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Natural infection of garlic (*Allium sativum* L.) by viruses in the Czech Republic

Natürliche Infektion von Knoblauch (*Allium sativum* L.) durch Viren in der Tschechischen Republik

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Summary

Garlic (*Allium sativum* L.) is one of the most important *Allium* crops. Significant yield and quality reduction due to virus infection is currently a serious economic problem. Garlic plants are usually infected by a mixture of viruses: *Onion yellow dwarf virus* (OYDV, Potyvirus), *Leek yellow stripe virus* (LYSV, Potyvirus), *Garlic common latent virus* (GarCLV, Carlavirus), *Shallot latent virus* (SLV, Carlavirus), and various allexiviruses. DAS-ELISA and RT-PCR were used to detect the above-mentioned viruses in five cultivars of garlic cultivated in the Czech Republic, and garlic cloves imported from both China and Spain. The results showed that Czech seed garlic (means from five cultivars) was infected on average at 75.4% by OYDV, at 31.2% by LYSV, at 99.6% by GarCLV, and at 81.1% by SLV. The cultivars Anton and Vekan were markedly less infected than other cultivars. Whereas LYSV was not detected in 'Vekan', 'Anin' had the highest LYSV infection rate among the cultivars studied. GarCLV was not detected in the imported cloves from China and OYDV with SLV were not detected in cloves from Spain. In total, 80.9% of the garlic plants tested exhibited virus symptoms on their leaves.

Key words: allexiviruses, ELISA, *Garlic common latent virus*, *Leek yellow stripe virus*, *Onion yellow dwarf virus*, RT-PCR, *Shallot latent virus*

Zusammenfassung

Knoblauch (*Allium sativum* L.) ist eine der wichtigsten Nutzpflanzen der Gattung *Allium*. Bedeutende Ertrags- und Qualitätsrückgänge durch Viruserkrankungen verursachen gegenwärtig ernst zu nehmende wirtschaftliche Probleme. Knoblauch wird in der Regel von einem Komplex verschiedener Viren infiziert: Zwiebelgelbverzwergungsvirus (OYDV, Potyvirus), Lauchgelbstreifenvirus (LYSV, Potyvirus), Latentes Knoblauchvirus (GarCLV, Carlavirus), Latentes Schalottenvirus (SLV, Carlavirus) und verschiedenen Alexiviren. DAS-ELISA und RT-PCR wurden zum Nachweis dieser Viren in fünf in der Tschechischen Republik kultivierten Knoblauchsorten und aus China und Spanien eingeführtem Knoblauch verwendet. Die fünf tschechischen Sorten waren im Schnitt zu 75,4% mit OYDV, 31,2% mit LYSV, 99,6% mit GarCLV und zu 81,1% mit SLV infiziert. Die Sorten „Anton" und „Vekan" zeigten merklich geringere Infektionsraten als die anderen tschechischen Sorten. Während LYSV in „Vekan" nicht nachgewiesen werden konnte, wies „Anin" die höchten LYSV-Infektionsraten aller untersuchten Sorten auf. GarCLV konnte nicht in den Importen aus China nachgewiesen werden und OYDV mit SLV nicht in denen aus Spanien. Insgesamt zeigten 80,9% der untersuchten Knoblauchpflanzen durch Viren hervorgerufene Blattsymptome.

Stichwörter: Allexiviren, ELISA, Latentes Knoblauchvirus, Latentes Schalottenvirus, Lauchgelbstreifenvirus, RT-PCR, Zwiebelgelbverzwergungsvirus

1 Introduction

Garlic (*Allium sativum* L.) is one of the most important *Allium* crops widely cultivated throughout the world. Since a significant reduction in both yield and quality due to virus infection is currently a serious economic problem, garlic viruses have been intensively studied. Garlic plants are usually infected by a mixture of viruses: *Onion yellow dwarf virus* (OYDV, Potyvirus), *Leek yellow stripe virus* (LYSV, Potyvirus), *Garlic common latent virus* (GarCLV, Carlavirus), *Shallot latent virus* (SLV, Carlavirus), and mite-borne mosaic viruses (allexiviruses). As the above-mentioned viruses appear to be economically important, prevention and control procedures have been suggested for them (Walkey et al. 1987; Szyndel et al. 1994; Fletcher et al. 1998). So far, potyviruses (OYDV, LYSV) have been the best studied *Allium* viruses. These viruses cause a bright yellow-green mosaic on leaves (Van Dijk 1993a; Takaichi et al. 1998), permitting their visual detection. Carlaviruses (GarCLV, SLV), although detected in diseased garlic plants, were not considered to cause severe symptoms in the event of single infection (Van Dijk 1993b).

