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Evaluation of inoculation techniques of ZYMV with a susceptible squash variety

George E. Boyhan*, Suzanne P. Stone, Cassandra A. Bure[1]

Department of Horticulture, University of Georgia, 1111 Miller Plant Science Building, Athens, GA 30602.
*[1]111 Fieldstone Farms Dr. Coweta, GA 30278, *E-mail: cassie.bure@yahoo.com*

Abstract

Inoculation techniques were evaluated for the transmission of Zucchini Yellow Mosaic Virus (ZYMV) to susceptible squash (*Cucurbita pepo* L.) variety, Gentry. Two different types of airbrush sprayers (gravity fed and siphon fed) were evaluated in comparison to the standard method of rub inoculating leaves dusted with carborundum abrasive. In addition, the number of inoculations (1-3) with the airbrush sprayers and whether the carborundum dust was directly applied to leaves or mixed with the inoculum were also evaluated. The standard method consistently had high infection rates of 90% or greater, whereas the airbrush sprayers had inoculation rates of 30%-97%. Whether the carborundum was dusted or applied in the inoculum solution had no impact on infection rate. The greater the number of inoculations the greater the infection rate in two out of three experiments. In conclusion, the airbrush sprayer may be helpful during preliminary screening; however, it should not be relied on as the sole method of inoculation.

Key words: Cucurbits, disease resistance, potyvirus, summer squash, *Cucurbita pepo*

Introduction

Viruses are an important class of plant pathogens, second only to fungi in total number of plant diseases causing economic damage. In Georgia losses were estimated at 16.5% for all horticultural and agronomic crops from all disease sources (Williams-Woodward, 2012). Viruses account for approximately 25% of these losses and are estimated to be $175 million.

Virus diseases are important constraints in crop production and effective methods of assessing damage and more importantly determining potential sources of disease resistance are essential. A number of different techniques have been proposed to accomplish this objective. For example, in sugar cane (*Saccharum officinarum*), the most satisfactory method of inoculation was to apply four drops of viruliferous juice to the axil and prick the axillary tissue 100 times. This resulted in an average infection rate of 95% (Liu, 1949).

A variety of environmental factors can influence virus inoculation particularly with recalcitrant viruses such as Tomato Spotted Wilt Virus (TSWV). Hutton and Peak (1951) found that TSWV lost effectiveness after 20 min and that concentration, as well as, abrasive could have an effect on infectivity.

The specific isolate used can affect inoculation. Back inoculation from herbaceous to woody material with Plum Pox Potyvirus Bor-3 isolate could be accomplished easily with either grafting or mechanical techniques (Monsion *et al.*, 2008). This was not the case with other isolates suggesting there are some genome differences for adaption for ease of inoculation.

In addition to the isolate used, the technique employed to inoculate material can have an effect. A review of four different inoculation techniques with Grapevine Fanleaf Virus (GFLV) was conducted (Valat *et al.*, 2003). These techniques included nematode-mediated inoculation, micrografting, leaf bombardment with virus coated gold particles, and electroporation of mesophyll protoplasts. This review paper has recommendations for the different methods based on research goals. Novel inoculation techniques have been developed for inoculating Soilborne Wheat Mosaic Virus (SBWMV) onto hard red winter wheat (*Triticum aestivum*) where rub-inoculated leaves had inconsistent results. This virus infection is vectored by the plasmodiophorid, *Polymyxa graminis*. Growing wheat plants in germination bags and including infected roots or mechanically inoculating the roots proved to be an effective method of inoculation (Driskel *et al.*, 2002). Maize (*Zea mays*) viruses have been effectively transmitted using a jeweler's engraving tool to push droplets of virus inoculum into the major vascular bundles (Redinbaugh *et al.*, 2001).

Bioassays have also been used to ensure virus inoculation and to assess types of resistance. Rajamony *et al.* (1999) used a back inoculation technique to investigate resistance in *Cucumis* spp. They found that *C. figarei* and *C. ficifolius* were immune to Green Mottle Mosaic Virus (CGMMV) whereas resistant cultivars of *C. melo* var. *momordica*, could be a source of CGMMV when back inoculated to a susceptible host.

