. The leaves of cucurbit plants also have large surface areas and are often soft. Therefore, although ZYMV concentration in leaves is lower than that of most other contact transmissible viruses, the large surface area and soft growth would provide increased opportunity for contact transmission by rubbing. Studies on whether ZYMV could be spread by leaf rubbing caused by air movement alone should be done in an aphid-proof environment so that infected and healthy pumpkin or melon plants intertwine and move by wind. ZYMV was also transmitted by crushing healthy leaves in presence of infective sap and when leaves had mild or extensive damage (lacerated and bruised), our crushing and trampling experiments still showed ZYMV transmission readily occurred. Such transmission can happen when plants are damaged by machinery, or when humans or other large mammals move through a crop.

Overall, there was little difference between ZYMV isolates Knx-1 and Cvn-1 in the contact transmissibility (leaf rubbing and crushing) or survival of infectivity in sap over time (6 h), although they represent two phylogenetic groups (B and A-I, respectively) (11). Isolate Vic-1 which belongs to group A-I was also readily contact transmitted and its infectivity survived in sap for up to 3 h. Interestingly, isolate Nt-1 belonging to phylogenetic group A-II was readily transmitted by leaf rubbing but its infectivity in extracted sap was lost rapidly (30 min after extraction). Further studies on the stability of infectivity of isolates from group A-II would be of interest.

As mentioned in the Introduction, contaminated cutting tools are known to spread a number of different viruses including PVS (16), HLFPV (23), CyMV, ORSV (19), TMV (25) and PVY (48). Cutting blades contaminated with infective sap also transmitted ZYMV, but at least 3 cuts of an infected leaf petiole or small fruit were needed before healthy test plants became infected. Little sap was expressed following each cut as zucchini leaf petioles are hollow and their fruit dense. This explains why several cuts were needed with the same blade so that sufficient infective sap coated it prior to cutting the healthy plant.

ZYMV remained infective for up to 6 h in sap contaminating seven different types of surfaces. With skin however, little infectivity survived. Possibly the virus was inactivated by previous use of skin care products or by rapid drying of sap within 5 min of application. With cotton and leather, absorption of sap started within 30 min and by 6 h it was completely absorbed and dried. With shoe sole, tyre, metal and plastic surfaces, sap started to dry out within 60 min and was completely dried by 6 h. These findings indicate that ZYMV could be spread on clothing, footwear and machinery, for up to 6 h after contamination with infective sap and up to 24 h on plastic. When such surfaces were tested under similar conditions, infectivity of PVX was retained for 10 sec on leather, 3 h on rubber and skin, and 6 h on cotton (49); and infectivity of PVS for 7 h on metal and 25 h on rubber (16). Moreover, TMV remained infective for several weeks on different surfaces (6).

Considerations in determining the appropriate disinfectant to use need to include: virus inactivation, ease of use (time of incubation), safety to personnel, availability, cost, and corrosiveness on equipment. For these reasons we included mostly household and commercially available products in our studies. Both 20% nonfat dry milk and 1:4 dilution household bleach (sodium hypochlorite) inactivated ZYMV infectivity. Similarly, Lewandowski et al. (25) found treating TMV contaminated cutting tools with a solution of nonfat dry milk plus Tween 20 or household bleach completely eliminated TMV transmission to petunia plants. When we tested nonfat dry milk with and without Tween 20, both solutions prevented ZYMV infection but Tween 20 alone did not. Sodium hypochlorite is considered extremely effective against all types of microorganisms and has low toxicity, is easy to use and is relatively low cost (20). It was also effective at inactivating PepMV from glasshouse surfaces (33), and PVY (48), HLFPV (23) and *Tomato chlorotic dwarf viroid* (29) from contaminated cutting blades. However, a drawback to sodium hypochlorite solutions is that they are corrosive. We found surface corrosion on steel blades started within 5 min of dipping in bleach containing sodium hypochlorite, but rinsing in water reduced this. Commercial products are often recommended for decontaminating tools and surfaces from plant viruses and viroids (33, 43). We found Menno-Florandes, Virkon and CeramaKlen did not completely inactivate ZYMV, but Farmcleanse was effective and did not corrode blades. Alcohols such as denatured ethanol (70%), hand sanitizer (62% ethanol), and Isowipes (70% isopropyl alcohol) were only partially effective.

This work raises the question as to whether contact transmissibility occurs with other *Potyviruses*. From our initial experiments with other potyviruses infecting cucurbits, *Papaya ringspot virus* (PRSV) and *Watermelon mosaic virus* (WMV) could both be transmitted readily when infected leaves were lightly rubbed on healthy leaves of cucurbit plants (10/16 and 14/16 plants infected respectively) (unpubl. data), and further research is underway on the contact transmissibility of these two viruses. It would also be of interest to examine the contact transmissibility other potyviruses that are known to reach high infection incidences in field crops. For example, *Pea seed-borne mosaic virus* can reach levels up to 100% within field pea crops with little evidence of colonisation by aphid vectors (12), so perhaps, in addition to transmission by non-colonising migrant aphids, leaf-to-leaf contact is providing another avenue for spread when plants intertwine and wind moves them.

Integrated disease management (IDM) strategies for control of ZYMV in Australian cucurbit crops were described previously (9, 10). Recommended measures include: isolation of new cucurbit plantings from older ones; removing any potential alternative virus reservoirs (weeds, volunteer cucurbit plants, old finished or abandoned crops) during and between growing seasons; roguing of plants with virus symptoms; use of reflective mulch; planting upwind of potential virus sources; manipulation of sowing

date; deployment of tall non-host millet barriers; and use of pumpkin and zucchini cultivars with *Zym* resistance gene and cucumber cultivars with *zym* gene. However, infection with ZYMV continues to be widespread in cucurbit crops in tropical and sub-tropical Western Australia with extensive crop losses reported (10). This study identifies a previously unknown method of ZYMV transmission that may contribute to within crop epidemics and is not being addressed by current IDM approaches. Thus, the ease that ZYMV was transmitted by contact was surprising and highlights the importance of sanitation and hygiene practices in cucurbit production. Measures that address contact transmission such as washing down machinery, disinfecting cutting tools and surfaces and limiting handling and movement within crops have now been included within a modified integrated management strategies for viruses of cucurbits.

Acknowledgements

We thank Eva Gajda for assisting with ELISA. This research was funded by Horticulture Australia Ltd projects VG06022 and VG01728.