Both potyviruses and carlaviruses have been previously detected in garlic plants from many countries. Details of the occurrence of OYDV and LYSV have been described in Greece (Dovas et al. 2001), Italy (Dovas and Vovlas 2003), Brazil (Daniels 1999; Fajardo et al. 2001), and Japan (Takaichi et al. 1998, 2001). Conci et al. (2002) studied LYSV in Argentina. GarCLV has been detected in Brazil (Daniels 1999), Italy (Dovas and Vovlas 2003) and, along with SLV, in Greece (Dovas et al. 2001). Dovas et al. (2001) detected allexiviruses in Greece and Takaichi et al. (1998) in Japan. In addition to potyviruses and carlaviruses, garlic plants have often been infected by allexiviruses. Allexiviruses cause light green stripes on garlic leaves (Barg et al. 1997; Takaichi et al. 1998; Daniels 1999).

Novák (1959) was the first to describe viral leaf spots on garlic plants in the Czech Republic. Later, Havránek (1971) studied the host range of a "garlic mosaic virus" using electron microscopy. In the 1960 s, a system of "symptom-free" seed garlic production was established using meristem culture.

The aims of this study are (i) to study the occurrence of selected viruses (OYDV, LYSV, GarCLV, SLV, and mite-borne mosaic viruses) infecting seed garlic in the Czech Republic, and associations among the viruses, (ii) to evaluate the visual symptoms on garlic leaves, and (iii) to compare the rate of infection of both Czech and imported garlic plants.

2 Materials and methods

2.1 Materials

In total, 463 bulbs of Czech origin from five cultivars ('Anin': 82, 'Anton': 129, 'Benátčan': 78, 'Bjetin': 87, and 'Vekan': 87 samples) were tested. Czech seed material was randomly selected from the production stock of Kozák Ltd. In addition,

garlic bulbs (53 samples) from ShanDong province in south-eastern China and 14 garlic bulbs from Spain were tested for virus incidence. These samples were imported into Czech supermarkets by Čerozfrucht Ltd. and Hortim International Ltd., respectively. All plants were grown hydroponically in a greenhouse at a temperature of 18°C over a photoperiod 16/8 hours (day/night). Plants were fertilized with Kristalon-start (5 g/10 l water) once a week. Leaf samples (0.5 g) were taken from garlic plants when they had three fully developed leaves.

2.2 Virus detection

OYDV, LYSV, GarCLV, and SLV were detected by DAS-ELISA using diagnostic kits produced by Bioreba AG according to the manufacturer's instructions. Mite-borne mosaic viruses were detected using RT-PCR in leaf samples from 39 plants with light green stripes. Total RNA was extracted using TRI REAGENT® (Sigma, T9424) as recommended by the manufacturer. The final RNA precipitate was resuspended in 25 µl of deionised water. The concentration of isolated RNA was determined spectrophotometrically and then diluted to a concentration of about 0.1 µg µl^{-1}. Reverse transcription was carried out in a 25 µl reaction volume consisting of 5 µl of total RNA, 0.5 µl of oligo (T)18 primer (20 pmol µl^{-1}), 5.0 µl of AMV RT 5x buffer (Promega), 2.0 µl of 10 mM dNTPs (Promega), 5U AMV Reverse transcriptase (Promega), 0.5 µl of 40U.µl^{-1} RNasin® RNase Inhibitor, and 11.5 µl of deionised water. RT was carried out at 42°C for 60 minutes. The resulting cDNA (4 µl) was amplified by PCR with the primers RT1/RT2 (Tsuneyoshi and Sumi 1996) in 21 µl reaction volume (2 µl of PCR buffer 10x (Sigma), 0.4 µl of each primer (20 pmol µl^{-1}), 2 µl of 1 mM dNTPs, 1U redTaq polymerase (Promega), and 16.0 µl of deionised water) in a thermocycler (Tpersonal, Biometra). Cycling parameters were as follows: pre-denaturation 95°C for 10 minutes; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min; elongation at 72°C for 45 sec, and a final extension of amplification products for 10 min at 72°C. The 0.2 ml tubes were used for two-steps RT-PCR. The PCR product (5 µl) was analyzed in a 2% agarose gel by electrophoresis and ethidium bromide staining. Virus-free garlic plants were used as negative controls and virus isolates (OYDV16, LYSV134, GarCLV51, and SLV137) as positive controls.

2.3 Symptom evaluation

Garlic plants were observed for leaf symptoms during development. Symptoms were evaluated on three-point scale: symptom-free leaves, light green stripes, and bright yellow green stripes.

