Virus testing of unhealthy *Gentiana* aff. *saxosa*

DOC RESEARCH & DEVELOPMENT SERIES 202

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Published by
Department of Conservation
PO Box 10–420
Wellington, New Zealand

DOC Research & Development Series is a published record of scientific research carried out, or advice given, by Department of Conservation staff or external contractors funded by DOC. It comprises reports and short communications that are peer-reviewed.
Individual contributions to the series are first released on the departmental website in pdf form. Hardcopy is printed, bound, and distributed at regular intervals. Titles are also listed in our catalogue on the website, refer http://www.doc.govt.nz under Publications, then Science and research.

ISSN 1176–8886
ISBN 0–478–22674–8

This is a client report commissioned by West Coast Conservancy and funded from the Science Advice Fund. It was prepared for publication by Science & Technical Publishing Section; editing and layout by Sue Hallas. Publication was approved by the Chief Scientist (Research, Development & Improvement Division), Department of Conservation, Wellington, New Zealand.

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ABSTRACT

Gentiana aff. *saxosa* plants to be used in a translocation showed strong leaf yellowing. Three methods (Enzyme Linked Immuno Sorbent Assay, transmission electron microscopy and sap transmission) were used to test two of these plants for the presence of three virus genera known to infect *Gentiana* species. No evidence of viral infection was found.

Keywords: gentian, virus, *Potyvirus*, ELISA, New Zealand.

Delmiglio, C. 2005: Virus testing of unhealthy *Gentiana* aff. *saxosa*. *DOC Research & Development Series 202*. Department of Conservation, Wellington. 9 p.

1. Introduction

A proposed translocation of the nationally critical, coastal gentian *Gentiana* aff. *saxosa*[1] (de Lange et al. 2004), was put on hold until the cause of observed leaf yellowing was determined. The School of Biological Sciences at the University of Auckland was asked to determine whether plant viruses were present in a sample of two of the plants.

2. Material and methods

2.1 PLANT MAINTENANCE

Two *Gentiana* aff. *saxosa* plants were received in June 2004 and transferred into 16-cm-diameter pots containing Debco indoor/outdoor potting mixture with Green Jacket Timecontrol® fertiliser, following arrival at the University of Auckland. Plants were kept in a greenhouse and watered every 3 days.

2.2 IDENTIFICATION OF POTENTIAL VIRAL SOURCE

A search was conducted on the VIDE (Virus Identification Data Exchange) database (http://image.fs.uidaho.edu/vide/) to identify potential virus candidates. The search named three virus genera as being able to affect members of the Gentianaceae plant family: *Potyvirus*, *Carlavirus* and *Ilarvirus*. To identify the presence of any of these viruses in the *Gentiana* samples three methods were used:

- ELISA (Enzyme Linked Immuno Sorbent Assay), for the detection of potyviruses.
- Transmission electron microscopy (TEM), for the non-specific detection of virus particles.
- Sap transmission from suspected hosts to known susceptible hosts, for the non-specific detection of viruses.

2.2.1 ELISA tests using broad-spectrum antisera

Leaf samples from the two plants were tested by standard ELISA, using the protocols provided by the antisera manufacturers (Agdia Inc., USA). The Poty-group antiserum (Agdia Inc., USA), a broad-spectrum monoclonal antibody that

[1] This species was named as *Gentianella scopulorum* in a recent revision of the genus (Glenny 2004).

reacts to a highly conserved sequence of amino acids on the virus coat protein, was used to detect members in the Potyviridae family. Detection was by a standard indirect-ELISA protocol (Agdia Inc., USA system 27200), also known as Antigen Coated Plate (ACP) ELISA. The ELISA test was carried out in 96-well polystyrene Nunc-Immuno™ plates with MaxiSorp™ surface (Nalge Nunc International, Denmark).

In the indirect-ELISA, the microtitre plate is coated with plant extracts and the antigen, if present, binds to the plate. During a second incubation step, an antibody raised against the virus is introduced to form an antigen–antibody complex. During the third incubation step, the antigen-antibody complex reacts with an alkaline phosphatase (AP)-labelled secondary antibody that binds to the Fc-region of the primary antibody. This is followed by an enzymatic assay in which the AP label reacts with 4-nitrophenyl-phosphate to yield free 4-nitrophenol, which is yellow in colour, and can be monitored by spectrophotometry. Optical densities (OD) were read at 405 nm using a BIO-RAD Microplate Reader Model 550 (Bio-Rad Laboratories). Positive results were defined as having OD readings greater than the mean of the negative controls plus three standard deviations. All tests were carried out in duplicate wells and contained both positive and negative controls, and buffer blanks.