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Table 1. Contact transmission of *Zucchini yellow mosaic virus* (ZYMV) to zucchini plants by leaf rubbing, cutting and crushing (experiments 1, 2 and 3)

Treatment [a]	ZYMV isolate	Number of plants ZYMV-infected/total number of plants [b]							
		Expt 1a	Expt 1b	Expt 2a	Expt 2b	Expt 3a	Expt 3b	Expt 3c	Expt 3d
Rub	Knx-1	6/6	10/10	-	-	5/5	5/5	-	-
	Cvn-1	5/6	10/10	-	-	-	-	5/5	5/5
	Vic-1	5/6	10/10	-	-	-	-	-	-
	Nt-1	nt	8/10	-	-	-	-	-	-
Cut x 1	Knx-1	-	-	0/5	0/5	-	-	-	-
x 3	Knx-1	-	-	1/5	2/5	-	-	-	-
x 5	Knx-1	-	-	3/5	2/5	-	-	-	-
Crush x 1	Knx-1	-	-	-	-	1/5	0/5	-	-
x 3	Knx-1	-	-	-	-	3/5	3/5	-	-
x 5	Knx-1	-	-	-	-	4/5	5/5	-	-
Crush x 1	Cvn-1	-	-	-	-	-	-	1/5	3/5
x 3	Cvn-1	-	-	-	-	-	-	1/5	2/5
x 5	Cvn-1	-	-	-	-	-	-	1/5	5/5
Control		0/6	0/10	0/15	0/15	0/15	0/15	0/15	0/15

[a] Rub = infected leaf gently rubbed onto healthy plant leaf; Cut = scalpel blade used to cut infected leaf petiole (experiment 2a) or small fruit (experiment 2b) before cutting healthy petioles; Crush = rubber mallet covered with parafilm used to crush infected leaf then crush healthy plant leaf.
[b] Tip leaves from all plants tested individually up to 39 days after inoculation by ELISA.

Table 2. Infectivity of *Zucchini yellow mosaic virus* (ZYMV) infective of sap incubated for different time periods before inoculation to zucchini (experiment 5)

Expt	ZYMV isolate	Number of plants ZYMV-infected[a]							
		Incubation time periods prior to inoculation[b]							
		5 min	30 min	60 min	2 h	3 h	6 h	24 h	48 h
a	Knx-1	4	4	4	nt	3	3	0	0
b	Knx-1	5	5	4	5	5	5	nt	nt
c	Cvn-1	5	3	4	3	3	5	nt	nt
d	Nt-1	1	0	0	0	0	0	0	nt
e	Vic-1	2	3	4	1	1	0	0	nt

[a] Each treatment consisted of 5 plants of zucchini cv. Blackjack. nt = not tested. Tip leaves from all plants tested individually up to 27 days after inoculation by ELISA.
[b] Infective sap left at room temperature in porcelain bowls or metal trays for the incubation period, then resuspended in distilled water (1:1).

Table 3. Infectivity of *Zucchini yellow mosaic virus* (ZYMV) infective sap after incubation on seven surfaces for different time periods (experiment 6)

Surface	Number of plants ZYMV infected [a]				
	Incubation time periods prior to inoculation[b]				
	5 min	30 min	60 min	6 h	24 h
Cotton	5	4	5	3	nt
Rubber shoe sole	3	3	4	3	nt
Tyre - inner tube	5	3	3	4	nt
Leather	5	5	5	1	0
Metal	5	5	5	3	0
Plastic	5	5	4	5	2
Skin	1	0	0	nt	nt
Fresh infective sap	5	-	-	-	-
Control	0	-	-	-	-

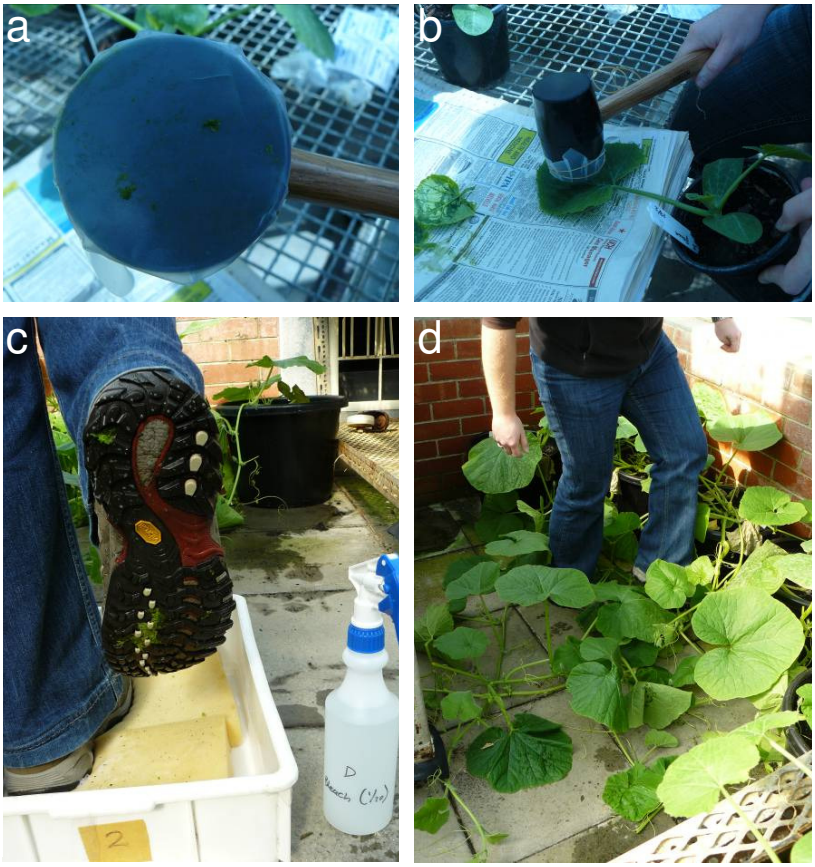
[a] Each treatment consisted of 5 plants of zucchini cv. Blackjack, isolate Knx-1 was used. Tip leaves from all plants tested individually up to 27 days after inoculation by ELISA. nt = not tested.
[b] Infective sap applied to surface, left at room temperature for each incubation period and then wiped onto leaves of test plants.

