Effect of Two *Allexivirus* Isolates on Garlic Yield

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ABSTRACT

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Garlic (*Allium sativum*) is infected by numerous viruses forming a viral-complex, which is widely distributed in the garlic production regions of Argentina. This work is the first report of the effect of two *Allexivirus* isolates, *Garlic virus A* (GarV-A) and *Garlic virus C* (GarV-C), on garlic yield. Garlic cvs. Morado-INTA and Blanco-IFFIVE were used in the experiments, and four treatments were evaluated: plants inoculated with GarV-A only, GarV-C only, virus-free plants (negative control), and plants infected with the virus-complex. Assays were performed in anti-aphid cages and in the field during 2002 and 2003. GarV-A caused significant reductions in bulb weight (14 to 32%) and diameter (6 to 11%) compared with the negative control in the two cultivars under both assay conditions. GarV-C caused less damage than GarV-A (15% in weight and 5% in diameter) with respect to the negative control in cv. Blanco-IFFIVE, and did not produce significant yield losses in cv. Morado-INTA in either year or under either assay condition.

Additional keywords: garlic mite-borne virus, garlic mosaic

Garlic (*Allium sativum* L.) is infected by numerous systemic viruses that are spread widely by the seed bulbs due to exclusive vegetative propagation of the crop. Thus, the viruses accumulate in plants over time and form a viral complex composed of different species producing a disease known as garlic mosaic. *Onion yellow dwarf virus* (OYDV), *Leek yellow stripe virus* (LYSV), *Shallot latent virus* (SLV), and *Garlic common latent virus* (GCLV) have been cited as aphid-borne viruses infecting garlic (2,10,17,18,34,35). Other garlic viruses are transmitted by mites (1,21,36,37) and have been grouped in the genus *Allexivirus* (38). Based on the nucleotide sequence, different virus species have been detected within the genus *Allexivirus*, such as *Garlic viruses A, B, C, D, E, and X* (GarV-A, B, C, D, E, and X) (7,8,23,29,31,33). The sequence of an *Allexivirus* species, *Garlic mite borne filamentous virus* (accession no. X98991) (21), detected in Argentina showed 91% homology of viral coat protein (CP) amino acid sequence and 98% homology of 3' noncoding nucleotide sequence with GarV-A from Japan (31). Given this high sequence homology, it was designated GarV-A. This virus was detected in most of the garlic production areas in Argentina, with an incidence

ranging from 35 to 64% depending on the region (26).

Another *Allexivirus*, serologically identified as GarV-C, has been isolated in Argentina by mechanical transmission from infected garlic to *Chenopodium murale* (3). The GarV-A and GarV-C tested in this work are widely distributed in the garlic production regions where *allexiviruses* were studied (3,8,19–21,30,33).

The greatest economic losses in garlic have been attributed to OYDV, and to a lesser degree, to LYSV (4,24,27). Reductions of the bulb weights of White garlic type and cv. Rosado Paraguayo in Argentina (60 and 53%, respectively) by virus-free plants and plants infected with the viral complex have been reported (4,12). Some reports suggest that mite-borne viruses may negatively influence yields of garlic (36), but this has not been demonstrated. The lack of information available about the effect of the viruses on garlic yield is due to the difficulty in isolating each viral species from the garlic virus complex and to the lack of specific antisera against each virus species. This paper reports the effect of two *Allexivirus* (GarV-A and GarV-C) isolates on garlic yield.

MATERIALS AND METHODS

Serological assays and antisera. Virus infection was assessed using the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), following the protocol described by Clark and Adams (9) and adjusted for garlic by Conci et al. (15). Immunosorbent electron microscopy plus decoration (ISEM-D) (28) was also used for selecting the virus-free plants and plants infected with GarV-A and GarV-C.

