

Serological Detection of Onion Yellow Dwarf Virus in Garlic

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ABSTRACT

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A highly sensitive antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) was developed to test for potyvirus in garlic leaves. Antiserum adsorbed with an equal volume of sap from healthy leaves is routinely used at 1:10,000 dilution and is able to detect virus at dilutions up to 1:40,000. Leaf sap can be diluted up to 1:512 when using antibody at the routine concentration. Virus was detected in plants 2 wk after inoculation via aphids. Immunosorbent electron microscopy showed that the potyvirus being tested was only onion yellow dwarf virus (OYDV). All field-grown plants tested were OYDV-infected.

Garlic (*Allium sativum* L.) is vegetatively propagated, and all traditional commercial clones are infected with one or more viruses (13). Some viruses, especially the potyviruses onion yellow dwarf virus (OYDV) and leek yellow streak virus (LYSV), cause mosaic symptoms in infected leaves and can cause yield reductions in excess of 25% (6,14).

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Other viruses, mainly carla- and rymoviruses, are latent in the leaves and cloves, and do not appear to affect yield significantly (11).

Programs to produce certified virus-free stocks of popular cultivars are presently being carried out in several countries (6). Such programs are time-consuming and expensive, as the new propagation material must be developed from excised meristems grown under conditions which protect against reinfection, and must be tested at every stage for the presence of each virus.

An alternative would be to develop cultivars resistant to damaging viruses, especially OYDV. Variations in symptoms among plants within single fields

have been reported and may be based on resistance to a virus (10). More recently, incorporation of genes for viral coat proteins or coat protein subunits into plant cells has been shown to impart resistance in tobacco to other potyviruses (7).

In either case, a sensitive method of detecting a virus is needed to identify resistant plants. The method must be able to distinguish these plants from tolerant plants, which allow the virus to multiply but do not express mosaic symptoms or suffer yield reduction. Knowledge of variation in viral concentration within the garlic plant parts and over the growing season is also needed to ensure that comparisons among cultivars and individuals reflect true differences in viral concentration rather than differences among sampling units and/or times.

A program was started to develop garlic resistant to OYDV. A highly sensitive antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) was adapted to detect OYDV in leaf sap. Methods and results for ACP-ELISA of OYDV in garlic are reported. The results indicate variation in virus levels within and among plants.

MATERIALS AND METHODS

Plant material and virus culture. The garlic cvs. Shevat and Frankon, selected by Z. Mitchnik at the Agricultural Research Organization and showing clear mosaic symptoms, were used as the original source of virus. Other local and imported garlic cultivars were sampled for the presence of OYDV. One bulb from an experimental plot and certified to be free of OYDV was the kind gift of H. Lot, INRA, Avignon, France. Plants from this material were grown in pots in an insect-proof greenhouse.

Antibody production and ELISA. OYDV was purified from frozen garlic leaves of Shevat by procedures used in our laboratory to purify other potyviruses (2). Virions (about 0.1 mg/ml) were emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly into both hind legs of a 6-wk-old rabbit. Injections were repeated weekly for 4 wk. The animal was bled from the ear vein weekly, starting 1 wk after the first injection. ACP-ELISA was performed as described by Joisson et al (4) with small modifications. To reduce unspecific binding, the rabbit antiserum was adsorbed (8 C, 18 hr) with an equal volume of OYDV-free garlic leaf sap. For plant testing, serum from the third bleeding was used at a final dilution of 1:10,000, and goat anti-rabbit IgG alkaline phosphatase conjugate (BioMakor, Rehovot, Israel) was used at 1:2,000 dilution. Reference antiserum against OYDV was obtained from H. Lot (3). Antiserum to LYSV was obtained from H. Vetten, Institute for Biochemistry and Plant Virology, Braunschweig, Germany.

Serial dilutions of both antisera and antigen were made to test the sensitivity of the assay. Antisera were diluted up to 1:80,000 and tested with expressed sap from 1–2 g of leaf material suspended in 2 ml of 0.05 M sodium carbonate buffer, pH 9.6. The experiment was repeated twice, and each dilution was tested in duplicate. Antigen dilution series were made by suspending expressed leaf sap at a ratio of 1 g leaf:2 ml sodium carbonate buffer and diluting this up to 1:2,048. The experiment was repeated four times, and each dilution was tested in duplicate.