Table 4. Inactivation of *Zucchini yellow mosaic virus* (ZYMV) in infective sap with various disinfectants

Experimental treatment	No. of plants ZYMV infected[a]			Corrosion on blades after 1 hr [d]
	Expt 7[b]	Expt 8a[c]	Expt 8b[c]	
Non-fat dry milk (20% wt/vol)	0	nt	nt	+
Non-fat dry milk (20% wt/vol) + Tween 20 (0.1%)	0	nt	nt	nt
Tween 20 (0.1%)	4	nt	nt	nt
Bleach (1:20) (42g/l sodium hypochlorite, 4% chlorine)	1	0	0	nt
Bleach (1:4) (42g/l sodium hypochlorite, 4% chlorine)	0	0	0	++
Menno (3% wt/vol)	3	0	0	nt
Dishwashing liquid (0.4% wt/vol)	2	1	nt	nt
Disinfectant (1:20) (benzalkonium chloride 1.5% w/w)	0	0	nt	+
Virkon (0.5% wt/vol)	1	0	0	nt
Virkon (1% wt/vol)	1			nt
Farmcleanse (1:10)	0	0	0	-
Hand sanitizer (62% ethanol)	2	nt	nt	nt
Denatured ethanol (70%)	nt	0	2	nt
Cerama Klen (2.5% wt/vol, 34g/kg chlorine)	nt	2	2	nt
Isowipe (70% isopropyl alcohol)	nt	0	2	nt
Undiluted infective sap	5	3	5	+
Distilled water	4	2	nt	-
0.1M phosphate buffer (inoculation buffer)	4	nt	nt	-
Healthy sap	0	0	0	nt

[a] Each treatment consisted of five plants of zucchini cv. Blackjack and isolate Knx-1; tip leaves from all plants tested individually by ELISA up to 37 days after inoculation.
[b] Infective sap diluted (1:1) with disinfectant and then used immediately to inoculate plants without buffer or abrasive.
[c] Infected leaf tapped with parafilm covered mallet five times, disinfectant then applied to parafilm as aerosol spray (3 sprays), healthy leaf then tapped five times.
[d] Scalpel blade dipped in disinfectant solution for 30 sec and left to air-dry. Area of corrosion on blade, ++++ = >75%; +++ = 50-75%; ++ = 25-50%; + = <25%; - = no damage.

Figure 1. a) Sap and crushed leaf material adhering to parafilm covering a rubber mallet after tapping ZYMV-infected zucchini leaves; b) contaminated rubber mallet used to tap healthy zucchini plant (experiments 3a-d); c) Sap and crushed leaf material adhering to rubber shoe sole following trampling of ZYMV-infected zucchini leaves before dipping in footbath containing bleach (1:20 dilution); and d) trampling healthy pumpkin plants with footwear contaminated with ZYMV (experiment 9).



SECTION 4

Contact transmission of *Papaya ringspot virus*, *Watermelon mosaic virus* and *Squash mosaic virus* in zucchini and lack of seed transmission in pumpkin and zucchini.

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Introduction

Plant viruses can be spread by a number of different methods, including vegetative propagation of infected plant material, by vector insects, mites, fungi or nematodes, through seed from infected plants and by contact of infective sap with wounded leaves (Astier *et al.* 2007).

The principal viruses infecting cucurbit crops in Australia are *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV), all from the genus *Potyvirus* (Greber 1969; Persley and Horlock 2003; Coutts and Jones 2005; Persley *et al*. 2010). These viruses are spread non-persistently by many aphid species (Buchen-Osmond *et al.* 1987). ZYMV is seed transmitted at low levels in some cucurbit types (Desbiez and Lecoq 1997; Coutts *et al.* 2011b), but there are no reports on seed transmission of PRSV or WMV. Apart from a few examples, *Potyviruses* are not usually considered to be contact transmitted. Studies undertaken in HAL projects VG01728 and VG06022 have shown ZYMV is transmitted from infected to healthy zucchini plants by leaf rubbing, crushing and on contaminated cutting tools. ZYMV was also found to remain infective for up to 6 hours after sap from infected leaves was placed on a number of different surfaces including metal, plastic, cotton and rubber. This supports the suggestion ZYMV may be transmitted by machinery within infected cucurbit crops (Fletcher *et al*. 2000). There is no information on the contact transmissibility of PRSV or WMV.

In addition to these three potyviruses, *Squash mosaic virus* (SqMV) a *Comovirus* is sometimes found infecting cucurbits in Australia (Greber 1969; Persley and Horlock 2003; Coutts and Jones 2005; Persley *et al.* 2010). It is transmitted by seed and various beetle vectors (Campbell 1971), and, although it is considered to be contact transmitted (Blancard *et al.* 2005), no experimental evidence has been reported supporting this. Initial experiments reported in HAL project VG01728 found SqMV was transmitted by leaf-to-leaf contact and by leaf crushing, but not on contaminated blades. However, the SqMV inoculum sources used was contaminated with ZYMV and so further work was needed using a SqMV pure virus culture.

This study describes glasshouse experiments undertaken to determine (i) if PRSV, WMV and SqMV can be transmitted by rubbing or crushing infected leaves onto healthy plants, (ii) if PRSV is seed transmitted and (iii) whether seed transmission of SqMV can be confirmed.

Materials and Methods

All experiments were done in an air-conditioned insect-proofed glasshouses kept at 18-25^{o}C. The virus isolates used were PRSV-Qld, WMV-Qld and SqMV-Bme1 which were maintained in zucchini cv. Blackjack. Leaf samples were tested for virus infection by ELISA as described by Coutts and Jones (2005). The polyclonal antiserum for SqMV, PRSV and WMV was obtained from Loewe Biochemica.

Experiment 1 investigated whether PRSV, WMV and SqMV could spread from an infected to a healthy plant by leaf-to-leaf contact. A leaves from a zucchini plant infected with one of PRSV, WMV or SqMV were lightly rubbed onto the leaf surface of healthy zucchini plants so that no visual damage to the test plant leaf surface occurred. For each virus, 16 plants were rubbed (2 leaves/plant). Plants rubbed with healthy leaves were the controls. Three weeks after rubbing, tip leaves were sampled from each plant and tested individually by ELISA.

Experiment 2 simulated virus transmission by leaf crushing such as when leaves are damaged by machinery tyres. A rubber mallet with parafilm secured with an elastic band to cover its head (5 cm diameter) was used to tap zucchini leaves infected with PRSV. This mallet was then used to tap the leaves of a healthy zucchini plant. The number of taps was 1, 3 or 5 on the infected plant leaf followed by the same number of taps on a leaf of the test plant. The parafilm was changed between each test plant. Five plants were used for each mallet treatment (2 leaves/plant). The negative control was a healthy leaf tapped 1, 3 or 5 times followed by the same of taps on a healthy plant leaf. The positive control was leaf-to-leaf rubbing following the procedure in experiment 1. Three to four weeks after rubber mallet inoculation, tip leaves were sampled from each plant and tested individually by ELISA for PRSV.

In experiment 3, seed transmission of SqMV and PRSV was examined. Virus-infected fruit of zucchini (PRSV), and butternut and Jarrahdale pumpkin (SqMV) (Fig. 1a) were harvested from cucurbit fields, the seed removed and sown in trays in an insect-proof glasshouse (Fig. 1b). The first true leaf from each seedling was sampled and tested for PRSV or SqMV in groups of 10 by ELISA.

Results

In experiment 1, when leaves infected with PRSV, WMV or SqMV were rubbed onto leaves of healthy plants, all plants became infected with SqMV (16/16), and most plants became infected with WMV (14/16) and PRSV (10/16). None of the control plants ever became infected.

