New Insights in Freesia Leaf Necrosis Disease

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Abstract

Although freesia leaf necrosis disease (FLN) is known in freesia cultures for over forty years, the causal agent(s) is/are still under investigation. In plants with FLN symptoms a virus belonging to the genus *Ophiovirus* was found; this virus is now known as *Freesia sneak virus* (FreSV). The virus was mechanically inoculated to and artificially maintained in host plants and purified from these plants. An antiserum was raised and an ELISA-based detection method for FreSV was developed. Mechanical inoculation from indicator plants to freesia seedlings was never successful. Transmission of FreSV using resting spores from *Olpidium brassicae* was tested to establish whether FreSV is the cause of FLN. The detection of FreSV was evaluated in several surveys using freesias with symptoms (66 lots), without symptoms (98 lots) and by random testing (45 lots). More than 40 different cultivars were surveyed. FreSV was found associated with FLN symptoms, although not in all the freesia lots with FLN-like symptoms. In such lots often *Freesia mosaic virus* (FreMV) was present and in some lots neither FreSV nor FreMV could be detected. Implications of these findings will be discussed.

INTRODUCTION

Freesia leaf necrosis disease (FLN) is known in freesia cultures for over forty years. The disease mainly affects the leaves, not the flowers. Symptoms, consisting of chlorotic spots and stripes, begin at the leaf tip and often spread over the whole leaf. Later these chlorotic areas turn grey-brown and become necrotic (Fig. 1A). Mildly affected plants only show light chlorotic symptoms on the lower (oldest) leaves (Van Dorst, 1973; Bouwen, 1994). In plants showing FLN symptoms a virus belonging to the genus *Ophiovirus* was found, provisionally called Freesia Ophiovirus, but now known as *Freesia sneak virus* (FreSV; Vaira et al., 2007).

FLN is soil-borne and is transmitted by the chytrid fungus *Olpidium brassicae* (Van Dorst and Peters, 1988). Resting spores of *O. brassicae* are very persistent and can survive for more than twenty years in soil without loosing the capacity to transmit the disease. Therefore we tested whether FreSV can be transmitted by *O. brassicae* to freesia seedlings and is able to induce FLN symptoms. A detection method suitable for screening will enable growers to select healthy freesia lines and reduce disease levels in the future. For this reason an antiserum against FreSV was developed and surveys were conducted to clarify disease symptoms and occurrence of the virus.

MATERIAL AND METHODS

Purification and Back Inoculation of FreSV

To link FreSV to FLN-symptoms several methods were applied. Firstly, sap of freesias with FLN symptoms (Fig. 1A) was inoculated on the indicator plants *Nicotiana occidentalis* P1 and *N. hesperis* 67A. After repeated subinoculations systemic symptoms developed (Fig. 1B). The virus was purified using a protocol described for *Mirafiori lettuce big-vein virus* (Roggero et al., 2000; Fig. 1C) and an antiserum was raised. An attempt to fulfil Koch's postulates was made by mechanical inoculation of the purified virus onto 400 freesia seedlings. Secondly, soil obtained from three different fields with a FLN-history was used for growing seedlings of freesia, *N. hesperis* and *N. occidentalis*. Later, these seedlings were tested with DAS-ELISA for the presence of FreSV and

symptom development was recorded. Roots of the seedlings which tested positive for FreSV were also examined for the presence of *O. brassicae*. Resting spores were isolated from the roots and used to infect freesia seedlings in a hydroponic system: 1 resting spore per tube, 9 tubes total; 1 resting spore per tube, 9 tubes total.

Surveys

To evaluate the DAS-ELISA and link the presence of FreSV results to the occurrence of FLN-symptoms in practice, freesia lots were sampled in the period 2005 - 2007 by Naktuinbouw inspectors. The 3 main questions were:

- Can FreSV be detected in all samples with FLN-like symptoms?

From 66 lots, representing 43 different cultivars, 8 to 13 samples with symptoms were taken from each lot and tested for the presence of FreSV, FreMV and *Bean yellow mosaic virus* (BYMV) in DAS-ELISA. In total 703 samples were tested. Of the samples which tested negative for the above viruses, a RNA-extraction was carried out using RNeasy Plant Minikit (Qiagen), followed by a general Ophiovirus RT-PCR (Vaira et al., 2003).

- Does FreSV occur in symptomless material (latent infections)?

In total 1960 samples were tested for FreSV. These samples were taken from symptomless plants belonging to the 20 most cultivated freesia cultivars in the Netherlands. 98 Lots were tested, 20 leaves per lot.