2.4 Statistics

Because samples from China and Spain were not used as seed in the Czech Republic, they were not statistically treated with Czech samples. Since a limited number of Czech garlic samples was analysed for mite-borne mosaic viruses infections, we statistically treated only poty- and carlaviruses. Cochran's Q-test (Zar 1996) was used for test of the null hypothesis that the proportion of samples infected is the same for each detected virus. For the analysis, blocks corresponded to the individual plants scored and treatments corresponded to the viruses detected. Each cultivar was analyzed separately. Log-linear analysis of contingency tables (Zar 1996) was used for a test of the null hypothesis of the homogeneity of virus infestation of those cultivars studied. Each virus was analyzed separately. To determine the degree of association among viruses, a phi coefficient of association (Zar 1996) was computed between all pairs of viruses. Due to several occurrences of marginal zero counts in partial two-by-two tables, data from all the cultivars were merged before analysis. NCSS 2001 software (Hintze 2001) was used for the analyses except for the Q-test which was computed by Statistica 6.0 software (StatSoft Inc.).

3 Results

3.1 Natural infection of the Czech samples

All tested viruses were detected in the Czech garlic material. Nevertheless, significant differences in infection rates were found both among viruses and among the cultivars analyzed.

Infection rates of GarCLV, SLV and OYDV were apparently higher than that of LYSV (Table 1). While GarCLV was the most common virus, LYSV was the rarest virus in the Czech samples. In general, the proportion of samples infected by

Table 1: Infection of Czech (A) and imported (B) garlic material with four different viruses as revealed by DAS-ELISA. Average frequency represents arithmetic mean of proportions (and coefficient of variation in %) of positive samples of respective cultivars

Cultivar/region	No. of tested garlic plants	Positive garlic plants (%)			
		OYDV	LYSV	GarCLV	SLV
(A) Czech material					
Anton	129	79.1	6.2	99.2	5.4
Anin	82	100.0	92.5	100.0	100.0
Benátčan	78	100.0	55.1	100.0	100.0
Bjetin	87	90.8	2.2	100.0	100.0
Vekan	87	6.9	0.0	98.9	100.0
Average frequency		75.4	31.2	99.6	81.1
(Coefficient of variation)		(52.0)	(131.8)	(0.5)	(52.2)
(B) Imported material					
Spain	14	0.0	28.6	85.7	0.0
China	53	96.2	94.3	0.0	100.0

OYDV, *Onion yellow dwarf virus*; LYSV, *Leek yellow stripe virus*; GarCLV, *Garlic common latent virus*; SLV, *Shallot latent virus*.

individual viruses mutually differed in each of screened cultivars (Cochran's Q-test; Vekan: Q = 246.1, Bjetin: Q = 233.9, Anton: Q = 301.8, Anin: Q = 18.0, Benátčan: Q = 135.0, all P < 0.005). Only a weakly positive significant association was found between OYVD and LYSV (phi = 0.34, $P = 0.001$). Associations among other viruses were insignificant ($P > 0.05$).

Cultivars significantly differed in virus infection levels for all ELISA-tested viruses (log-linear analysis; OYDV: $\chi^2 = 282.5$, LYSV: $\chi^2 = 318.6$, SLV: $\chi^2 = 470.4$; all $P << 0.001$), except for GarCLV ($\chi^2 = 0.65$, $P = 0.96$; Table 1). The cvs. Anton and Vekan were markedly less infected than other cultivars. 'Anin' was the most infected cultivar showing by far the highest level of LYSV infection among the cultivars studied.

The occurrence of mite-borne mosaic viruses was also confirmed in all screened cultivars. In total, 31 positive RT-PCR reactions of 39 tested samples were recorded. The cvs. Bjetin and Benátčan were markedly more infected than other cultivars.

3.2 Occurrence of viruses in imported garlic

The pattern of virus infection identified in the garlic samples from China and Spain differed from that found in the Czech samples (Table 1). OYDV, LYSV and SLV were detected in almost 100% of the analyzed samples from China. On the other hand, GarCLV commonly recorded in Czech garlic plants was not detected in samples from China. Garlic plants from Spain showed low infection level. OYDV and SLV were not detected in Spanish samples.

3.3 Symptoms on leaves

Most of the garlic plants tested (80.9%) exhibited virus-like symptoms. Single infections with either OYDV or OYDV in combination with other viruses always caused bright yellow-green stripes on garlic leaves (92.8% of infected plants). Light green stripes were visible in the rest of the infected garlic plants. Mite-borne mosaic viruses in combination with carlaviruses were detected in these plants. Only carlaviruses were detected in garlic plants without visual symptoms (19.1% of tested plants).