In experiment 2, when healthy leaves were lightly crushed (1 tap) with a rubber mallet contaminated with PRSV, no plants became infected. When moderately crushed (3 taps), or heavily crushed (5 taps) 2/5 and 3/5 plants became infected, respectively. None of the healthy control plants became infected, and all plants rubbed with PRSV infected leaved became infected.

In experiment 3, none of the 1030 zucchini seedlings tested were PRSV infected. Also, none of the 630 pumpkin seedling tested were found to be SqMV infected. Many of the zucchini and pumpkin seeds collected were shrivelled and failed to germinate.

Discussion

This study shows PRSV, WMV and SqMV can be spread readily from infected to healthy cucurbit plants by direct leaf contact. This provides new information on the epidemiology of these viruses. Although further work is needed, for example to determine the stability of PRSV, WMV and SqMV in infective sap, as we have done for ZYMV (Coutts *et al.* 2011a), these results help explain why some virus epidemics occur rapidly in cucurbit crops. Cucurbit plants have leaf surfaces with long, sharp hairs that can easily cause fine scratches, so infection probably occurs through minute wounds caused by abrasive contact between leaves of healthy and infected plants such as when plants move by wind. Before this study, the only proven method of natural spread of PRSV and WMV was via aphid vectors, and for SqMV was by beetles and seed. Experimentally these viruses can be mechanical transmitted to soft plants in presence of an abrasive and buffer. We found PRSV could readily be spread by crushing healthy leaves in presence of infective sap as may happen when plants are handled or when machinery travels through a crop. It would be of interest to complete the same experiments (crushing, trampling, cutting) for PRSV, WMV and SqMV as have been done for ZYMV (Coutts *et al.* 2011a).

For PRSV and SqMV, no seed transmission was found, but only a small proportion of seeds collected from infected fruit germinated. Many of the seeds were shrivelled and had failed to fill out, probably due to virus infection. When zucchini plants were grown in the glasshouse and infected with PRSV, WMV or SqMV although the plants produced fruit it was often small and misshapen and no viable seed was ever produced even when flowers were hand-pollinated. Further seed testing is needed to determine the seed transmissibility of these viruses. For ZYMV, 3 of 430 zucchini seedlings tested were found infected (Coutts *et al.* 2011b), but no pumpkin seedlings (9560) were infected. Seed was collected from field grown infected-fruit.

The ease that PRSV, WMV and SqMV could be transmitted by contact was surprising and although further work is needed, it highlights the importance of sanitation (hygiene) practices in cucurbit production. Measures such as washing down machinery, disinfecting cutting tools and limiting handling and movement within crops need to be included in a modified integrated management strategies for viruses of cucurbits to address contact transmission.

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Fig. 1. a) Pumpkin (*Cucurbita maxima*) infected with SqMV with uneven skin colour and distortion (right) and healthy fruit (left); b) seedlings used for SqMV testing from seed collected from SqMV-infected pumpkin fruit.

SECTION 5

Survey for alternative hosts of potyviruses infecting cucurbits in Queensland

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Introduction

Cucurbit crops in Queensland are often infected with *Zucchini yellow mosaic virus* (ZYMV) and *Papaya ringspot virus* (PRSV) (Greber 1969, Persley and Horlock 2003). ZYMV and PRSV epidemics occur in many cucurbit growing areas of the world, both viruses are spread by aphids and ZYMV is seed transmitted at low levels in some cucurbit types. When cucurbit crops are infected early severe yield and quality losses occur. Few naturally occurring alternative hosts have been found these include wild cucurbits and some non-cucurbitaceous weed species (Desbiez and Lecoq 1997).

Identifying alternative hosts of PRSV and ZYMV in Queensland would provide information as to how these viruses survive between growing seasons.

Methods and results

In this survey, weeds were collected from within and adjacent watermelon, pumpkin and zucchini crops at Ayr and Clare in north Queensland. These crops had high incidence of leaf symptoms typical of potyvirus infection. When random samples from these crops were collected and tested by ELISA, both ZYMV and PRSV were detected, with many plants being infected with both viruses.

All weed samples were tested by ELISA using specific antibodies for PRSV, ZYMV and WMV. At least 10 samples of each of the following weed species were tested: *Trianthema portulacastrum, Solanum nigrum, Sonchus oleraceus, Chenopodium album, Nicandra* physalodes and *Passiflora suberosa.*
None of the weed samples were positive for the three viruses tested while PRSV and ZYMV were detected from all cucurbit crop samples tested.

Further samples of *Sonchus oleraceus* (sowthistle) were collected from adjacent a 100% PRSV-W infected zucchini crop at Bundaberg. No PRSV was detected in any of the weed samples tested.

Conclusion

In this study non-cucurbitaceous weeds were tested from areas of high virus pressure and none were found infected. From previous work, weedy cucurbit species such as burr gerkin (*Cucumis anguria)* and paddy melon (*Citrullus lanatus*) are occasionally found infected with PRSV, ZYMV and WMV (Persley and Horlock 2003). Studies in Western Australia

identified two new alternative hosts of ZYMV, the Australian native cucurbit *Cucumis maderaspatanus,* and the naturalised legume species *Rhyncosia minima* in addition to Afghan melon (*Citrullus lanatus*) but incidences were low (Coutts et al 2011).

These species may play a role in virus survival between cucurbit growing seasons but their abundance and incidence of virus infection suggests that they play a relatively minor role in the epidemiology of potyvirus infection of cucurbits in Queensland.

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SECTION 6

Assessment of zucchini cultivars for tolerance to potyviruses

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Introduction

The main viruses infecting cucurbit crops in Queensland are *Papaya ringspot virus* – type W (PRSV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) (Greber 1969; Persley and Horlock 2003). Cucurbit plants infected with these viruses have distorted leaves with mosaic and fruit are small, lumpy and distorted. When plants are infected early, there are substantial yield and quality losses as the fruit produced is unmarketable (Greber *et al*. 1988; Herrington 1987; Coutts and Jones 2005; Coutts *et al*. 2011). The three viruses are non-persistently aphid transmitted with *Myzus persicae* (green peach aphid) and *Aphis gossypii* (melon aphid) being the main vectors. They also infect volunteer cucurbits and wild cucurbits including Afghan or paddy melon (*Citrullus lanatus*) and wild gerkin (*Cucumis angurin*) (Persley and Horlock 2003; Coutts and Jones 2005).

Strategies used to manage virus diseases in cucurbit crops includes destroying old cucurbit crops, weeds and volunteer cucurbits that act as virus and vector reservoirs, avoid overlapping plantings, use of reflective mulch to deter aphid landing, planting upwind of virus sources, use of non-host barriers and use of resistant varieties. Insecticide application is ineffective at decreasing their spread within cucurbit crops (McLean *et al.* 1982; Coutts and Jones 2005; Persley *et al*. 2010; Coutts *et al*. 2011). Host resistance genes against ZYMV have been introduced into commercial cultivars of cucumber, pumpkin and zucchini. The gene *zym* in cucumber was effective against ZYMV, but *Zym* in zucchini and pumpkin delayed ZYMV infection (Coutts *et al*. 2011). Commercial zucchini cultivars are available with tolerance to PRSV.