Western blots and electron microscopy. Western blots of leaf sap from the original source plants were prepared according to the technique described by Salomon (8). The blots were tested with both our antiserum and OYDV-specific antiserum (3).

Samples for electron microscopy (EM) were prepared as described by Cohen et al (1) using both full-strength extract (1 g leaf tissue:2 ml sodium carbonate buffer) and this extract diluted at a 1:1 ratio with buffer. Preparations were decorated with locally produced anti-

serum and reference antisera against OYDV and LYSV. Antiserum was applied at three dilutions (1:10, 1:100, and 1:1,000), and two grids per antigen-antiserum combination were examined, for a total of 12 grids for each antiserum.

Virus distribution within a plant. To assess variations in relative viral content within a plant, all fully green, nonsenescent leaves per plant of four plants from each of four cultivars/lines were tested for OYDV coat protein (CP) 1 mo before harvest. All leaves per plant of the sampled plants were numbered according to position, with the topmost, tightly curled leaf being number 1, following the convention of Jones and Mann (5). Top, middle, and basal sections from the fourth fully developed leaf of each of four plants of Shevat were also tested for OYDV CP concentrations to assess variations in viral distribution within a leaf.

Changes in viral CP content over time. Fifty plants derived from meristem and callus culture of Frankon were transplanted to an insect-proof greenhouse. Four months after transplanting, plants were tested visually and by ACP-ELISA for the presence of OYDV. Apparently healthy plants were retested 1 mo later. Three days after the second test, four plants which were negative for OYDV were reinoculated using green peach aphids (*Myzus persicae* (Sulzer)). Aphids were starved 2 hr then allowed to feed 10 min on a field-grown OYDV-infected plant (Shevat). About 10 aphids were transferred to each of the four OYDV-

free plants and allowed to feed for 18 hr. Plants were then sprayed twice with 0.1% nicotine sulfate to eliminate the aphids. Inoculated plants were maintained in an unheated insect-proof greenhouse until mosaic symptoms developed.

RESULTS AND DISCUSSION

ELISA procedure. Serial dilutions of the antisera were made from 1:1,250 to 1:40,000. As the antisera had been adsorbed with an equal volume of leaf sap from an OYDV-free plant, these dilutions were equivalent to 1:2,500 to 1:80,000 final concentrations. Absorption values decreased linearly with the log₁₀ of the antiserum dilution (Fig. 1).

Serial dilutions up to 1:2,048 were made of sap from leaves of infected Shevat. The ELISA readings decreased with the log₁₀ of the antigen dilution (Fig. 2). The largest decrease was seen between undiluted leaf sap and sap diluted 1:4. The slight rise in A_{405} reading from 1:4 to 1:16 dilution indicates that virus aggregates may be dispersed or that new binding sites are exposed by the low-level dilution of the sap. Viral antigen in sap diluted more than 1:1,024 was indistinguishable from the virus-free sap of healthy plants.

We routinely use antibody diluted 1:10,000, with undiluted sap, to test garlic plants for the presence of OYDV.

Antibody specificity. Western blots of sap from the original source were prepared and tested with our antiserum, OYDV-specific antiserum (Fig. 3), and LYSV antiserum (not shown). Banding