- What is the distribution of FreSV in randomly sampled freesia lots?

The freesia lots had no or only a low percentage of virus symptoms (low: 1 to 2% of the plants showed symptoms). Per lot 48 samples were taken. In total 45 lots were surveyed representing 31 different cultivars. The 1632 samples were tested on FreSV, FreMV and BYMV using DAS-ELISA.

RESULTS

Purification and Back Inoculation of FreSV

DAS-ELISA, using the newly developed antiserum against FreSV (Prime Diagnostics, Wageningen, the Netherlands), was well suited for detecting FreSV in leaves, flowers, stems and corms of infected freesia (data not shown). No cross reactivity was observed with other viruses occurring in freesia like FreMV and BYMV. Healthy freesia material did not react with the antiserum (Fig. 2).

The DAS-ELISA was used to detect FreSV in the 400 freesia seedlings which were mechanically inoculated with purified FreSV. None of the seedlings showed symptoms of FLN or tested positive with ELISA.

FreSV was detected in freesia seedlings which were grown in one particular soil with a FLN-history; all 10 tested seedlings tested positive. None of the freesias grown in the other two soils and none of the *Nicotiana* plants tested positive for FreSV. In freesia tested positive with ELISA, FreSV was detected before symptoms became apparent. *Olpidium* resting spores were isolated from the roots of symptomatic freesia plants; no resting spores were observed in the *Nicotiana* plants. Eighteen *Olpidium* cultures were set up to infect freesia seedlings via water culture (Fig. 3A). After two days on this hydroponic system, the freesia seedlings were planted in steam-sterilized soil. After four months FreSV was detected in one plant; the symptoms were relatively mild compared to common FLN symptoms (Fig. 3B).

Surveys

In total 703 samples with FLN symptoms were taken from 66 different freesia lots. In 642 samples a virus was detected (91%). 365 Samples tested positive for FreSV (52%): 268 for FreMV (38%). In only one sample a mixed infection of FreMV and BYMV was detected. Also mixed infections of FreSV with FreMV were rare (14 samples; 2%). Per lot the percentages of FreSV, FreMV and mixed infections were calculated as shown in Figure 4. In 20 lots, several to all samples tested negative for FreSV, FreMV and

BYMV (8% of all samples). These samples tested also negative with the general Ophiovirus RT-PCR.

Of the samples taken from lots with no symptoms, only 3 of the 98 lots tested positive for FreSV. Infection percentages of these 3 lots were low: 5-15%. These samples were re-examined for symptoms: a few chlorotic spots were observed. These symptoms were comparable to those in Figure 3B, but the number of spots was less.

In 26 of the 45 randomly tested lots, low percentages of leaf necrosis (FLN) or mosaic symptoms (FreMV) were observed. In 13 lots FreSV was found varying from 4-20% of the samples; 11 were double infected (FreSV and FreMV).

DISCUSSION

Mechanical inoculation of purified FreSV to healthy freesia seedlings seemed not to be feasible. It is unclear whether this is due to difficulties in inoculation system – FreSV is vectored by *O. brassicae* in nature, not by mechanical transmission – or to changes in FreSV during the passages through *Nicotiana* spp. Transmission through *O. brassicae* was shown in hydroponic culture. The induced symptoms were relatively mild compared to common symptoms of FLN. However, since only one plant became infected, results are preliminary. More research will be needed to fulfil Koch's postulates.

In 8% of the samples with FLN-symptoms no virus was detected with both ELISA and RT-PCR. This may indicate the presence of another unknown virus. This virus should also be vectored by *O. brassicae*, based on our own experience and on results of Van Dorst and Peters (1988). In 1994 a varicosavirus has been detected in freesia (Bouwen, 1994). At that time it was questioned whether this virus was responsible for FLN disease. Of other varicosaviruses, like *Lettuce big vein associated virus*, it is shown that it can be transmitted by *O. brassicae*. In case of lettuce big vein disease, two viruses often co-occur at the same time (Navarro et al., 2004). A comparable situation may exist in freesia with FLN.

The high percentage of FreMV (ca. 40%) in plants with FLN-disease indicates that symptoms caused by FreMV are difficult to distinguish from leaf necrosis. This is not surprising; since over 600 different cultivars are cultivated in the Netherlands, variation in symptoms is to be expected. Only a few cases of double infection of FreSV and FreMV were detected. A double infection – in the past described as "severe leaf necrosis" (Van Dorst, 1973) – is also known as "complex disease". This complex disease is progressive and plants may die before flowering. This complex disease is easily recognized by the growers and plants are removed from the freesia lots.

