Iris Yellow Spot Virus in the Netherlands: Occurrence in Onion and Confirmation of Transmission by *Thrips tabaci*

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Abstract

Since its first detection in the Netherlands in 1992, *Iris yellow spot virus* (IYSV, genus *Tospovirus*) has been reported worldwide in *Allium* crops, in a few ornamentals and in a small number of weeds. After recent findings of IYSV in *Alstroemeria* and *Eustoma* in the Netherlands, a number of neighbouring onion fields were surveyed. In 2005 and 2006, only few infected plants were found with obvious symptoms of IYSV. In 2007, after sampling and testing small leaf samples with various types of damage, including small brown-yellow colored spots and spots with thrips feeding damage, a high percentage of plants were found with positive IYSV scores in ELISA. Infection by IYSV could be confirmed in most ELISA-positive samples by RT-PCR. Under laboratory conditions, evidence was obtained that *Thrips tabaci* acts as a vector for this virus. Acquisition of the IYSV from infected *Datura stramonium* plants resulted in virus uptake and replication in over 60% of the thrips exposed, as determined by Western blotting and immunolocalisation of the virus in the foregut, and in epithelial and midgut muscle cells using antibodies against both the viral N and NSs proteins. Successful transmission of IYSV to seedlings of *Emilia sonchifolia* was observed, however, only at low frequency under the laboratory conditions used.

INTRODUCTION

Iris yellow spot virus (IYSV), belonging to the genus *Tospovirus* of the family *Bunyaviridae*, was probably for the first time isolated in 1981 in onion (*Allium cepa* L.) in Brazil (Avila et al., 1981) and later also in the United States, where it caused straw-coloured, dry, necrotic lesions with a spindle or diamond shape (Hall et al., 1993). An isolate found in iris (*Iris hollandica* Tub.) in the Netherlands in 1992 was described and characterized by Cortês et al. (1998). Since these first reports, IYSV has been detected worldwide in *Allium* crops in several countries, including Australia (Coutts et al., 2003), India (Ravi et al., 2006), Israel (Gera et al., 1998), Peru (Mullis et al., 2006), Reunion Island (Robène-Soustrade et al., 2005), Spain (Córdoba-Sellés et al., 2005) and the USA (Gent et al., 2007, Pappu et al., 2008). In addition to a number of *Allium* species, several ornamental plant species, such as lisianthus (*Eustoma russellianum*) and alstroemeria (*Alstroemeria* sp.) (Kritzman et al., 2000; Okuda and Hanada, 2001), and several weeds also appeared to be susceptible to IYSV.

So far, *Thrips tabaci* is the only reported vector of IYSV. Using *Nicotiana benthamiana* as acquisition and inoculation host, the virus was transmitted rather efficiently by *T. tabaci* (Nagata et al., 1999a). Transmission by *T. tabaci was* confirmed in a study in Israel. *T. tabaci* reared on infected plants in the laboratory or collected from infected onions in the field were able to transmit the virus to onion seedlings (Kritzman et al., 2001). This study also indicated that *Frankliniella occidentalis,* another important tospovirus vector, failed to transmit IYSV.

IYSV infections cause considerable economic losses in Israel (Kritzman et al., 2000) and the United States (Pappu et al., 2006), but only minimal losses have been reported from the Netherlands (Cortês et al., 1998). In September 2004, IYSV was detected in two alstroemeria plants grown in a greenhouse. The virus was detected a few weeks after a high number of thrips had flown in, probably from recently lifted onion

crops. The same phenomenon occurred in lisianthus grown in a greenhouse in 2006 and 2007. Surveys in 2005 and 2006 resulted in the detection of only a few infected onion plants with symptoms of IYSV. More elaborate samplings made in 2007 resulted in the detection of IYSV in many plants, even though obvious characteristic symptoms of IYSV were lacking. The results of these samplings will be described in this report and, in addition, ample evidence will be presented that IYSV replicates in *T. tabaci*.

MATERIALS AND METHODS

Collecting and Maintaining Infected Onion Plant Material and Thrips

Onion plant material was collected at four different fields in the Netherlands at the end of August and in early September, 2007. One field was located at Luttelgeest near the greenhouse in which IYSV-infected alstroemeria plants were detected in 2004. Two fields were located at Mijnsheerenland, close to the greenhouse with infected lisianthus plants found in 2006. In addition, samples were collected in a field with no history of IYSV infections near Zevenhuizen. None of the onions in these parcels showed the characteristic symptoms of IYSV, but many if not all onions exhibited small yellow-brown spots, leaf bendings in which thrips often accumulated and spots with thrips feeding damage (Fig. 1). Small leaf samples, of approximately 1-2 cm^2 were randomly collected and tested for the presence of IYSV.

Thrips present on the onion plant material from Luttelgeest and Mijnsheerenland were collected and maintained on leek in several separate colonies in a growth chamber (25^{o}C) with a light/dark regime of 16 and 8 h (Peters et al., 1997). The collected thrips belonged to the species *Thrips tabaci*.