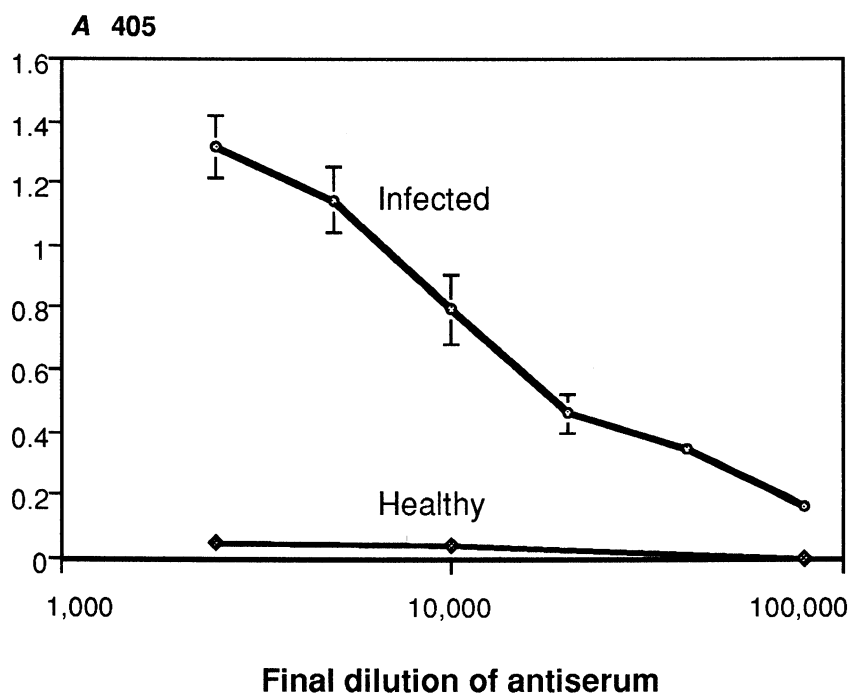


Fig. 1. A_{405} absorption curve (45 min) for antiserum against onion yellow dwarf virus, at dilutions between 1:5,000 and 1:80,000, when challenged with undiluted leaf sap (1 g leaf:2 g carbonate buffer). Antiserum was diluted in nonfat dry milk dissolved in phosphate-buffered saline and applied to garlic leaf sap-coated plates.

patterns of our antiserum and the OYDV antiserum were identical, indicating that our source material contains mainly, if not exclusively, OYDV. No reaction was seen with LYSV antiserum, indicating that this virus is not present in our sample of Shevat.

Electron microscopy of extracts from field-grown, virus-infected plants (Shevat) showed all virus particles on all grids were decorated with the OYDV-specific antiserum. A decorated virion with a gap in the decoration shows the width of the undecorated particle (Fig. 4A). Particles treated with LYSV antiserum were not decorated (Fig. 4B).

Viral distribution. Comparison among leaves of a plant (Table 1) shows that the OYDV level in the apical, physiologically youngest leaf is relatively low. More mature leaves showed increasing levels of virus up to about the fourth or fifth leaf below the apex. In Shevat (the only cultivar which had more than five fully green leaves at the time of sampling), virus content in older leaves (numbered 6-9) leveled off. Leaf tips were found to have more virus ($A_{405} = 0.274$) than middle ($A_{405} = 0.160$) and basal ($A_{405} = 0.079$) sections of leaves.

The possibility of testing dormant cloves of garlic for presence of OYDV should also be explored (9,12). We are now investigating whether our present system is sensitive enough to detect virus in the clove storage leaf or in the leaf initials of the dormant plantlet. Such tests could be used to screen during the summer season, before the bulb is separated into planting material. This procedure may save testing of all ensuing plants, since in a normal field planting it is impossible to tell which plants are derived from the same bulb. Since one bulb yields about eight to 10 cloves suitable for planting, testing in the dormant clove would reduce the number of tests to be carried out by at least 80%. However, testing one clove per bulb will only be suitable if all cloves of a bulb show similar reactions.

Changes in viral concentrations over time. Mosaic symptoms were visible about 1 wk after aphid inoculation of in vitro-grown, OYDV-free plants. Levels of OYDV increased rapidly between the first and the second week after inoculation (Table 2). By the second week, all four test plants showed A_{405} readings above that of the healthy control.

Ten marked plants derived from meristem and callus culture of Frankon and tested twice for the presence of virus appeared free of OYDV at the first test on 9 May 1993 (mean $A_{405} = 0.065$), but appeared infected at the second test on 11 June (mean $A_{405} = 0.207$). Two plants with equally low readings in May (mean $A_{405} = 0.082$) had low readings (mean $A_{405} = 0.098$) in June and were rated free of OYDV.

The rapid increase in relative viral content between the two readings may have been due to the sharp temperature rise in the screenhouse during the month of May. Effects of temperature on the recorded levels of virus, both in plants grown from infected cloves and in plants from meristem culture, need to be further tested to ascertain the proper time and conditions to test material.