Insect mediated inoculation techniques have also been used. Three different inoculation techniques, mass inoculation, cage inoculation, and natural field inoculation techniques were used with whiteflies (*Bemisia tabaci*) to inoculate *Solanum* spp. with Tomato Yellow Leaf Curl Virus (TYLCV). Mass inoculation was inadequate to evaluate for resistance as susceptible material may be selected because of inadequate disease pressure. Cage inoculation was the best as it insured 100% infection with susceptible material (Picó *et al.*, 1998). In another study, various techniques were evaluated for the transmission of Sorghum Stripe Virus (SStV) to sorghum (*Sorghum bicolor)* (Narayana and Muniyappa, 1996). Ninety-five percent infection was possible using the planthopper (*Peregrinus maidis*) at a rate of two viruliferous planthoppers per seedling at the 2-leaf stage when confined for 48 h in a cylindrical cage.

A simple handheld device for inoculating soft plant material with cDNA of a potyvirus is described by GalOn *et al.* (1997). This device was found to be very effective at inoculating squash (*Cucurbita pepo*) plants when bombarded with gas propelled tungsten or gold particles.

Not all inoculation techniques have proven to be effective and efficient. Kryczyn´ski and Szyndel (1987) found that both potato (*Solanum tuberosum*) and *Physalis floridana* could be successfully inoculated with Potato Leaf Roll Luteovirus (PLRV) by tissue implantation and by sap, but both methods were time consuming and inefficient. Both grafting and mechanical techniques were tested using two strains of Potato Virus Y against several genotypes with differing degrees of susceptibility (Kameníková, 1987). Stem grafting was the most effective with 100% infection, but was not as efficient in terms of labor compared to mechanical inoculation. Mechanical inoculation; however, could be as low as 30% depending on the genotype. Using a spray gun to inoculate potato seedlings with Potato X Potexvirus (PVX) or Potato Y Potyvirus (PVY) was successful at a 96% rate with 88% of seedlings showing symptoms (Fernandez-Northcote, 1991). Mechanical inoculation of grapes (*Vitis vinifera*) has been effective only under limited conditions, therefore grafting techniques have been investigated as an alternative (Lahogue *et al.*, 1995). It was found that dormant grafting with the rootstock as the inoculum source was the most effective method; however, green grafting with unrooted rootstock as the inoculum, could be used with a concomitant elimination of large tracks of land, is less time consuming, and can be done throughout the year.

Additional items such as poly-L-ornithine may be required for successful virus transmission (Nienhaus *et al.*, 1990). Cherry Leaf Roll Nepovirus (CLRV) transmission to *Betula* spp. only occurred after poly-L-ornithine was added to the inoculum.

The objective of this study was to evaluate different air-brush configurations and inoculation techniques for their effectiveness in transmitting Zucchini Yellow Mosaic Virus (ZYMV).

Materials and methods

The experiments were conducted in the greenhouses at Durham Horticultural Research Farm of the University of Georgia in Watkinsville, GA. Three different experiments were conducted using squash variety Gentry, a known variety susceptible to ZYMV. These experiments used a randomized complete block design with six replications arranged as a 2 x 2 x 3 factorial with two additional treatments, a standard method and a water only treatment. The first factor consisted of either a gravity-fed or siphon-fed airbrush (TCP Global, San Diego, CA, Master Airbrush models E91 and G22, respectively). The second factor consisted of carborundum dust either applied as a dust to the leaves or mixed in the inoculum solution to form a slurry. The third factor consisted of the number of applications (1, 2, or 3) within 3-5 days. The inoculum solution consisted of ZYMV infected squash leaves and 0.05 M phosphate buffer (0.05M mono and dibasic KPO_4).

Each experimental unit consisted of six seedlings sown individually in 3 inch pots with soil media (Fafard 3B, Conrad fafard, Inc., Agawam, MA). Inoculations began with the first true leaves. Table 1 lists the dates of sowing, inoculation, and evaluation of the experimental material.