Antisera against OYDV, LYSV, GarV-A, and MIX-antiserum obtained from garlic plants infected with the mixture of viruses that naturally affect garlic were used, taken from the stock of IFFIVE-INTA, and obtained as described in Conci et al. (14), Lunello et al. (25), and Helguera et al. (21,22), respectively. Mixed antiserum basically detects *Allexivirus* and *Carlavivirus* (V. C. Conci, unpublished results). GCLV and GarV-D antisera were kindly provided by D. E. Lesemann, H. J. Vetten, and E. Barg from BBA, Braunschweig, Germany; SLV by L. Bos and D. Z. Maat from the Research Institute for Plant Protection, Wageningen, Netherlands; and GarV-C and GarV-A by S. Sumi from the Institute for Biotechnology Research, Wakunaga Pharmaceutical. Another antiserum obtained from a mixture of garlic viruses was kindly provided by M. Carvalho from UFV from Viçosa, Brazil, and R. Shepherd from the University of California, Davis (5,6). It was demonstrated that this antiserum basically detects *Allexivirus* (V. C. Conci, unpublished results).

Isolation of *Allexivirus*. GarV-C and GarV-A viruses were isolated from garlic plants chronically infected by the virus complex. Leaves were macerated in a borate buffer solution (0.05 M, pH 8.1) and mechanically transmitted with 300 mesh Carborundum to *Chenopodium murale* plants, in which *Allexivirus* produces local lesions (37). Plants were checked periodically for symptom expression. Tissue from each lesion obtained was macerated in the same buffer solution and used as inoculum to infect new leaves of *C. murale*, one leaf with each lesion. This procedure was repeated six times to obtain virus isolates. Sixteen possible isolates were obtained, from which one was identified as GarV-C and another as GarV-A. To confirm the purity of the isolates, they were tested by ISEM-D with dilution of 1:500 for the coating antiserum and serial double dilutions ranging from 1:25 to 1:6,400 for decoration of the homologous antiserum to detect possible differences in the intensity of the viral particles decoration. Each virus isolate was maintained and multiplied in *C. murale* through mechanical inoculations. The identity of the viral isolate was confirmed periodically by ISEM-D with the specific antisera mentioned.

Plant material. Two garlic cultivars were used: Morado-INTA, a selection from Asiatic garlic with short growth cycle (230 days), belonging to the germ plasm bank of La Consulta INTA Experimental

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Fig. 1. Differences between garlic bulbs: **A**, negative control; **B**, plants inoculated with *Garlic virus C* only C-treatment; **C**, virus-complex treatment, and **D**, plants inoculated with *Garlic virus A* only, A-treatment in cv. Blanco IFFIVE.

Agricultural Station from Mendoza, Argentina; and Blanco-IFFIVE, a selection of the Institute of Phytopathology and Plant Physiology, National Agricultural Technology Institute (IFFIVE-INTA) from Córdoba, Argentina, belonging to the White garlic type with a crop cycle of 210 to 230 days. Both cultivars are cultivated in the most important Argentine garlic producing regions.

Virus-free garlic plants (negative controls) used as healthy controls were obtained by thermotherapy followed by meristem culture (11,16). These plants were hardened and multiplied in anti-aphid cages and analyzed by ISEM-D using the antisera mentioned above. Garlic plants chronically infected with virus complex positive to OYDV, LYSV, GarV-A, GarV-C, GarV-D, and GCLV (virus-complex treatment) were used as positive controls.

GarV-C and GarV-A isolates were transmitted to virus-free garlic planted in separate anti-aphid cages (C and A treatments, respectively). Plants at the three-leaf stage were inoculated mechanically with each virus isolate. After 6 months, each inoculated plant was tested by ISEM-

Fig. 2. Effect of *Garlic virus A* (GarV-A) and *Garlic virus C* (GarV-C) isolates on weight and diameter of garlic cv. Blanco-IFFIVE in the anti-aphid cage for years 2002 and 2003, respectively. Abbreviations of treatments: NC, negative control; C, C-treatment; A, A-treatment; VC, virus-complex treatment. Bars with different letters are significantly different ($P < 0.05$) based on LSD.