The rapid increase in virus level allowed separation of clearly infected plants from prospective OYDV-free plants using small amounts of leaf material, such as is available from plantlets within 4 mo after they have been removed from in vitro culture, or perhaps even in culture if the culture vessel is large enough. A second reading after 1 mo allowed further elimination of infected

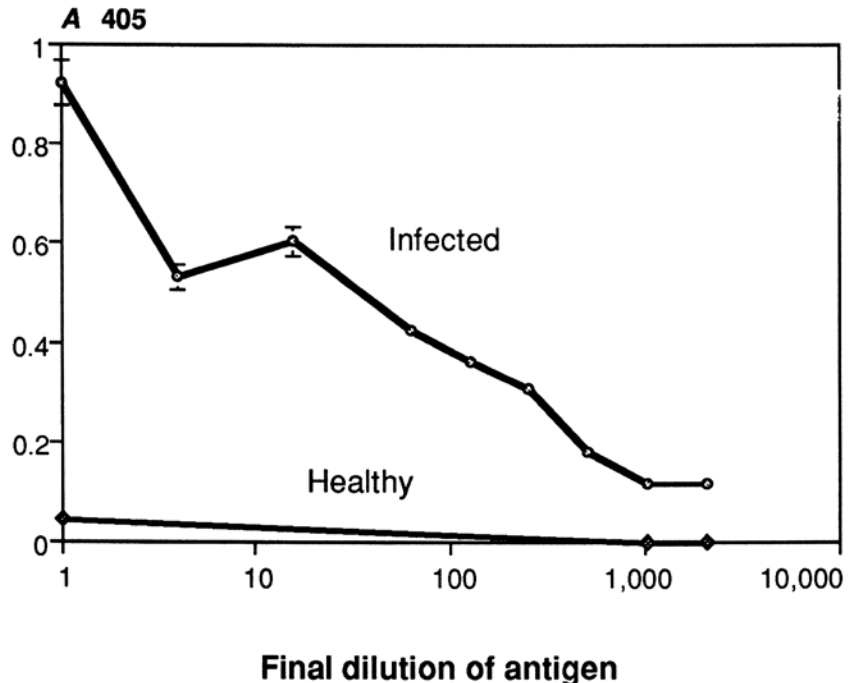


Fig. 2. A_{405} absorption curve (15 min) for antigen-coated plate enzyme-linked immunosorbent assay of garlic leaf sap from onion yellow dwarf virus infected plants and from healthy plants, used at dilutions between 1:0 and 1:2,048 with carbonate buffer.

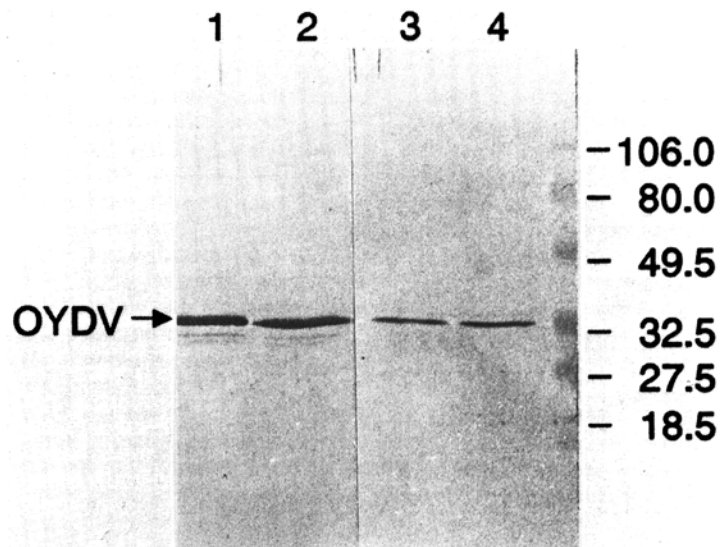


Fig. 3. Western blot of extract from garlic leaves of cv. Shevat. Sap was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with several antibodies. Lanes 1 and 2 show our onion yellow dwarf virus (OYDV) antiserum (diluted 1:4,000). The faint bands of lower molecular weight may be the products of partial proteolytic cleavage of the coat protein during extraction. Lanes 3 and 4 show OYDV antiserum from H. Lot (diluted 1:1,000). Two additional lanes where reaction was not detected (not shown) were treated with leek yellow streak virus antiserum from H. Vetten. Prestained molecular weight markers are on the far left.

plants. This rapid elimination allows us to concentrate attention on the prospective OYDV-free plants, which we multiply as quickly as possible to build up stock.

