European Journal of Plant Pathology **103:** 373–378, 1997.

Some characteristics of the transmission of grapevine leafroll associated virus 3 by *Planococcus citri* Risso

Cristina Cabaleiro[1] and Antonio Segura[2]
[1] *Departamento de Ingenieria Agroforestal y Producción Vegetal, Universidad de Santiago de Compostela, 27002 Lugo, Spain (Fax: 34-82-241835);* [2] *Departamento de Biología Vegetal, Universidad de Santiago, 15706 Santiago de Compostela, Spain (Fax: 34-82-596904)*

Accepted 28 January 1997

Key words: virus-vector, mealybugs, closterovirus, grapevine, transmission

Abstract

Some characteristics of the acquisition and transmission of GLRaV-3 by *Planococcus citri* were determined by ELISA testing and transmission experiments. Groups of five insects were used, i.e. the advisable minimum group size suggested by the results of ELISA of insect groups of various sizes. The virus was transmitted to only 1/10 test plants each of which had been exposed to a group of insects fed on GLRaV-3 infected plants for at least three days, eventhough more than 80% of the insect groups were expected to contain viruliferous individuals under these conditions. Viruliferous mealybugs transferred to potato plants could retain the virus for up to 24 h, but lost the capacity for effective transmission to vines within 1 h after transfer. In newly infected vines, the virus remained latent or undetectable by ELISA for at least 13 months.

Introduction

Leafroll is one of the most widespread viral diseases of grapevine. Its expansion has been mainly due to movement of infected plant material, especially asymptomatic rootstocks. Its aetiology has not been fully clarified, but there are at least seven leafroll associated closteroviruses (GLRaVs). Furthermore, several trichoviruses are associated with both leafroll and the rugose wood complex (Walter and Martelli, 1996). The trichoviruses GVA and GVB have been transmitted mechanically to herbaceous hosts (Conti et al., 1980; Engelbrecht and Kasdorf, 1985; Monette et al., 1990; Hu et al., 1990). GVA was first transmitted by *Pseudococcus longispinus* Targioni Tozzetti in controlled conditions from infected vines to *Nicotiana clevelandii* Gray (Rosciglione et al., 1983) and several research groups have reported experimental vectorial transmission of GVA, GVB, and GLRaV-3 from vine to vine (Engelbrecht and Kasdorf, 1985, 1987, 1990a; Rosciglione and Gugerli, 1989; Tanne et al., 1989; Boscia et al., 1993; Belli et al., 1994; Garau et al., 1995). At the moment, four pseudococcids (*Pseudococcus longispinus* Targioni Tozzetti, *Planococcus ficus* Signoret, *Pseudococcus affinis* and *P. citri* Risso) and at least one coccid (*Pulvinaria vitis* L.) have been reported as vectors of GLRaV-3 (Engelbrecht and Kasdorf, 1984; Rosciglione and Gugerli, 1989; Tanne et al., 1989; Belli et al., 1994; Golino et al., 1994; Cabaleiro, 1995). The relationship between spread of leafroll in the vineyards and the presence of mealybugs was confirmed in Europe (Rosciglione and Gugerli, 1989; Belli et al., 1994; Cabaleiro and Segura, 1997; N. Ioannou, A. Hadjinicoli and A. Hadjinicoli, pers. comm.), in New Zealand (Jordan et al., 1993) and South Africa (Engelbrecht and Kasdorf, 1985, 1990a, 1990b). In other countries no coccid or pseudococcid vectors have been identified as associated with the spreading of the disease in the field (Dimitrijevic, 1973; Caudwell et al., 1983; Legin et al., 1986, Teliz et al., 1989; Habili et al., 1995).

Notwithstanding the evidence on transmission of leafroll associated viruses by mealybugs, little is known on the characteristics of this process. In most

experiments on GLRaV-3 transmission, large numbers of mealybugs have been used in order to ensure that even poorly efficient vectorial activity would be detected (Engelbrecht and Kasdorf, 1984; Rosciglione and Gugerli, 1989; Tanne et al., 1989; N. Ioannou, A. Hadjinicoli and A. Hadjinicoli, pers. comm.). In these reports vectorial efficiency itself was not quantified. Preliminary findings by P. Lanotte and A. Minafra (pers. comm.) indicate that GVA may be transmitted semi-persistently by mealybugs, just the same as badnaviruses (Lockhart et al., 1995). Following the finding that *Pl. citri* acts as a vector of GLRaV-3 under both controlled and field conditions (Cabaleiro and Segura, 1997), experiments were carried out to determine the acquisition and retention of the virus by the mealybug and the efficiency of transmission, using serological analysis of both mealybugs and innoculated vines (Campbell and Madden, 1990).