D with the antisera mentioned above. Uninfected plants were eliminated. Bulbs produced by infected plants were harvested and stored in a dry place at room temperature (17 to 27°C range) until the start of the next crop cycle.

One year before starting the assays, the four treatments (negative control, virus-complex, C, and A) were planted and multiplied in individual anti-aphid cages (2 by 1 m), one for each treatment, 10 cm distant within rows, with 30 cm between rows in order to obtain bulbs under the same growing and environmental conditions. The sanitary condition of each treatment was tested again by DAS-ELISA and ISEM-D.

Yield assays. The four treatments were compared during two consecutive years (2002 and 2003) in parallel assays, in anti-aphid cages and in the field. Cloves of equal size were planted for the assays. Clove size was standardized using two sieves (1.5 and 1 cm²), which resulted in three size categories. Cloves retained by the second sieve (category 2) were used for treatments and assays. Three days prior to planting, cloves were disinfected by immersion for 15 min in a suspension of captan (Captan Tomen WP 80%, 25 g),

procymidone (Sumilex 50 SC 50%, 15 g), pentachloronitrobenzene (PCNB, WP 78, 2%, 15 g), and benomyl (Benlate WP 50%, 25 g) in 25 liters of water. The assays were conducted in the research field of the IF-FIVE-INTA, Córdoba, Argentina.

Anti-aphid cage assays. Assays were conducted in a randomized plot design, one plot per treatment. Each plot consisted of an anti-aphid cage (3 × 1 m). Cloves were planted in each anti-aphid cage in three rows 30 cm apart and at 10-cm intervals. Plants continued to be infected until the end of the crop cycle, as confirmed by DAS-ELISA and/or ISEM-D.

Field assays. The trials were carried out in four randomized complete blocks for each cultivar and for each year of assay. Each block contained four plots, one for each treatment. The cloves were planted in furrows 60 cm apart and at 15-cm intervals. The cloves used in the assays were obtained from the anti-aphid cages in the previous crop cycle.

Each experimental plot in each block included 20 cloves. Random samples of 28 plants in 2001 and 25 plants in 2002 were taken from each treatment to evaluate virus infection by DAS-ELISA with OYDV,

LYSV, and GarV-A antisera. The relative virus concentration (RC) was calculated for each year and treatment to compare the DAS-ELISA absorbance values for different viruses and at different times (15). The RC was calculated as the ratio between the DAS-ELISA absorbance value (measured at 405 nm) of the sample and the mean plus twice the standard deviation of the DAS-ELISA absorbance of five healthy control samples tested in the same plate. Absorbance readings were measured with a Dynatech MR 4000 spectrophotometer (Dynatech, Guernsey Channel Islands, UK).

The plants were irrigated every 7 to 10 days. After harvest, bulbs were stored dry at room temperature. Bulb weight and diameter were recorded 30 days after harvest. Bulb weight was measured with a precision balance, and diameter was recorded at the greatest equatorial diameter of the bulb with a digital caliper. The treatments were compared by ANOVA. When needed, LSD-Fisher multiple comparisons test was applied. The statistical analyses were carried out with InfoStat statistical software (Version 2004, Grupo InfoStat, Facultad Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

Fig. 3. Mean weight and diameter of garlic bulbs in treatments of cv. Blanco-IFFIVE in field assays for 2002 and 2003, respectively. Abbreviations of treatments: NC, negative control; C, C-treatment; A, A-treatment; VC, virus-complex treatment. Bars with different letters are significantly different ($P < 0.05$) based on LSD.

RESULTS

Results varied with the virus isolate tested (Fig. 1). Bulb weight and diameter were reduced significantly by GarV-A in the cv. Blanco-IFFIVE in anti-aphid cage assays, in the 2 years of assays (Fig. 2). A reduction of 32 and 14% in bulb weight and of 11 and 6% in diameter was observed for each year, respectively. A marked reduction in A-treatment yield with respect to the negative control was also observed for the two variables evaluated in both years in the field assays (Fig. 3). Losses of 25 and 17% in bulb weight and 10 and 5% in bulb diameter were detected for 2002 and 2003, respectively.