The ACP-ELISA for OYDV-infected garlic plants proved very sensitive. The fourth and fifth leaves from the apex were the most appropriate for sampling viral infection in mature, field-grown

Table 2. The increase in onion yellow dwarf virus (OYDV) concentrations at 0, 1, and 2 wk following aphid transmission to tissue culture-propagated garlic plants

Test plant	A_{405}		
	0	1	2
Healthy control	0.067 ^c	0.056	0.105
Infected cv. Shevat	0.773	0.739	0.731
24	0.064	0.032	0.165
30	0.071	0.100	0.298
31	0.078	0.062	0.396
36	0.043	0.035	0.130

^c Values represent means of four readings.

plants. The ACP-ELISA was also useful in identifying viral-infected plants among meristem-grown plants shortly after transfer from in vitro culture.

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LITERATURE CITED

- Cohen, J., Loebenstein, G., and Milne, R. G. 1982. Effect of pH and other conditions on immunosorbent electron microscopy of several plant viruses. *J. Virol. Methods* 4:322-330.
- Cohen, J., Salomon, R., and Loebenstein, G. 1988. An improved method for purification of sweet potato feathery mottle virus directly from sweet potato. *Phytopathology* 78:809-811.
- Conci, V., Nome, S. F., and Milne, R. G. 1992. Filamentous viruses of garlic in Argentina. *Plant Dis.* 76:594-596.
- Joisson, C., Dubs, M. C., Briand, J., and van Regenmortel, M. 1992. Detection of potyviruses with antisera to synthetic peptides. *Res. Virol.* 143:167-178.
- Jones, H. A., and Mann, L. K. 1963. Onions and Their Allies. Leonard Hill, Ltd., London.
- Messianen, C., Youcef-Benkada, M., and Beyries, A. 1981. Rendement potentiel et tolerance aux virus chez l'ail. *Agronomie* 1:759-762.
- Namba, S., Ling, K., Gonsalves, C., Slightom, J., and Gonsalves, D. 1992. Protection of transgenic plants expressing the coat protein gene of watermelon mosaic virus II or zucchini yellow mosaic virus against six potyviruses. *Phytopathology* 82:940-946.
- Salomon, R. 1989. Partial cleavage of sweet potato mottle virus coat protein subunit by an enzyme in extracts of infected symptomless leaves. *J. Gen. Virol.* 70:1943-1949.
- Stein, A., Salomon, R., Cohen, J., and Loebenstein, G. 1986. Detection and characterization of bean yellow mosaic virus in corms of *Gladiolus grandiflorus*. *Ann. Appl. Biol.* 109:147-154.
- van Dijk, P. 1993. Survey and characterization of potyviruses and their strains of *Allium* species. *Neth. J. Plant Pathol. Suppl.* 2:1-48.
- van Dijk, P., Verbeek, M., and Bos, L. 1991. Mite-borne virus isolates from cultivated *Allium* species and their classification into two new rymoviruses in the family Potyviridae. *Neth. J. Plant Pathol.* 97:381-399.
- Vunsh, R., Rosner, A., and Stein, A. 1991. Detection of bean yellow mosaic virus in *Gladiolus* corms by the polymerase chain reaction. *Ann. Appl. Biol.* 119:289-294.
- Walkey, D. G. A. 1990. Virus diseases. Pages 191-212 in: *Onions and Allied Crops*, vol. 2. H. D. Rabinowitch and J. L. Brewster, eds. CRC Press, Boca Raton, FL.
- Walkey, D. G. A., and Antill, D. N. 1989. Agronomic evaluation of virus-free and virus-infected garlic (*Allium sativum* L.) and shallot (*A. ascalonicum* L.) by meristem-tip culture. *J. Hort. Sci.* 62:211-220.