Materials and methods

Vector. *Pl. citri* from a vineyard in which leafroll was spreading were reared on potato sprouts for several generations; potatoes are not susceptible to GLRaV-3.

Plant material. Potted plants of *Vitis vinifera* cv. Albariño (clone C1) infected with GLRaV-3 and free from other viral diseases (field indexed for GFLV, fleck and Stem pitting; ELISA negative for GLRaV-1, 2, 5) were used as the source of virus. Healthy (field indexed) Albariño and Cabernet franc cuttings rooted in vermiculite were potted for use as recipient in transmission tests; Cabernet franc was used due to its sensitivity to leafroll.

Determination of the experimental group size. In each of two replicate experiments, mealybugs born and reared on GLRaV-3-infected grapevines were analyzed for virus presence by DAS-ELISA with repeated tests at concentrations of 1, 2, 3, 4, 5 and 10 insects per well, with 10 wells for each concentration. Additional DAS-ELISAs were performed for concentrations of 1 and 5 insects per well. Experimental group size was determined as described in Results following estimation of the probability of acquisition of GLRaV-3 by a single insect.

Acquisition access time (AAT). Nymphs of *Pl. citri* reared on potato plants were transferred to GLRaV-3 infected vines and allowed to feed for 1 h, 1 day, 3 days or one week; mealybugs from potato were used as negative controls. For each treatment, 10–12 groups of five mealybugs each were analysed by ELISA for the presence of GLRaV-3 and 10 groups of 5 mealybugs each were transferred to healthy Albariño or Cabernet franc grapevine plants. Mealybugs were removed from recipient plants after 15 days, the plants were then sprayed with dimethoate (1.2 ml/l of the 40% commercial product), and transferred to a greenhouse. Adult leaves were analysed by ELISA for the presence of GLRaV-3 about every 3 months between month 4 and 18 following exposure to vectors.

Retention of infectivity. Nymphs of *Pl. citri* born and reared on GLRaV-3 infected grapevines were transferred to potato plants for 1 hour, 1 day, 3 days or one week; Nymphs kept on infected vines were used as positive controls, and mealybugs born and reared on potato as negative controls. For each treatment 10 groups of 5 mealybugs each were analysed by ELISA and 10 groups of 5 mealybugs each were transferred to healthy Albariño or Cabernet franc grapevine plants, which were subsequently treated as in the AAT experiment.

Detection of GLRaV-3 by ELISA. DAS-ELISA was performed essentially according to Clark and Adams (1977) with a commercial kit (Bioreba AG, Basel) following the manufacturer's instructions. The insects were ground in 100 μl of the extraction buffer, the same as for grapevines: 0.5 m Tris HCl pH 8.2 with 1% polyethyleneglycol (MW 6000), 0.8% NaCl, 2% polyvinylpyrrolidone (MW 24000) and 0.05% Tween 20 (Gugerli et al., 1984). Absorbance at 405 nm was recorded using a microplate reader (Titertek Uniskan). Samples with absorbance readings more than three times that of the healthy control and higher than 0.100 were considered positive.

Estimation of individual infection or infectivity rates from group rates. The probability that a single insect was a virus-carrier or vector was calculated using the estimator proposed by Swallow (1985):

$$p_s = 1-(R/N)^{1/k}$$

and the corrected formula by Burrows (1987):

$$p_B = 1-\{(2kR+k-1)/(2kN+k-1)\}^{1/k}$$

where

N = total number of test plants or mealybug groups

R = number of negative plants or negative mealybug groups

k = number of individuals per plant or per mealybug group

Results

Determination of the experimental group size. The ELISA results for mealybug groups of various size reared on GLRaV-infected vines are presented in Table 1. The virus was detected in 19% of single insects (8/42), in 66% of five-insect groups and in the 100% of 10-insect groups. Individual infection given by Swallow's and Burrows's estimators using groups of two to five insects was between 0.13 and 0.19, in agreement with the results of testing single mealybugs. Using ten insect groups the uncorrected estimator p_S was 1 and the corrected estimate p_B was 0.31, in disagreement with single mealybug analysis. On the basis of these data, and bearing in mind that *Pl. citri* is already known to be a vector of GLRaV-3 and that the maximum manageable number of test plants in our working conditions was ten, it was decided to perform transmission assays with groups of 5 insects, i.e., 1–2 insects more than the minimum advisable number suggested by Swallows's abacus (Swallow, 1985).