In Queensland since 2004, PRSV-W has been one of the main factors limiting zucchini production in the cucurbit growing areas of Bundaberg, Lockyer valley and areas of south Queensland. This study describes two field trials done in Queensland at (i) Ayr in 2007, and (ii) Gatton in 2009 that investigated the effectiveness of commercial zucchini cultivars with tolerance to PRSV and ZYMV in limiting virus spread and increasing marketable yield.

1) Zucchini Cultivar Trial at Ayr Research Station (2007).

Five zucchini cultivars (Table 1) were grown and virus spread from naturally occurring external virus sources by aphids. For each cultivar, 11 to 14 plants were sampled. A single young leaf was collected from each plant in October. All leaf samples were tested individually by ELISA using antibodies specific to PRSV, WMV and ZYMV.

Results

- WMV was not detected in any of the zucchini plants tested.
- PRSV was detected in 82% of plants, and ZYMV in 72% of plants.
- Most plants were infected by at least one virus
- 60% of plants were infected by both viruses.
- Cv. Quirinal had the lowest number of plants infected with PRSV and ZYMV.
- Cv. Paydirt had only 5/12 plants infected with ZYMV, but all plants were infected with PRSV.
- Similar numbers of plants became infected with PRSV and ZYMV for cvs Mamba, Calida and Congo.
- Virus concentrations in ELISA varied considerably among and between cultivars with a tendency for absorbance values to be lower in infected plants of tolerant cultivars compared with the highly susceptible cultivars.

Table 1. Incidence of PRSV and ZYMV in plants of five zucchini cultivars

Cultivar	Resistance present*	PRSV^	ZYMV
Mamba	unknown	10/12	12/12
Quirinal	ZYMV, WMV	6/12	6/11
Calida	ZYMV, WMV	11/11	9/11
Congo	none	11/14	12/14
Paydirt	ZYMV, WMV	12/12	5/12

* Resistance present according to seed company.
^ Number of plants positive by ELISA/ total tested

Conclusions

- The results indicate that cultivars with mild leaf and fruit symptoms are virus tolerant and give economic yields, despite supporting virus replication.
- We have established that several zucchini cultivars have tolerance to PRSV and ZYMV and can provide economic yields under high virus pressure.

2) Yield and quality benefits from sowing of virus-tolerant zucchini cultivars – Field trial at Gatton Research Station (2009).

The trial consisted 14 zucchini cultivars (Table 2) grown in of single row plots, with four replicates/cultivar, one row per replicate (Table 3). Spreader rows of virus susceptible squash cv. Green Ruffles and zucchini cv. Black Adder alternated with each test cultivar row. A guard row was planted on each side of the trial area. The trial was hand sown on 16 March 2009 and then thinned soon after germination to10 plants/plot with plant spacing of 0.5 m.

The spreader row acted as a uniform virus source within the trial. Every second plant in each spreader row was sap inoculated with PRSV-W infective sap / cold water and celite on 27 March 2009. Efficiency of this inoculation was 99%. Infection of test rows was via natural spread by aphids (virus was not introduced into the test rows by manual inoculation).

On four occasions (29 April, 6 May, 13 May and 27 May) fruit of marketable size and greater were picked at each harvest, discarding fruit too large to market. All fruit with dead flowers even if a little small to be marketable were picked at harvest.

At each harvest leaf symptoms were rated, the number of marketable fruit and their weights recorded, along with the number of unmarketable fruit due to small size or deformity (due to virus infection or other causes eg. insect damage) were counted and weighed. Large unmarketable fruit were counted separately and discarded.

Leaf samples from plants selected at random from each test row were collected and tested by ELISA on 5 June 2009 for PRSV, ZYMV and WMV. Plants were also assessed for virus symptoms.

Table 2. Details of zucchini cultivars used, seed company and resistance present.

Treatment	Cultivar	Type	Resistance present*	Company
1	Goldsmith	yellow	WMV, ZYMV	SPS
2	Litani	Lebanese	WMV, ZYMV	SPS
3	Nitro	Green	PRSV, WMV, ZYMV	SPS
4	Hummer	Green	PRSV, WMV, ZYMV	SPS
5	Dakota	green	unknown	SPS
6	Calida	Green	WMV, ZYMV	Clause
7	Sintia	Green	WMV, ZYMV	Clause
8	Amanda	Green	ZYMV	Clause
9	Golden Arrow	Yellow	PRSV, WMV, ZYMV	Clause
10	Crowbar	Green	WMV, ZYMV	SG
11	Houdini	Green	WMV, ZYMV	SG
12	Paydirt	Green	WMV, ZYMV	SG
13	Mamba	Green	unknown	Terranova
14	Black adder	green	WMV, ZYMV	Terranova

*Resistance present according to seed company.

Table 3. Field Trial Layout

Replicate 1	Replicate 2	Replicate 3	Replicate 4
1-1 (7) Sintia	2-1 (1) Goldsmith	3-1 (8) Amanda	4-1 (2) Litani
1-2 (1) Goldsmith	2-2 (6)Calida	3-2 (6) Calida	4-2 (13) Mamba
1-3 (10) Crowbar	2-3 (7)Sintia	3-3 (12) Paydirt	4-3 (10) Crowbar
1-4 (6) Calida	2-4 (11)Houdini	3-4 (2) Litani	4-4 (4) Hummer
1-5 (9) Golden arrow	2-5 (13) Mamba	3-5 (13) Mamba	4-5 (1) Goldsmith
1-6 (1Black adder4)	2-6 (8) Amanda	3-6 (11) Houdini	4-6 (5) Dakota
1-7 (Paydirt12)	2-7 (4) Hummer	3-7 (5) Dakota	4-7 (12) Paydirt
1-8 (3)Nitro	2-8 (9) Golden arrow	3-8 (9) Golden arrow	4-8 (3) Nitro
1-9 (5)Dakota	2-9 (14) Black adder	3-9 (1) Goldsmith	4-9 (14) Black adder
1-10 (4)Hummer	2-10 (3) Nitro	3-10 (14) Black adder	4-1- (6) Calida
1-11 (13)Mamba	2-11 (5) Dakota	3-11 (7) Sintia	4-11 (8) Amanda
1-12 (11)Houdini	2-12 (10) Crowbar	3-12 (3) Nitro	4-12 (7) Sintia
1-13 (2)Litani	2-13 (2) Litani	3-13 (4) Hummer	4-13 (11) Houdini
1-14 (8)Amanda	2-14 (12) Paydirt	3-14 (10) Crowbar	4-14 (9) Golden arrow

Results:

- PRSV was detected in all leaf samples tested, WMV was detected in 2 samples, ZYMV was not detected in any sample.