Table 1. Sowing, inoculation, and evaluation dates of 'Gentry' squash inoculated with ZYMV[z]

Experiments	Sowing date	Inoculation date[y]	Evaluation dates
1	20 Feb. 2014	6 Mar. 2014	2 Apr. 2014
		7 Mar. 2014	
		10 Mar. 2014	
2	7 Aug. 2014	19 Aug. 2014	8 Sept. 2014
		20 Aug. 2014	
		21 Aug. 2014	
3	26 May 2015	10 June 2015	29 June 2015
		11 June 2015	22-23 June 2015
		12 June 2015	(ELISA screening[x])

[z]Experiments were a factorial arrangement of two types of air brush applicators (siphon or gravity fed), two application methods of carborundum dust (leaf dusted or as part of the inoculation solution), and inoculum applied one, two, or three times. In addition, a standard method of rub inoculated carborundum dusted leaf tissue and a water only inoculation were included.

[y]Inoculations were done 1, 2, or 3 times based on treatment. Standard and water inoculations were done on the first inoculation date.

[x]Along with the visual evaluations, the third experiment also used enzyme-linked immunosorbent assay (ELISA) for ZYMV.

Plant material was evaluated visually as with or without symptoms and reported as percent of infected plants. In addition, a double antibody sandwich enzyme-linked immunosorbant assay (DAS-ELISA) (Agdia Inc., Elkhart, IN) was used to evaluate the third experiment. The absorbance reading from this test was evaluated at 405 nm on a microplate reader (Model infinite M200Pro, Tecan Group Ltd., Männedorf, Switzerland). Data were analyzed using a factorial design with Stata 14.1 (StataCorp, College Station, TX).

Results

There was a significant by date interaction across all factors, so the results are presented separately for each date (Table 2). For the April 2, 2014 evaluation date, the standard method was significantly better at infecting squash tissue than either the gravity or siphon fed airbrush sprayers with over 90% infection compared to less than 40% for the airbrush sprayers. There was no difference between dusting the leaves with carborundum prior to inoculation or incorporating the carborundum in the inoculum solution. The number of inoculations with the airbrush sprayer had a significant effect in the first experiment. The infection rate ranged from just over 9% for one inoculation to over 50% with three inoculations.

On September 8, 2014, the standard method at 97.2% infection did not differ from the gravity fed airbrush sprayer with 96.8% infection (Table 1). Both methods, however, performed significantly better than the siphon fed airbrush sprayer with 75% infection.

On June 29, 2015, the standard method was significantly better than either the gravity or siphon fed airbrushes. The standard method had a 91.7% infection rate compared to 45.4 and 35.6% for the gravity fed and siphon fed airbrushes, respectively (Table 2). Again, the application of carborundum, whether dusted on the leaves or added to the inoculum solution made no difference

Table 2. Percent infected ‘Gentry’ yellow squash based on different airbrush types, method of carborundum application, and number of applications of ZYMV inoculum[z]

	Date		
	2 .04.2014	8.09.2014	29.06.2015
Airbrush type			
Gravity fed	35.6a[x]	96.8a	45.4b
Siphon fed	30.0a	75.0b	35.6b
Standard[y]	93.3b	97.2a	91.7a
Carborundum			
Dusted	42.3	82.4	55.6
Slurry	23.3	89.4	25.5
Number of inoculations			
1	9.3a	80	30.6a
2	34.0b	88	43.1ab
3	55.1c	90	47.9b
Probabilities			
Airbrush type	0.000	0.000	0.000
Carborundum application	0.000	0.082	0.000
Number of applications	0.000	0.100	0.020
Airbrush type x Carborundum	0.112	0.906	0.060
Airbrush type x Application No.	0.450	0.353	0.301
Carborundum x Application No.	0.030	0.846	0.734
Airbrush type x Carborundum x Application No.	0.060	0.946	0.383

[z]Inoculum: 0.05M phosphate buffer (mono & dibasic KPO_4) with ZYMV infected macerated leaves, 2:1 V/W ratio.

[y]Standard: Leaves were dusted with carborundum and inoculum was rub applied with a cotton swab.