Acquisition access time (AAT). The minimum access time required for infection of any of the members of a five-insect group was 3 days (Table 2). After one week the virus was detected in 11/12 groups of 5 insects, giving a p_s value of 0.39, higher than for insects reared since birth on infected plants. However, the virus was detected in only 1 of 10 plants expposed to insects given a 3-day acquisition time, and the transmission rate was again 1/10 for an AAT of 1 week ($p_S = 0.02$). The first positive ELISA results were recorded only 13 months after transmission, and the plants remained positive from then on and developed lefroll symptoms.

Retention of infectivity. Table 3 shows the results of the retention experiments. It was not possible to detect GLRaV-3 in pre-infected mealybug groups fed on potato for more than 1 day, by which time p_S has already fallen to 0.04. Although 45% of insect groups were still carrying virus after 1 h feeding on potato, all the test plants remained ELISA negative for 18 months.

Discussion

Pl. citri is a GLRaV-3 vector able to transmit the virus under controlled conditions (Cabaleiro and Segura, 1995; N. Ioannou, A. Hadjinicoli and A. Hadjinicoli, pers. comm.) and quite efficient in the field (Cabaleiro and Segura, 1997). Transmission experiments with this virus/vector system are hampered by the very long time needed for plants to give positive ELISA responses. Moreover, the comparison of such results is complicated by the apparent dependence of the duration of the incubation period in plants on the vector species and the number of vectors per recipient plant. In our previous study using as vectors groups of about 20–30 *P. citri virus* transmission of the virus to 5/5 plants was detected only after 8 months (Cabaleiro and Segura, 1997) while in the present work it was ascertained only about 13 months after inoculation with groups of five-insect/plat. Rosciglione and Gugerli (1989) detected the antigen after 5 months in plants inoculated each by 50 *Pl. ficus* or *Ps. longispinus* and N. Ioannou, A. Hadjinicoli and A. Hadjinicoli. (pers. comm.) obtained the first positive responses only four months after inoculation using as vectors 75 *Pl. ficus* individuals per plant. The use of more sensitive detection methods such as nucleic acid extraction and hybridization and PCR allowed Belli et al. (1994) to detect GLRaV-3 in grapevine plants four months after inoculation with *Pulvinaria vitis* when no positive results could be obtained by ELISA.

In this work, the number of vectors per recipient plant was determined by ELISA testing of variously sized groups of insects. Although it was found that not all viruliferous mealybugs were necessarily inoculative, all acquisition access periods allowing infection of the vectors also proved sufficient for effective, albeit poorly efficient, transmission to plants. The inferred probability of a sufficiently exposed individual insect transmitting GLRaV-3 to a vine was 0.02, which is low but similar to that reported for many other vector/plant systems (Berger and Ferris, 1988) and is higher than the 0.01 value implied for *P. ficus* by the results of Rosciglione and Gugerli (1989) and the value of 0.008 obtained by N. Ioannou, A. Hadjinicoli and A. Hadjinicoli, (pers. comm.) using 75 *P. citri* individuals for each of 15 plants. However, all these figures differ markedly from our previous 0.07–0.11 with 20–30

Table 1. ELISA detection of GLRaV-3 in variously sized groups of mealybugs (*P. citri*) hatched and raised on infected vines. The inferred probability of acquisition of the virus by a single insect is estimated (p_S, Swallow's estimate; p_B, Burrow's estimate)

k	Positive/total number of groups analysed					
	1st series	2nd series	other series	Total (%)	p_S	p_B
1	1/10	4/10	3/22	8/42 (19)	0.191	0.191
2	2/10	3/10		5/20 (25)	0.134	0.132
3	4/10	5/10		9/20 (45)	0.181	0.177
4	3/10	6/10		9/20 (45)	0.139	0.136
5	7/10	7/10	11/18	25/38 (66)	0.193	0.190
10	10/10	10/10		20/20 (100)	1	0.317

k = insects/well in 100 μl extraction buffer

Table 2. Results obtained when potato-reared mealybugs (*P. citri*) were fed for various periods of time on GLRaV-3 infected grapevine plants and then either checked by ELISA in groups of five or transferred in groups of five to test plants which were analysed by ELISA periodically for up to 18 months. Probability (Swallow's estimator, p_S) that a single insect was a GLRaV-3 carrier or had acquired and transmitted GLRaV-3