The yields of plants infected with GarV-C in anti-aphid cages were significantly smaller than the yields of negative control in the 2002 assay. A 15% reduction in bulb weight and 5% in diameter were observed. In both years, C-treatment produced bulbs of significantly greater weight and diameter than A-treatment (Fig. 2). In the field assay, no significant differences were detected between C-treatment and negative control in 2002; however, differences were significant in 2003 assays, with a 9% reduction in bulb weight and 4% in diameter. The isolate GarV-C caused significantly less effect on yield than isolate GarV-A for both yield variables evaluated, except for

diameter in the second year in the field assay (Fig. 3).

The virus-complex treatment caused the greatest damage to yield, and the losses were statistically significant for all treatments tested in both years. Weight losses of up to 61% and diameter reductions of up to 30% with respect to the negative control were detected (Figs. 2 and 3).

In cv. Morado-INTA in anti-aphid cages, GarV-A significantly reduced bulb weight and diameter compared with the negative control during the 2 years of assay (Fig. 4). Reductions of 25 and 14% in weight and 11 and 7% in diameter were detected in 2002 and 2003, respectively. In field assays, a significant bulb weight and diameter reduction was observed in A-treatment with respect to the negative control (Fig. 5), with losses of 27 and 31% in weight and of 8 and 10% in diameter in 2002 and 2003, respectively. Losses in bulb weight and diameter in A-treatment in field assays were higher than those detected for the virus-complex treatment, taking into account that differences in the virus-complex treatment with respect to negative control were nonsignificant in 2003. The A-treatment was not significantly different from virus-complex treatment only in bulb diameter in 2002.

The C-treatment did not differ significantly from negative control in yield in

either of the two assay conditions tested (anti-aphid cages and field assays) in the 2 years (Figs. 4 and 5). The bulbs in the C-treatment were significantly heavier and larger than in the A-treatment.

High percentages of OYDV infection were detected in plants in the field assays. In the 2002 experiment, 100% of the plants of all treatments of the two cultivars were infected with this virus. In 2003, however, percentages ranged from 36 to 88%. LYSV infection was lower than that recorded for OYDV. The percentages of LYSV infection ranged from 7 to 75% in 2002, and from 4 to 56% in 2003. Infections with GarV-A were different in the 2 years of assays. The extreme infection values in 2002 ranged from 4 to 36%, whereas in 2003 GarV-A infection was very low, being almost absent in some treatments, such as C-treatment in cv. Blanco-IFFIVE and negative control in cv. Morado-INTA (Table 1).

The analysis of viral concentration (evaluated through RC) in cvs. Blanco-IFFIVE and Morado-INTA in 2002 revealed a high concentration of OYDV in all treatments; however, in 2003 RC values were lower (Table 1).

DISCUSSION

This work is the first report in which the effect of two isolates of *Allexivirus*, GarV-

Fig. 4. Effect of *Garlic virus A* (GarV-A) and *Garlic virus C* (GarV-C) isolates on weight and diameter of garlic cv. Morado-INTA in the anti-aphid cage for 2002 and 2003, respectively. Abbreviations of treatments: NC, negative control; C, C-treatment; A, A-treatment; VC, virus-complex treatment. Bars with different letters are significantly different ($P < 0.05$) based on LSD.