IYSV samples collected in 2006 and 2007 were inoculated to *Nicotinana benthamiana* plants and stored at -80°C.

DAS-ELISA and RT-PCR

In total, 102 small samples from Luttelgeest, 52 from Mijnsheerenland and 68 from Zevenhuizen were tested. They were ground in 1 ml of 1 x PBS-0.05% Tween 20 (PBS-T). Double antibody sandwich-ELISA (DAS-ELISA) was performed using 200 µl of the extracts; the remaining parts of the extracts were stored in Eppendorf tubes at -80^{o}C. Antibodies targeting the N protein of IYSV were used to confirm infection with the virus (Cortês et al., 1998). Due to the lack of onion plants with the characteristic symptoms or of healthy plants, no convincingly positive and negative controls could be included in the DAS-ELISA. Samples with readings higher than twice the average ELISA value of the 10% lowest scoring samples were considered to be infected. The positive samples were inoculated to *N. benthamiana*.

In order to confirm and compare the results obtained by DAS-ELISA, 30 samples from Zevenhuizen with the highest signals in ELISA and 7 samples that were considered negative based on ELISA were additionally tested using reverse-transcriptase PCR (RT-PCR). RNA was isolated from 200 µl of the remaining extract using TRI reagent (Ambion, Austin, USA), a phenol-based solution for the isolation of RNA. The isolated RNA was dissolved in 10 µl of TE-buffer (0.01 M Tris, 0.001 M EDTA, pH 7.5). The N-gene of IYSV was reverse transcribed from this RNA using AMV reverse transcriptase (Sigma-Aldrich, St. Louis, USA) and a reverse primer targeting the 5' side of the N-gene of IYSV. This cDNA was subsequently multiplied using primers as described by Cortês et al. (1998) by PCR and analyzed by gel electrophoresis

Transmission of IYSV by *Thrips tabaci*

Young (0-4 h old) larvae of *T. tabaci* were transferred to disks from inoculated *Datura stramonium* leaves showing the characteristic yellow spots evoked by IYSV. Late second instar larvae and the pupae were transferred to non-infected *D. stramonium* disks. After pupation, cohorts of ten pupae were then transferred to a young, non-infected *Emilia sonchifolia* plant. The plants were then covered individually with Plexiglas cages

to prevent thrips from escaping. After two weeks, two or three pieces of leaves showing feeding damage by thrips were collected, washed with water to remove the thrips and ground in 200 µl of 1 x PBS-T. A 5 µl sample of the macerates was analyzed by Western blotting using IYSV N protein antibodies.

During the acquisition and the transmission, the thrips were kept in a growth chamber (25^{o}C) with a light/dark regime of 16 and 8 h.

Testing Replication of IYSV in *Thrips tabaci* Using Western Blotting

Young larvae of *T. tabaci* (0-4 h old) were collected and transferred to disks from the inoculated leaves of *D. stramonium* showing symptoms of IYSV. After two days, the larvae were transferred to leaf disks of non-infected *D. stramonium*. Second instar larvae (3 days old), pupae and at least 2-day-old adults were collected at random from these cohorts and macerated in 5 µl of 1 x PBS. Purified N-protein of IYSV (50 ng) was used as a positive control. These samples were analysed by Western Blotting using antibodies against the N protein of IYSV and subsequently using monoclonal antibodies against the NSs protein of *Watermelon silver mottle virus*, which also reacts with IYSV NSs (Chen et al., 2006). These proteins were visualized using chemiluminescent peroxidase substrate (CPS-350, Sigma-Aldrich, St. Louis, USA).

Whole Mount Immunofluorescent Staining of IYSV in Tissues of *Thrips tabaci*

Larvae were collected and treated as described in the previous paragraph. The gut and salivary glands were dissected from adult *T. tabaci* (3 to 6 days old) in 0.5 x PBS. These tissues were prepared and analyzed by the whole mount immunofluorescent staining technique as described by Nagata et al. (1999b) using antibodies against the N protein of IYSV. The specimens were scanned with a Zeiss LSM 510 Laser Scanning Microscope.

RESULTS

Presence of IYSV in Onions in the Netherlands

Leaf samples from Luttelgeest and Mijnsheerenland were tested only with DAS-ELISA. IYSV could be detected in 20% (20 of 102) of the samples from Luttelgeest, in 29% (15 of 52) of the samples from Mijnsheerenland and 44% (30 of 68) of the samples from Zevenhuizen (Fig. 2). RT-PCR analysis of the 30 positive samples from Zevenhuizen confirmed the presence of IYSV in 22 samples and also in one of the 7 negative samples.

Transmission of IYSV by *Thrips tabaci*

A total of 480 thrips was transferred to 48 *E. sonchifolia* plants. Two weeks after inoculation, none of these test plants showed the characteristic symptoms of an IYSV infection. The symptoms might, however, have been obscured by the feeding damage. Indeed, 11 of the exposed plants (23%) appeared to be infected as shown by Western Blotting.