Meanwhile, the test developed for FreSV is included in the quality system of Naktuinbouw, Select Plant® freesia. In the quality system, random testing is combined with visual inspection of freesia lots at several times a year. In this way latent infections and symptoms which might not be covered by the various tests are detected. To elucidate the epidemiology and causal agent(s) of FLN further investigation is still needed.

ACKNOWLEDGMENTS

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Figures

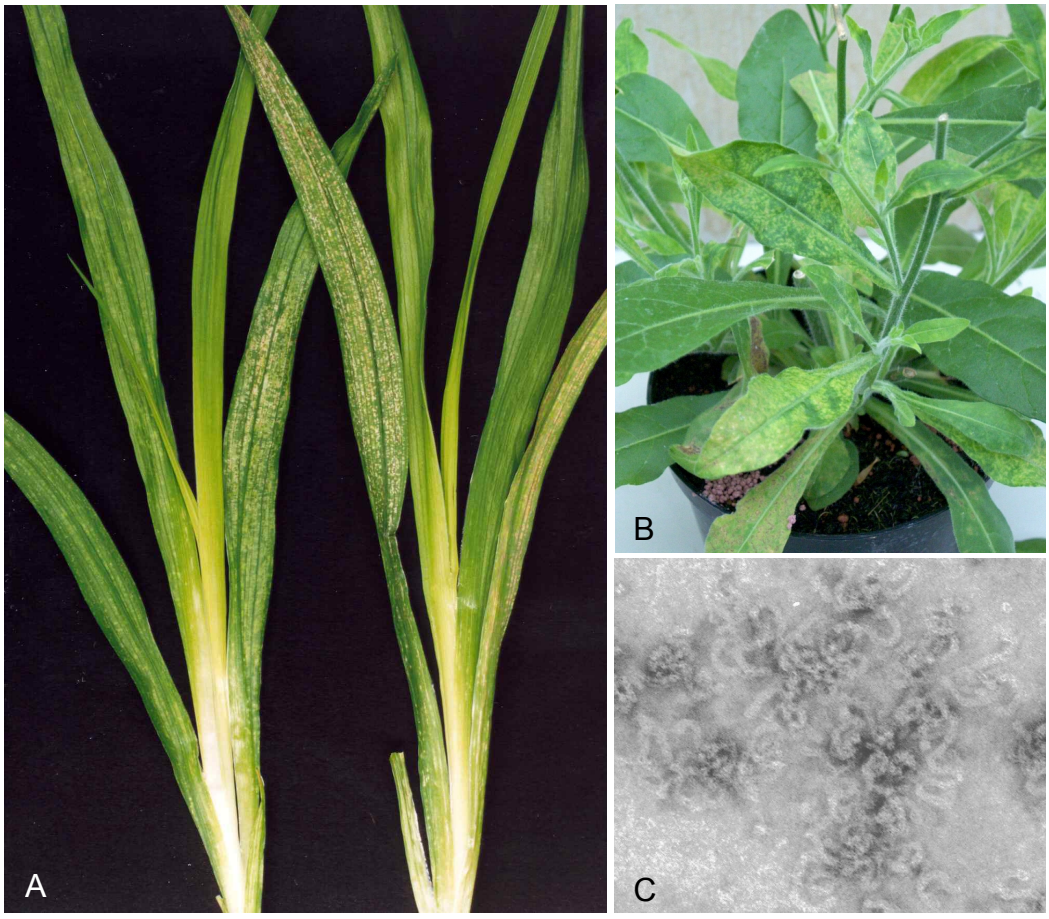

Fig. 1. Freesia leaf necrosis symptoms in freesia (A); Systemic symptoms of FreSV in *N. occidentalis* P1 after repeated subinoculations (B); electron microscopic image of ophiovirus particles purified from P1 (C).

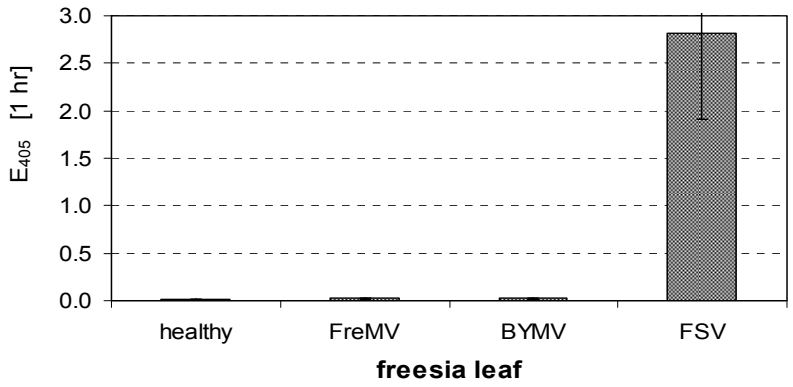

Fig. 2. ELISA readings with FreSV antiserum (A405, 1 hr, DAS ELISA) of healthy, FreMV-, BYMV- or FreSV-infected freesia leaves.