	Groups of 5 mealybugs		Plants	
Acquisition time	+/total (%)	p_S	+/total (%)	p_S
1 h	0/10 (0)	0	0/10 (0)	0
24 h	0/11 (0)	0	0/10 (0)	0
3 days	9/11 (82)	0.29	1/10 (10)	0.02
1 week	11/12 (92)	0.39	1/10 (10)	0.02
Control (–)	0/10 (0)	0	0/10 (0)	0

Table 3. Results obtained when mealybugs (*P. citri*) reared on GLRaV-3 infected grapevines were fed for various periods of time on potato and than either checked by ELISA in groups of five or transferred in groups of five to test plants which were analysed by ELISA periodically for up to 18 months. p_S, probability (Swallow's estimate) that a single insect had acquired and retained, or retained and transmitted GLRaV-3

	Groups of 5 mealybugs		Plants	
Time on potato	+/total (%)	p_S	+/total (%)	p_S
1 hour	5/11 (45)	0.11	0/10 (0)	0
24 hour	2/10 (20)	0.04	0/10 (0)	0
3 days	0/10 (0)	0	0/10 (0)	0
1 week	0/10 (0)	0	0/10 (0)	0
Control (–)	0/10 (0)	0	0/10 (0)	0
Control (+)	7/10 (70)	0.21	1/10 (10)	0.02

insects and 5 plants (Cabaleiro, 1995) or the values of 0.16–0.29 calculated after the results reported for *P. ficus* by Tanne et al. (1989). These higher values, however, are similar to the 0.2–0.3 reported for the transmission of cacao viruses by mealybugs under optimal conditions (Roivainen, 1976).

The 3-day acquisition threshold recorded in this work, is probably an overestimate, not only because no access times between 24 and 72 h were tested, but also because it does not take into account the time taken for newly transferred mealybugs to settle and recommence feeding. Settling time could probably be reduced by a pre-exposure fasting period, as it seem to pappen with cacaoviruses (Roivainen, 1980) and GVA (P. Lanotte and A. Minafra, pers. comm.).

After 3 days, the proportion of exposed insects that acquired virus continued to increase with exposure time, here again in accordance with the findings relative to cacao viruses (Roivainen, 1976) and BVA (P. Lanotte and A. Minafra, pers. comm.).

In this work, the virus was retained by infected mealybugs for up to 24 h after their removal from infected plants. However, because of their poor transmission efficiency or the low infectivity of the viral particles, infection of recipient vines only occurred after direct transfer of mealybugs from the virus source. For cacao viruses, retention times of up to 96 h have been reported, regardless of whether the insects have been fasted or not before acquisition (Roivainen, 1976), and GVA vectors retained infectivity for up to 48 h when kept fasting after removal from the virus source (P. Lanotte and A. Minafra, pers. comm.).

In general, semipersistently transmitted viruses are not retained by their vectors through molting (Berger and Ferris, 1988), although there are some doubts in the case of the transmission of cacao viruses by mealybugs (Roivainen, 1980). Although this aspect of transmission was not investigated in detail in this work, post-molt ELISA of virus exposed individual

mealybugs was always negative (results not shown). In view of the long acquisition threshold observed in this work, and of the post-transfer delay before viruliferous mealybugs begin feeding on the recipient plant, the possibility of virus loss through molting suggests that one cause of discrepancy between the number of ELISA-positive and that of virus-transmitting mealybugs may have been the occurrence of molting between transfer and recommencement of feeding.

Acknowledgements

We thank Amparo Blay and Pedro del Estal for identification of *Pl. citri* Risso and Fernando García-Arenal for critical reading of the manuscript. This work was partly funded by a grant from the Xunta de Galicia (XUGA200074A93).

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