C and GarV-A, on garlic crop yield is evaluated. The development of this kind of study has been hampered by the difficulties in isolating viruses from the garlic viral complex. In fact, there are no differential hosts that facilitate isolation of either virus easily and quickly. Furthermore, maintaining and multiplying virus isolates on garlic plants for comparative yield assays is also difficult. The greatest difficulty lies in the lack of plants naturally free of virus. Because garlic is propagated vegetatively, viruses persist over successive generations. Virus-free garlic plants are obtained through meristem culture (16,40), a long process that requires at least 1 or 2 years to produce virus-free plants in vitro, transfer them to ex vitro conditions, and harden them so that they readapt to normal crop conditions. These stages are highly risky, since plants are easily reinfected when they are exposed to the normal cultivation conditions in the field near infected garlic plants. Of the viruses affecting this crop, potyviruses and carlaviruses are transmitted by aphids in a nonpersistent manner, and allxiviruses are transmitted by eriophid mites, which are common pests in bulbs, making it easier to reinfest the crops. Quick reinfection was observed in this work in the negative control and in the A and C treatments in the field assays. Despite these difficulties, it is important to

determine the effect of each garlic-infecting virus on yield for the certification programs of bulb-seed garlic to efficiently control garlic mosaic. In this work, it was found that GarV-C, under the conditions studied and in the cultivars tested, caused mild or no damage on garlic yield. In cv. Blanco-IFFIVE, the effect on yield was significant in anti-aphid cages in one year (2002), with reductions in bulb weight and diameter of 15 and 5%, respectively, compared with the negative control, but in the second year (2003), no effect on the yield was observed. When C-treatment was evaluated in the field, in spite of the fact that plants became naturally infected, in the 2003 assay, significant losses were detected in weight and diameter in low percentages (9 and 4%, respectively). In cv. Morado-INTA, no significant effects were detected on the yield variables evaluated in anti-aphid cages and in field assays in both years. The results in C-treatment were similar to those reported by Takaichi et al. (32), who mentioned little yield reduction by virus infection with a single unidentified mite-borne virus species. The virus evaluated by Takaichi et al. (32) probably was GraV-C, considering the low effect on yield found.

GarV-A was the *Allexivirus* species that most affected yield in this work. In both

cultivars and both years, this virus caused losses that ranged from 32 to 14% in bulb weight and from 11 to 6% in diameter with respect to negative control, observed in anti-aphid cage assays. This is the first time that an *Allexivirus* has been reported to cause a severe effect on yield. This is important considering that GarV-A is a widespread virus in the garlic production regions (8,20,21,26,31,33).

Important yield losses were detected in the virus-complex treatment in cv. Blanco-IFFIVE ranging between 61 and 57% in bulb weight and between 30 and 26% in diameter in 2002 and 2003, respectively. This marked reduction in yield in chronically infected bulbs compared with virus-free plants was also observed by other authors in different garlic cultivars (4,24,39). However, it was also observed that in cv. Morado-INTA, losses caused by viruses of the virus-complex treatment were not so marked. In fact, a 19% loss in diameter and a reduction in bulb weight that ranged from nonsignificant to 37% were detected. These differences in yield between cultivars caused by the effect of viruses were also detected by Walkey and Antill (39), who observed a low yield loss in the cv. Fructidor.

When the viruses infecting each treatment in the field assays were identified, it was observed that all treatments had been

Fig. 5. Mean weight and diameter of garlic bulbs in treatments of cv. Morado-INTA in field assays for 2002 and 2003, respectively. Abbreviations of treatments: NC, negative control; C, C-treatment; A, A-treatment; VC, virus-complex treatment. Bars with different letters are significantly different ($P < 0.05$) based on LSD.

reinfecting in high percentages. These infections might explain some results, such as the lack of yield differences observed in the negative control and the C-treatment in cv. Blanco-IFFIVE in the first year (2002). In contrast, when the assay was repeated in 2003, C-treatment was significantly lower in bulb weight and diameter with respect to negative control. It was also found that in cv. Blanco-IFFIVE, the percentage of plants infected by OYDV in the first year was 100% in all treatments. Moreover, the analysis of RC of each virus during 2002 showed that the OYDV concentration in the negative control was very high (RC = 12.4), and higher than that detected in the C-treatment. Considering that OYDV has been cited in previous work causing the greatest yield losses (4,18,24), it can be assumed that the absence of significant difference might be due to the high OYDV concentrations detected in the negative control plants that year. In contrast, in the second year, when significant differences were detected between the two treatments, 64% of the negative control plants were infected by OYDV, whereas 88% were infected in the C-treatment. In addition, RC in the negative control plants was very low (RC = 1.2) compared with RC detected in C-treatment (RC = 2.8). This probably caused a yield reduction in the C-treatment, and the results were significantly lower than for the negative control.