- For yield data, the total yield for each cultivar was combined for the 4 replicate.
- Total yield of marketable fruit ranged from 2 kg (cv. Hummer) to 25.7 kg (Golden Arrow) (Table 4).
- Cvs Goldsmith and Golden Arrow had highest yield with more than 23 kg of marketable fruit, and symptoms were mild (1). Plants of cvs Hummer, Dakota, Amanda, Houdini, Mamba and Black Adder had lowest yields with less than 6 kg of marketable fruit, they also had severe symptoms (ratings of 4).
- Of the fruit harvested plants of cv. Paydirt had the highest % of fruit that was marketable

Table 4. Symptom ratings and yield data of marketable fruit in zucchini cultivars

Cultivar	Leaf symptom rating (1 mild – 4 severe)*	Total yield of marketable fruit (kg)	Per cent marketable fruit
Golden Arrow	1	25.7	59
Goldsmith	1	23.8	62
Nitro	3	20.0	56
Crowbar	2	18.3	58
Paydirt	2	16.9	72
Calida	2	13.9	40
Sintia	2	12.3	39
Litani	3	6.2	14
Amanda	4	6.0	17
Mamba	4	4.4	13
Black Adder	4	3.7	16
Dakota	4	2.5	9
Houdini	4	3.0	9
Hummer	4	2.0	5

*Leaf symptom ratings: 0 = no symptoms, 1 = very mild symptoms, 2 = mild mosaic type symptoms, 3 = severe mosaics and leaf "bubbling", 4 = severe mosaics and leaf deformation.

Conclusion:

- Under high virus pressure all cultivars became infected with PRSV although the symptom severity, affect on fruit yield and quality varied depending on cultivar.
- Generally an increase in symptom severity related to a decrease in marketable fruit produced.
- Five cultivars had useful tolerance to PRSV having at least 50% of the fruit produced being marketable under conditions of high virus pressure and could be recommended for use in areas with PRSV epidemics.

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Persley D, Cooke T, House S (2010) Diseases of vegetable crops in Australia. CSIRO Publishing, Collingwood, Vic.

SECTION 7

TECHNOLOGY TRANSFER

Communication and extension activities

(i) Articles in grower newsletters/magazines

- Agricultural Memo (Kununurra) April 2007, Growing virus-resistant pumpkins
- Agricultural Memo, (Kununurra) December 2007, Further work on growing virus resistant pumpkins
- Agricultural Memo, (Kununurra) April 2008, Why insecticides are not effective in controlling cucurbit virus diseases
- Agricultural Memo, (Kununurra) August 2008, Virus diseases in cucurbit crops
- Agricultural Memo, (Kununurra) June 2009, Virus resistant varieties
- Agricultural Memo, (Carnarvon) June 2009, Virus resistant varieties
- Agricultural Memo, (Kununurra) December 2009, Slowing the spread of virus into cucurbit crops
- WA Grower (December 2009) Understanding a devastating virus disease of cucurbits.
- Vegetables WA – Good practice guide (2009) Virus diseases in vegetable crops on the Swan Coastal Plain
- AgFlash, (Carnarvon) June 2010, Virus diseases in vegetable crops
- AgMemo (Kununurra) November 2010, Zucchini yellow mosaic virus and aphid trial results.
- Media release December 2010, Crop hygiene the key to managing virus diseases
- WA Grower December 2010, Crop hygiene the key to managing virus diseases

(ii) Grower/consultant/industry meetings and field days

During the project life cucurbit growing properties in Western Australia and Queensland were regularly visited each year to discuss the project objectives and virus management strategies with growers. In addition, growers in Victoria and northern New South Wales were visited.

Western Australia – Brenda Coutts

Industry seminars/meetings attended by cucurbit growers and horticulture consultants.

- Carnarvon (26 March 2008)
- Carnarvon (9-11 September 2008)
- Kununurra (17-19 September 2008)

- Perth (February 2009) Project summary presented to Horticultural researchers
- Perth (April 2009) Update of key biosecurity issues to the cucurbit industry
- Carnarvon (April 2010)
- Carnarvon (14 September 2010)

Field walks and demonstrations

- Carnarvon Horticultural Field Day (19 May 2009)
- Kununurra (September 2009) - spread of ZYMV in pumpkins.
- Kununurra Agricultural Show (8-9 July 2010) Virus in cucurbit crops
- Kununurra field day (14 July 2010) non-host barrier and time of sowing for delaying spread of ZYMV
- Carnarvon (14 September 2010) – ZYMV in zucchini

Queensland – Denis Persley

Industry seminars/grower meetings.

- Bowen (11 Feb 2008) –attendees 25
- Gumlu (12 Feb 2008) – attendees 10
- Ayr (13 Feb 2008) - attendees 40
- Bundaberg (27 May 2008) - attendees 40
- Bowen (31 March 2009) –attendees 25
- Ayr (1 April 2009) - attendees 40
- Mareeba (2 April 2009) –attendees 25
- Swan Hill (Vic) (March 2010)
- Bundaberg (9 August 2010) – attendees 50
- Gatton (12 August 2010) –attendees 40

Field walks

- Gatton Research Station (April 2009) - PRSV tolerance in zucchini varieties.

Northern Territory – Barry Conde

- Growers workshop in Darwin (December 2010)

(iii) Radio interviews

Western Australia – Brenda Coutts

- Radio interview on ABC Rural Report (July 2008) 'Virus resistant pumpkins'
- Radio interview on ABC Rural Report (November 2010) 'Virus and aphids'
- Radio interview on ABC Rural Report (December 2010) 'Managing virus diseases in vegetable'

(iv) Brochures and Technical publications

Queensland

- Persley DM and Gambley CF (2008) Virus diseases in cucurbits DEEDI Note
- Persley DM and Gambley CF (2009) Viruses in vegetable crops in Australia DEEDI Refnote PR09/4502
- Persley DM and Gambley CF (2010) Aphid transmitted viruses in vegetable crops. DEEDI Refnote PR10/ 5254
- Persley DM, Akem C and Martin H (2010) Cucurbit diseases in: Diseases of vegetable crops in Australia. (eds Persley, Cooke, House). CSIRO Publishing, pp113-138

Western Australia

- Australasian Plant Pathology Society – pathogen of the month 'Zucchini yellow mosaic virus'
- Coutts B (2009) Virus diseases of cucurbits DAFWA Farmnote revision No 166.

(v) Conference abstracts and presentations

Coutts B, Kehoe M, Jones RAC (2008) Studies on *Zucchini yellow mosaic virus* in cucurbits. In: Proceeding of Horticulture program 5th Biennial workshop, Mandurah, Australia. pp. 62.