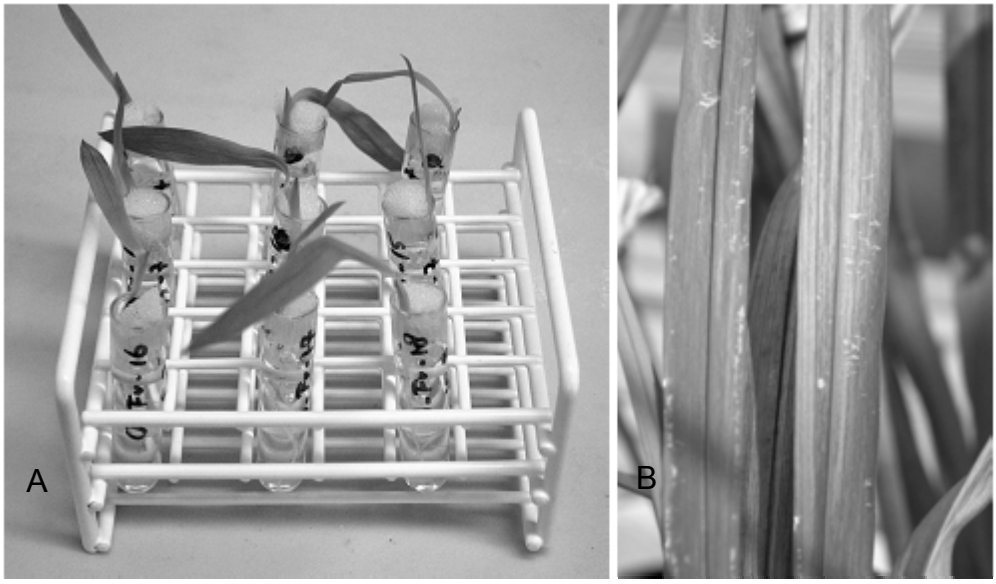

Fig. 3. Inoculation of freesia seedlings via *Olpidium* resting spores using water culture (A), symptoms after inoculation via *O. brassicae* resting spores, 16 weeks after sowing freesia (B).

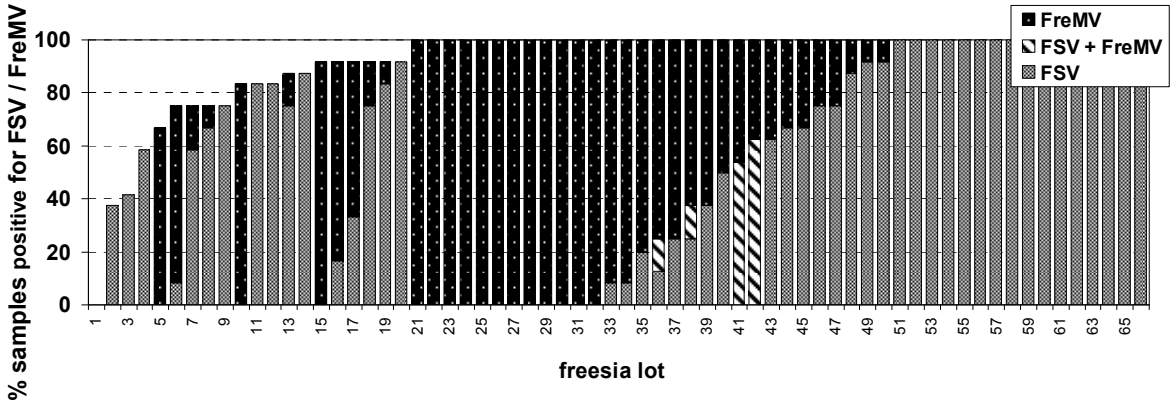

Fig. 4. Percentage of samples with freesia leaf necrosis symptoms in which FreSV (indicated as FSV in figure) and / or FreMV was detected using DAS-ELISA; 8-13 samples taken from 66 freesia lots representing 43 different cultivars.