The effect on yield of viruses GarV-A and GarV-C can be clearly observed under anti-aphid cages; those differences were maintained under natural field conditions, although the plants were infected by other

viruses. This indicates that although plants were infected by viruses, yield was not reduced immediately, or that probably the yield reduction depends on certain virus combinations. Yield reduction does not appear to occur in a short time. Conci et al. (13) also detected that when virus-free plants become infected, yield declines over successive crop cycles, and that even after five crop cycles exposed to infection, the plants still do not suffer the yield loss values of virus-complex plants. The reason why yield losses have not been detected despite the occurrence of reinfection has not been elucidated yet. Results of the assays conducted in this work have shown that, apparently, the viruses that previously infected the plants had a greater influence than those viruses more recently acquired, since the effects on yield were similar to those caused by treatments in the anti-aphid cage. GarV-C did not affect yield, or reduced it by a low percentage in the anti-aphid cage; the C-treatment showed a similar behavior in field assays, despite the infection produced. In contrast, GarV-A caused statistically significant yield losses with respect to the negative control in anti-aphid cages; in the field assays, the same differences were observed although negative control plants were infected. Further studies are necessary to elucidate the relationship between yield losses and infection with the different viral species, their combinations and concentrations. The evaluation of the effects of the two *Allexivirus* isolates studied on bulb weight and diameter, and the significant yield loss caused by GarV-A emphasizes the need to use certi-

fied material and to discriminate the viral species present, since not all of them affect yield similarly.

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Table 1. Percentage of virus-infected plants (%) and relative concentration (RC) of viruses in field assay plants

Cultivar	Treatment ^a	Antisera ^b	No. ^c	2002		No. ^c	2003	
				VIP ^d (%)	RC ^e		VIP ^d (%)	RC ^e
Blanco-IFFIVE	A	OYDV	28	100	9.9	25	60	1.7
		LYSV	28	75	1.8	25	20	0.7
	C	OYDV	28	100	7.7	25	88	2.8
		LYSV	28	39	1.1	25	8	0.6
	NC	GarV-A	28	36	0.9	25	0	0.3
		OYDV	28	100	12.4	25	64	1.2
		LYSV	28	21	0.9	25	12	0.4
		GarV-A	28	25	0.9	25	4	0.4
	A	OYDV	28	100	6.1	25	36	1.0
		LYSV	28	71	3.5	25	12	0.3
Morado-INTA	C	OYDV	28	100	4.8	25	80	1.7
		LYSV	28	61	3.7	25	56	1.3
	GarV-A	GarV-A	28	11	0.7	25	28	0.9
		OYDV	28	100	4.0	25	76	2.4
	NC	OYDV	28	7	0.7	25	4	0.3
		LYSV	28	7	0.7	25	4	0.3
	GarV-A	GarV-A	28	4	0.4	25	0	0.3
		OYDV	28	4	0.4	25	0	0.3

^a A = inoculated with *Garlic virus A* (GarV-A); C = inoculated with *Garlic virus C* (GarV-C); NC = negative control.

^b OYDV = *Onion yellow dwarf virus*; LYSV = *Leek yellow stripe virus*; antisera to GarV-C not available.

^c Number of plants tested.

^d Virus-infected plants.

^e Relative concentration was calculated as the ratio between the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) absorbance value (measured at 405 nm) of the sample and the mean plus twice the standard deviations of the DAS-ELISA absorbance of five healthy control samples tested in the same plate.

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