Coutts B, Jones R, Kehoe M. (2008) Studies on the epidemiology of *Zucchini yellow mosaic virus* in Western Australia: patterns of spread, virus-tolerant cultivars, alternative hosts and lack of seed transmission. In: Proceedings of 8th Australasian Plant Virology Workshop. Lake Okataina, Rotorua, New Zealand. pp. 24.

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(vi) Scientific referred publications

Coutts, B. A., Kehoe, M. A., and Jones, R. A. C. 2011. Minimising losses caused by *Zucchini yellow mosaic virus* in tropical, sub-tropical and Mediterranean environments through cultural methods and host resistance. *Virus Research* (in press)

Coutts, B.A., Kehoe, M. A., Webster, C. G., Wylie, S.J., and Jones, R. A. C. 2011. *Zucchini yellow mosaic virus*: biological properties, detection procedures and comparison of coat protein gene sequences. *Archives of Virology* (submitted)

Coutts, B. A., Kehoe, M. A., and Jones, R. A. C. 2011. *Zucchini yellow mosaic virus*: contact transmission, stability on surfaces and effective inactivation with disinfections. *Plant Disease* (submitted)

SECTION 8

RECOMMENDATIONS

(i) Scientific

- There are a number of complex factors that influence development of aphid vectored virus epidemics in cucurbit crops in Australia and the development of a predictive model to increase the understanding of these epidemics and guide management decisions is needed. Development of such a model requires accumulation of epidemiological information before the actual model can be devised, and then validation.

- Further studies to determine the extent to which PRSV, WMV and SqMV can be transmitted by crushing or trampling and on contaminated cutting blades is required. Also, the infectivity of sap containing PRSV, WMV and SqMV over time and on how long these viruses survive on different surfaces, as is determining the best disinfectants to inactivate them is needed.

- The potential roles of native plant species as virus reservoirs and hosts of aphid vectors requires further investigation.

- Studies to determine if ZYMV and PRSV are seed transmitted in cucurbit alternative hosts including wild *Citrullus lanatus* (Afghan melon, paddy melon, prickly melon), wild *Cucumis anguria* (burr gerkin) and endemic *Cucumis maderaspatanus* would help identify how these viruses survive between growing season.

(ii) Industry

The project modified existing integrated disease management strategies for virus diseases for vegetable cucurbits and extended it to growers. Further promotion of these strategies to the vegetable cucurbit industry is needed to increase its uptake and adoption in virus-affected cucurbit growing areas.

SECTION 9

APPENDIX A

Integrated disease management strategies to minimise virus infection in field grown vegetable cucurbit crops. For *Papaya ringspot virus*, *Squash mosaic virus*, *Watermelon mosaic virus* and *Zucchini yellow mosaic virus.*

Control measure	How achieved	Mode of action
Avoid spread from finished crops	Promptly destroy harvested crops with herbicide, burn, plough under, or cut and remove.	Removes potent external sources of virus infection and aphid infestation for spread to new crops.
Avoid spread from nearby crops	Plant upwind of potential infection sources. Avoid overlapping crop sowings in close proximity or sequential plantings side-by-side. Use intervening non-host crops or fallow between crops.	Minimises a major external source of virus infection and aphid infestation for spread to cucurbit crops.
Minimise spread from cucurbit weeds or 'volunteer' crop plants	Control weeds and crop 'volunteers' by spraying with herbicides before re-sowing land with new crop, on nearby unused land and along fence-lines. Hand-weed organic crop sites.	Removes a major internal and external source of virus infection and aphid infestation for spread to other plants.
Sow non-host barrier crops	Surround crop with tall non-host barrier crop such as millet or sorghum. Plant 4 weeks prior to cucurbit crop.	Aphids probe on non-host lose the virus before reaching cucurbit crop, tall barrier re-directs incoming aphids away from crop area.
Manipulate planting date and monitor aphid flights	Select planting dates to avoid exposure of young plants to peak aphids populations and flight times. Use sticky traps to monitor aphid flights prior to planting.	Diminishes infection of plants at their vulnerable young growth stage. Plants becoming virus-infected later are less damaged and yield more. Decreases virus spread and its final incidence.
Use roguing within crop	Remove crop plants with visible virus symptoms. Most effective if removed before virus spread starts. Bag, burn or bury infected plants	Helps remove a key internal source of virus infection for spread to other plants.
Use healthy seed	Purchase certified seed	Avoids virus infection of seedlings.
Use mulches or minimum tillage	Cover soil around plants with silvery reflective plastic mulch or spread straw mulches on soil surface at sowing time. Sow directly into crop debris or stubble without cultivation.	Reflective mulch diminishes aphids landing rates. Straw mulch, stubble and dry crop debris groundcover decrease aphids landing rates.
Use virus-tolerant cultivars	Plant tolerant pumpkin, zucchini and cucumber cultivars.	Delays virus infection.
Employ good sanitation practices	Use disinfectant (eg bleach) in footbaths, on cutting tools, for machinery and equipment wash down. Avoid moving machinery from old to new crops.	Viruses spread by contact and on contaminated surfaces, disinfectants inactivate virus decreasing spread
Institute 'susceptible crop and weed-free period' [Ultimate measure when all else fails]	Neighbouring properties in production district co-operate to provide weed and susceptible crop-free period over whole area, including fence-lines, road verges. Leave district fallow for at least 6 weeks. Plant non-host crops eg. green manure or *Solanaceous* vegetable.	Breaks infection cycle over entire area by removing all herbaceous growing plants that could be virus sources.

Zucchini yellow mosaic virus in cucurbit crops

Brenda Coutts and Monica Kehoe, Plant Virologists, Department of Agriculture and Food.

Zucchini yellow mosaic virus (ZYMV) is an aphid-borne virus that causes yield losses and fruit quality defects in cucurbit crops. ZYMV infects all cultivated cucurbit types including cucumber, pumpkin, rockmelon, squash, watermelon and zucchini. It does not infect other horticultural crops. Two strains of ZYMV occur in Western Australia: i) the strain found in Kununurra causes severe symptoms on leaves and fruit and is able to infect a number of commercially available virus 'tolerant' varieties of zucchini and pumpkin; and ii) the strain found in Carnarvon tends to cause milder symptoms and virus 'tolerant' varieties are less likely to become infected, unless under high disease pressure. ZYMV infection is widespread in the cucurbit growing areas of northern Western Australia (Carnarvon and Kununurra) as well as in Northern Territory and Queensland.