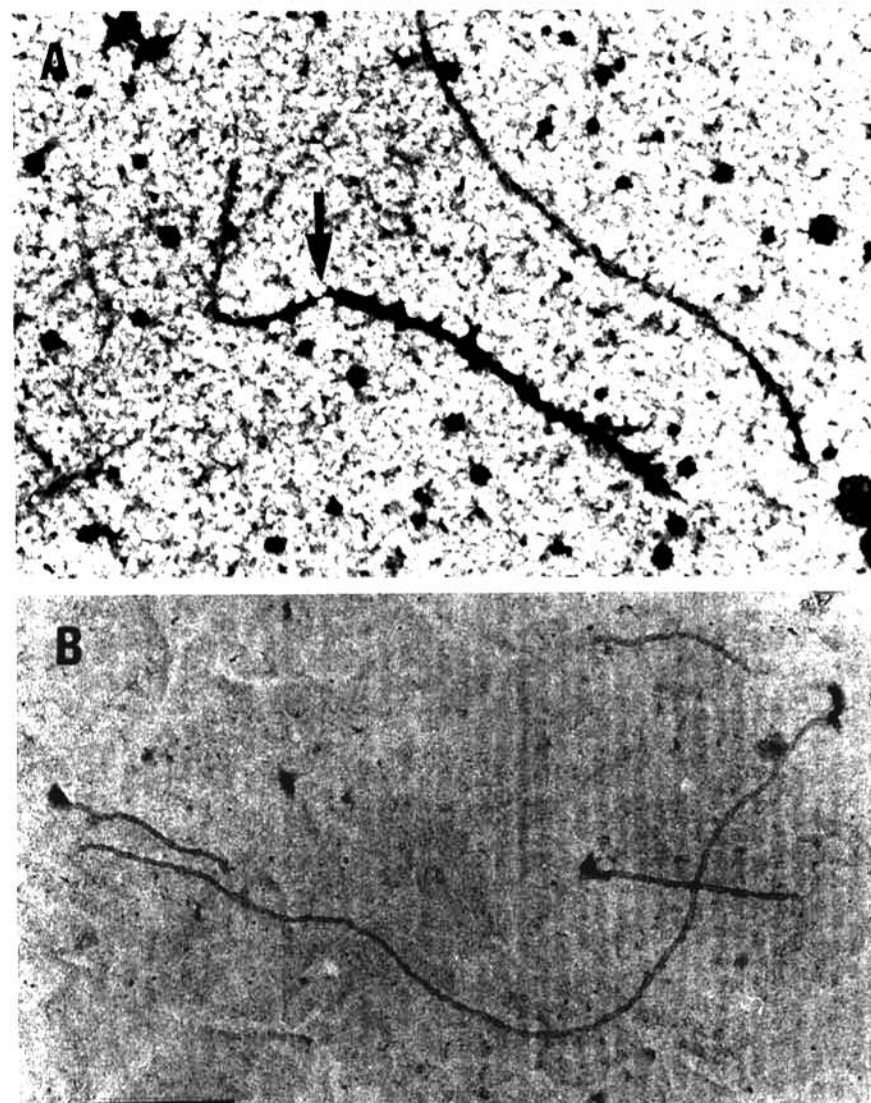


Fig. 4. (A) An onion yellow dwarf virus (OYDV) particle decorated with antiserum specific to OYDV. Arrow shows width of undecorated segment. (B) Undecorated OYDV particle from preparation challenged with leek yellow streak virus specific antiserum. (bar = 0.5 μ m, final magnification $\times 40,000$.)

Table 1. A_{405} readings (30 min) for onion yellow dwarf virus (OYDV) concentrations in leaves of various leaf positions (top leaf = 1) from four cultivars/lines of garlic

Leaf position ¹	Cultivar				
	Frankon	O. Frankon	No. 140	Shevat	Shevat
1	0.049 a ²	0.083 a	0.051 a	0.077 a	0.114 a
2	0.093 a	0.046 a	0.088 ab	0.235 b	0.131 a
3	0.147 a	0.127 a	0.180 b	0.379 b	0.197 ab
4	0.394 b	0.303 b	0.153 b	0.276 b	0.224 abc
5	0.361 b	0.360 b	0.193 b	0.332 b	0.359 c
6	0.252 abc
7	0.171 ab
8	0.299 bc
9	0.250 ab
SE	0.089	0.047	0.025	0.051	0.036

¹ Healthy control from greenhouse (leaf 3) A_{405} = 0.069 (for columns 1-4), 0.072 (for column 5).

² Numbers followed by the same letter within a column are not significantly different (P = 0.05, Duncan's test for least significant differences).