2.2.2 Negative staining transmission electron microscopy (TEM)

Transmission electron microscopy was used as a non-specific method to visualise virus particles (if present) from the samples from *Gentiana* aff. *saxosa*, using direct negative staining of plant extracts.

Sap from each plant was extracted by squashing a small piece of tissue (c. 0.5 × 0.5 cm) in 40 µL of sterile distilled water (pH 7), and a duplicate in 40 µL of 0.01M Sørensen phosphate ($Na_2HPO_4.2H_2O$ and KH_2PO_4) buffer (pH 7.4), with an ethanol-flamed glass rod. A drop of each extract was placed on carbon-stabilised, collodion-coated 400-mesh copper grids and excess liquid removed by touching the edge of the drop with filter paper. The samples extracted in water were then negatively stained with 3% potassium phosphotungstate (PTA; pH 7), while the samples extracted in buffer were stained with both 3% PTA and 2% uranyl acetate (UA; pH 7). Excess stain was removed using filter paper and the grid allowed to air dry prior to examination with TEM. A total of six grids were prepared and examined at 13500× and 44000× magnification, using a Fei/ Philips Tecnai 12 transmission electron microscope.

2.2.3 Sap transmission protocol

Indicator plants such as *Chenopodium quinoa* (Chenopodiacea), *Spinacia oleracea* (Chenopodiacea), *Nicotiana glutinosa*, *N. clevelandii* and *N. tabacum* (Solanacea) were dusted with 400-mesh carborundum powder to assist the penetration of virus particles. Two expanded leaves were chosen and marked using a small sterile cork borer. A small piece (2 × 2 cm) of each gentian suspected of being virus infected was ground in 2 ml (c. 1 : 10 w/v) of 0.01M Sørensen phosphate buffer (pH 7.4), using sterile mortars and pestles. The marked leaves were inoculated by gently stroking with a forefinger dipped in the sap extract. The healthy control plants were inoculated with Sørensen phosphate buffer only. Following inoculation, the leaves were gently rinsed

under tap water to remove any excess sap and plant tissue residues that might inhibit infection. The plants were observed for symptom development 2 and 4 weeks post inoculation.

3. Results

3.1 ELISA TESTS

The *Gentiana*. aff. *saxosa* tested negative for the presence of members of the Potyviridae virus family. The OD readings are summarised in Table 1.

3.2 TRANSMISSION ELECTRON MICROSCOPY

No virus particles were seen in any of the grids prepared with the samples of *Gentiana* aff. *saxosa*.

3.3 SAP TRANSMISSION

The inoculated indicator plants did not show any local or systemic symptoms and were comparable to control plants (inoculated only with buffer), at 2 and 4 weeks post inoculation.

3.4 OTHER OBSERVATIONS

The *Gentiana* aff. *saxosa* no longer showed yellowing symptoms 2 weeks after they were transferred to larger pots containing fertiliser.

TABLE 1. OPTICAL DENSITY READINGS AT 405 nm AFTER 2 HOURS' INCUBATION FOLLOWING ELISA.

SAMPLE NAME	WELL 1	WELL 2	MEAN READING
Gentiana aff. *saxosa* 1	–0.005	–0.006	–0.0055
Gentiana aff. *saxosa* 2	–0.002	–0.002	–0.002
–ve control 1: Oat (*Avena sativa*)	0.003	0.001	0.002
–ve control 2: Wheat (*Triticum aestivum*)	0.005	0.002	0.0035
–ve control 3: Tobacco (*Nicotiana tabacum*)	0.005	0.005	0.005
+ve control 1: Taro Unitec (*Colocasia* sp.), DsMV-infected	0.646	0.626	0.636
+ve control 2: Taro AS5 (*Colocasia* sp.), DsMV-infected	1.694	1.722	1.708
+ve control 3: Ryegrass (*Lolium multiflorum*), RGMV-infected	0.295	0.303	0.299

DsMV = *Dasheen mosaic virus* (*Potyvirus*, Potyviridae), RGMV = *Ryegrass mosaic virus* (*Rymovirus*, Potyviridae).

4. Conclusion

Results from the virus tests (ELISA, TEM and sap transmission) indicate that the original yellowing of *Gentiana* aff. *saxosa* was not due to a plant virus. Other possibilities include environmental conditions, nutrient imbalances or natural dieback at this time of year (J. Santos, DOC Motukarara Nursery, pers. comm.). Nutrient imbalance is partially supported as a cause by the fact that the plants no longer showed yellowing once transferred to larger pots containing fertiliser. However, other causes cannot be ruled out without further tests.

5. References

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