Symptoms

The leaf symptoms of ZYMV infection are severe mosaic, deformation, blistering and reduced size (Fig 1). Infected plants are stunted. Fruit symptoms of pumpkin, zucchini, squash, watermelon and cucumber include mottled and discoloured skin and knobbly areas which cause prominent deformations. Infected rockmelon fruit often have poorly formed surface 'netting' (Fig 2). Symptoms are similar to those caused by infection with *Papaya ringspot virus* and *Watermelon mosaic virus*.

Sources of virus

The virus needs living plants to survive and cannot live in soil or dead plant material. ZYMV infection is usually confined to plants in the cucurbit family. The main sources of ZYMV are old diseased cucurbit crops, volunteer cucurbits and cucurbit weed species such as Afghan or paddy melons. ZYMV also infects native cucurbit species including *Cucumis maderaspatanus* (Fig 3). These plants are found growing within crops, on roadside verges, and along fence-lines. Infected old crop plants, weeds and native cucurbit species allow the virus to survive between growing seasons. ZYMV is spread from these infected plants to young crops by aphids.

ZYMV can be transmitted at very low levels (<1%) in the seed of zucchini and pumpkin.

Aphid vectors and transmission

ZYMV is primarily spread by aphids, including melon (*Aphis gossypii*) and green peach (*Myzus persicae*) aphids, as well as many other species which are migrating through the crop. ZYMV is transmitted non-persistently, that is, an aphid picks up the virus after probing for 1-2 seconds on an infected plant and is then transmitted with 1-2 seconds of probing of a healthy plant, but the virus lost when the aphid probes one or two healthy plants. A small number of aphids are able to spread the virus to a large number of plants in a short time as they search for a suitable host plant to colonise.

Contact transmission

ZYMV is readily spread between plants when infected leaves are damaged by footwear, cutting implements or machinery and the infective sap then contacts or rubs on a healthy plant. Using a 1:4 dilution of household bleach, or 1:10 dilution of

'Farmcleanse', or 20% solution of skim milk powder in footbaths, to clean implements and machinery helps in reducing virus spread.

Yield and quality losses

When cucurbit plants become infected early (prior to flowering), yield losses can be up to 100%. If infection occurs before or at fruit set then most fruit will have quality defects and be unmarketable. When plants are infected after fruit set, the yield and quality losses can be reduced. The severity of the symptoms and magnitude of the losses depends on the time of infection, strain of the virus and variety grown.

Management

An integrated management approach using multiple control measures are aimed at delaying and minimising the level of virus in crops.

- *Removal and destruction of old cucurbit crops immediately after the final harvest* – to minimize virus spread to new crops
- *Destroy any wild or volunteer cucurbit plants before planting* – to reduce any potential virus sources for new crops
- *Remove any cucurbit plants showing virus symptoms, particularly before fruit set* – removing internal crop sources of infection may help to slow down the spread of the virus within the crop.
- *Plant a tall non-host border crop around the cucurbit crop approximately 4 weeks before planting* – a non-host border acts as a cleansing barrier. Infective aphids that feed on it will lose they virus they are carrying when they feed and will no longer be infective when they land on the cucurbit crop.
- *Plant cucurbits upwind from other crops, avoid sequentially planting downwind* – there is less infection upwind from infection sources as aphids can be blown along with the wind.
- *Employ good hygiene practices* - Use 1:4 dilution of household bleach for footbaths, and to wash equipment and machinery tyres
- *Avoid moving machinery from old crops to new ones*
- *Use virus tolerant cucurbit varieties when they are available* –the most effective strategy when they are available.

Although ZYMV is spread mainly by aphids, insecticide use is ineffective as a control measure. The insecticides do not work fast enough to prevent the aphid from feeding on and infecting a healthy plant before it is killed.

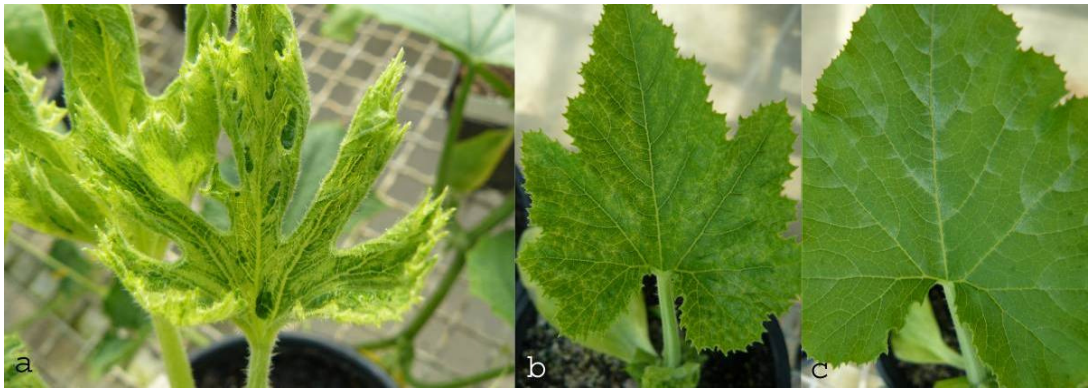

Figure 1. Symptoms of ZYMV infection on zucchini a) severe symptoms – mottling, bubbling and leaf deformation, b) mild symptoms – mottle, yellowing, c) healthy leaf.

Fig 2 Symptoms of ZYMV infection on various cucurbits a) yellow zucchini (left, infected fruit distorted, discoloured, lumpy; right, healthy); b) green zucchini (left, infected fruit severe distortion and knobbliness and skin mottle; right, healthy); c) lebanese zucchini (right, infected fruit severe distortion and knobbliness and skin mottle; left, healthy); d) Jarrahdale grey pumpkin (severe distortion and knobbliness); e) butternut pumpkin (mild mottle); f) watermelon (distorted, uneven skin colour and surface); g) cucumber (distorted, skin mottle and lumps); h) squash (discoloured with uneven skin surface); and i) rockmelon (reduced netting on surface).

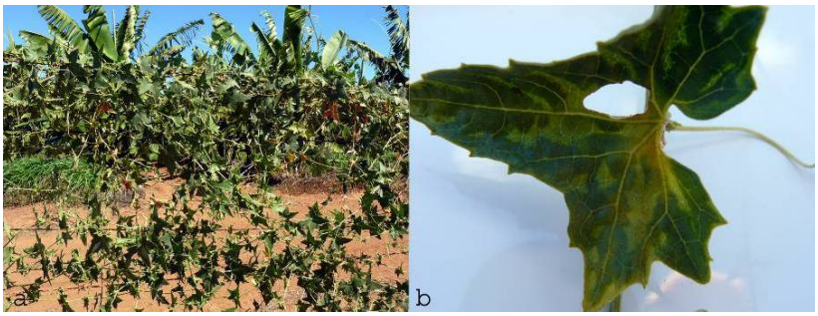

Fig 3 a) ZYMV infected *Cucumis maderaspatanus* growing on a fenceline b) Leaf mottle caused by ZYMV infection.