4 Discussion

Although garlic is a crop traditionally grown in the Czech Republic, the presence of viruses detected by serological and molecular techniques has only been reported for the last three years (Klukáčková et al. 2004). In general, our results show that garlic is heavily infected with viruses in the Czech Republic. Nevertheless, we found different patterns of infection both among viruses and among cultivars. In contrast to the accepted view that potyviruses infect garlic more frequently than carlaviruses because of the more effective transmission of potyviruses by aphids (Van Dijk 1993a), we found that the incidence of potyviruses in the Czech Republic is, on average, lower than that of carlaviruses. Almost all the garlic plants analyzed were always infected by at least one *Carlavirus* species, but some garlic cultivars showed rather low infection by potyviruses (Table 1). It is possible that carlaviruses are spread mainly by vegetative propagation (Dovas et al. 2001) as, under some circumstances, they do not cause visual symptoms on leaves. Therefore, negative selection of plants infected by carlaviruses cannot be applied during garlic seed production without serological (ELISA) or molecular (RT-PCR) detection of these viruses.

The high infection rates of the Czech garlic cvs. can probably be influenced by wild *Allium* species (e.g. commonly occurring and related *Allium vineale*; Duchoslav 2001) that could serve as virus inoculum sources for the cultivated ones. Although older studies had not reported wild *Allium* species as viral infection sources for *Allium* crops (Van Dijk 1993a,b), Dovas et al. (2001) detected LYSV and TuMV in some wild *Allium* species in Greece.

Our results show that OYDV is a more common potyvirus than LYSV in the Czech Republic. We found 100% infection by OYDV in two cultivars (Anin, Benátčan) and only cv. Vekan showed a low infection rate (6.9%). In Europe, Dovas et al. (2001) and Dovas and Vovlas (2003) detected OYDV in almost 100% of garlic plants tested in both Greece and Italy. We observed a high incidence of OYDV in imported bulbs from China. Similarly, Sutarya and Van Dijk (1994) detected OYDV in 87% of the garlic samples in Java. On the other hand, Daniels (1999), Fajardo et al. (2001), and Takaichi et al. (1998, 2001) described a low frequency (up to 40%) of OYDV in Brazil and Japan, respectively.

LYSV was detected at low frequencies in three of five Czech cultivars. This is in contrast to reports on the common occurrence (80–100%) of LYSV in garlic, e.g., from Brazil (Daniels 1999; Fajardo et al. 2001), Argentina (Conci et al. 2002), Greece (Dovas et al. 2001), Italy (Dovas and Vovlas 2003), and Japan (Takaichi et al. 1998; 2001).

GarCLV was the most common virus in the Czech samples irrespective of tested cultivars and in samples from Spain. Nevertheless, a comparison of published data shows that infection rates of GarCLV display the highest variation among the four viruses studied. GarCLV varied in incidence from 0 to 98% of garlic plants in different regions in Greece (Dovas et al. 2001) and from 23 to 98% in Italy (Dovas and Vovlas 2003). On the other hand, in our study and in that of Daniels (1999) GarCLV was detected in 0% and 4% of garlic samples from China and Brazil, respectively.

Hitherto published data (Dovas et al. 2001; Dovas and Vovlas 2003) report the very rare occurrence of SLV in garlic in southern Europe (Greece, Italy). We obtained similar results concerning garlic imported from Spain. However, Czech samples were 100% infected by SLV, except the cultivar Anton (5.4%). Diekmann (1997) stated that aphid transmission of SLV is less effective than for other viruses.

Garlic is also infected by mite-borne allexiviruses (Sumi et al. 1993; Helguera et al. 1997; Conci et al. 2003). We identified high infection rates (79.5%) of allexiviruses in limited sample (39) of our cultivated garlic plants. Similar results were noted by Takaichi et al. (1998), who detected mite-borne mosaic viruses in 20 to 65% of the garlic samples from Japan.

Van Dijk (1993a), Diekmann (1997), and Takaichi et al. (1998) described visual symptoms on the leaves of various *Allium* species caused by potyviruses and/or allexiviruses. They noted that OYDV caused bright yellow-green stripes and LYSV caused irregular light and dark green striping on the young leaves. Aggravation of the symptoms was reported when plants were co-infected with SLV and/or GarCLV (Van Dijk 1993b). Our observations showed that the presence of OYDV, irrespective of co-infecting virus(es), is responsible for the bright yellow-green stripes. We cannot comment on any visual symptoms caused by LYSV (Diekmann 1997) as we did not detect LYSV without the presence of OYDV. We can confirm an association between the presence of light green stripes on leaves and the occurrence of allexiviruses as previously reported by Barg et al. (1997), Diekmann (1997), Takaichi et al. (1998), and Daniels (1999). We found that the occurrence of carlaviruses alone does not lead to visual symptoms in garlic plants which agrees with data of Takaichi et al. (1998) for Japanese garlic cultivars.

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