[x]Percentages followed by the same letter within a factor are not significantly different based on Fisher’s protected least significant difference ($P \leq 0.05$).

in terms of infection rate. The number of inoculations also was significant with three applications having a significantly greater percent infection (47.9%) compared to one application (30.6%).

On June 29, 2015, the plant material were evaluated with ELISA for the presence of ZYMV (Table 3). There were differences between the inoculation technique used with the highest absorbance values (*i.e.*, highest virus concentration) for the gravity fed airbrush sprayer and the standard method with values of 1.270 and 1.094, respectively. The gravity fed airbrush sprayer also had a higher absorbance reading than the siphon fed sprayer. There were no differences in absorbance values between the carborundum application method or the number of inoculations.

Discussion

The purpose of the airbrush sprayer was to have a more efficient and less time consuming method of inoculating plants. In addition, it was hoped that it would offer a more uniform and operator independent method of application. It has been noted that depending on the individual applying the inoculum, there can be differences in the infection rate (Cecilia McGregor, personal communication) when using the standard method.

No method has 100% infection success even when applied to known susceptible material (Hutton and Peak, 1951; Monsion *et al.*, 2008; Nienhaus *et al.*, 1990). This can complicate screening programs for disease resistance. There is the chance of selecting material as being resistant that is merely an escape. This certainly emphasizes the need to do multiple screenings and perhaps to use multiple techniques to ensure that the resistance is real.

Table 3. Evaluation of ZYMV infected squash by enzyme-linked immunoabsorbant assay (June 29, 2015)[z]

Airbrush type	
Gravity fed	1.270a[x]
Siphon fed	0.892b
Standard[y]	1.094ab
Uninoculated	0.278c
Carborundum	
Dusted	1.407
Slurry	0.756
Application Number	
1	0.931
2	1.181
3	1.132
Probabilities	
Airbrush type	0.002
Carborundum application	0.000
Number of applications	0.092
Airbrush type x Carborundum	0.967
Airbrush type x Application No.	0.251
Carborundum x Application No.	0.149
Airbrush type x Carborundum x Application No.	0.620

[z]Inoculum: 0.05M phosphate buffer (mono & dibasic KPO_4) with ZYMV infected macerated leaves, 2:1 V/W ratio.

[y]Standard: Leaves were dusted with carborundum and inoculum was rub applied with a cotton swab.

[x]Absorbance values followed by the same letter within a factor are not significantly different based on Fisher’s protected least significant difference ($P \leq 0.05$).

The siphon air brush method was more problematic than the gravity fed method. Care had to be exercised to avoid clumps of tissue in the solution because the siphon air brush was prone to clogging requiring it be cleared. This was not as much of a problem with the gravity fed air brush sprayer. It is evident with lower percent infection with the second experiment on 8 Sept. 2014 and the ELISA test results from June 29, 2015 (Tables 2 and 3).

There may be an important environmental effect on successful inoculation. In this study, inoculations in September were more successful with infection rates at 75% or greater for all methods. In contrast, April 2014 and June 2015 experiments had infections rates below 50% for the airbrush sprayers compared to the standard method, which was above 90%.

Interestingly, in June 2015 experiment, the visual evaluation had the airbrush sprayer infection rates below 50% and significantly less than the standard rate whereas the ELISA absorbance was greater with gravity fed airbrush sprayer than the siphon airbrush sprayer, but was not statistically different from the standard method. This was so even though the ELISA test was performed a week earlier than the visual evaluation. This may indicate greater sensitivity for the ELISA test, the more likely interpretation, or the virus was somehow affected during the week between ELISA and visual screening.

In conclusion, the airbrush sprayer, particularly the gravity fed unit, may offer some value during preliminary screening since it is relatively easy to set up and use. There is some evidence that more inoculations result in better infection rates and inoculating more than three times may have additional benefits. Although there may be diminishing returns on efficient use of time and effort with increasing numbers of inoculations. Finally, using the airbrush sprayer cannot be the sole method of evaluating germplasm. The standard method will clearly have to be employed at some point and more sophisticated methods of screening such as ELISA or polymerase chain reaction techniques will have to be employed.

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