Uptake and Replication of IYSV in *Thrips tabaci*

Uptake and active replication of IYSV in *T. tabaci* was first monitored by Western blotting. Both the viral N and NSs proteins were detected in larvae, pupae and two or three day old adults, after having fed at least one day on healthy non-infected leaf material (Fig. 3), confirming replication of the virus in thrips. Viral antigen was detected in 21 out of the 32 thrips tested (11 larvae, 10 pupae and 11 adults), implying that IYSV multiplication occurred in 66% of the thrips. The applied technique enabled the simultaneous detection of both the N protein and the NSs protein in the thrips. The relative amounts of these proteins differed between the various developmental stages, as well as between specimens of the same stage. Both the N and the NSs proteins are

abundant in the larval stage, but present in much lower amounts in the pupae and the young adults.

Replication of IYSV was also confirmed using whole mount immunofluorescent staining. A total of 196 fore- and midguts of 3- to 6-day-old adults was surveyed. Positive N protein signals were found in the foregut and in the epithelial and midgut muscle cells of 69% of the thrips. The salivary glands of 18 out of 32 infected thrips contained the viral N protein (Fig. 4). In most of these salivary glands the signals were very low. The glands of only four thrips were heavily infected.

DISCUSSION

The 2007 onions surveys revealed unexpectedly high IYSV infestation rates, ranging from at least 20 to 44% and, moreover, the virus was geographically widespread. The infected plants, however, did not show the characteristic disease symptoms as reported by Avila et al. (1981). Most, if not all, onion leaves had small yellow-brown necrotic lesions that may be caused by fungal infections, hail damage and thrips feeding. These unspecific and nonobvious lesions were often not discerned on leaf bendings infested by thrips. Samples exhibiting these unspecific lesions and non-symptomatic samples taken from leaf bendings had an equally high infection percentage in the Mijnsheerenland samples and, therefore, no visible damage could convincingly be correlated to the virus infections (results not shown). The characteristic symptoms described by Avila et al. (1981) may depend on onion cultivar or represent an early stage of symptom expression. Recently, Pappu et al. (2008) published a series of photos suggesting that the diamond-like spots were seen on recently infected plants and that symptoms developed progressively into yellow-brownish, necrotic spots during the growing season.

Both ELISA and immunoblotting of entire leaves (results not shown) show that within a single leaf, small infected areas occur next to healthy areas, suggesting that IYSV is restricted to localized spots in onion. This implies that ELISA testing of extracts of entire leaves may result in false negatives due to dilution effects.

The ELISA values recorded showed a progression from hardly detectable to strong positive reactions. Therefore, ELISA may not unequivocally differentiate between virus-free and infected samples. A sample that was considered negative in DAS-ELISA appeared positive using RT-PCR and another seronegative sample caused an infection when inoculated on *N. benthamiana*. These two samples are an indication that the calculated borderline to differentiate healthy and positive samples was too high. The use of real-time RT-PCR may be a more reliable technique to get accurate data on the infection of onion plants with IYSV (Pappu et al., 2008).

Transmission experiments using Dutch populations of *T. tabaci*, sampled from onion fields and maintained in the laboratory for less than a year, resulted in a 23% transmission to *E. sonchifolia* when plants were exposed to cohorts of ten adult viruliferous thrips. Similar rates were obtained when onion seedlings and *D. stramonium* were used as test plants (results not shown). This outcome definitely demonstrates that *T. tabaci* is a vector of IYSV in the Netherlands, but the transmission rate is still low considering the high infection rate in onions. The transmission under our experimental conditions may therefore not reflect the rate of transmission under field conditions due to the experimental conditions or test plant systems used.

The low transmission rate might be due to the use of three different plant species in our transmission experiments, some of which may be less preferred by the thrips. *Allium porrum* was used to rear the thrips, *D. stramonium* as virus acquisition host and *E. sonchfolia* as inoculation host. IYSV infection remains localized in the inoculated leaves of the acquisition host *D. stramonium*, which necrotize rapidly leading to virus inactivation. These characteristics of our experimental system may lower the chance that the thrips will ingest an infective dose of virus. Furthermore, replication of IYSV is slow in *E. sonchifolia* when the virus is inoculated mechanically and sometimes systemic infection is hampered.

Experiments on replication of the virus in *T. tabaci* showed that the N and NSs protein could be detected in 66% of the thrips analysed by western blotting. The high percentage of infected thrips with a detectable virus load does not reflect the efficiency by which the virus is transmitted, possibly because the virus does not reach the salivary glands. Infection of the salivary glands of the thrips is a prerequisite for transmission of a tospovirus as shown by Nagata et al. (1999b), Moritz et al. (2003) and Assis Filho et al. (2005). The salivary glands of 18 out of 32 IYSV infected thrips contained the viral N protein, but only 4 of these were heavily infected, indicating that only these individuals were possible transmitters. In all, the presence of viral antigen in midgut and in salivary glands provides firm evidence that *T. tabaci* is capable of vectoring IYSV.

The high infection rate of onions with IYSV and the confirmation of *T. tabaci* as vector suggest that in the Netherlands the virus is transmitted to ornamentals, such as alstroemeria and lisianthus, by *T. tabaci* that has acquired the virus from onions. It remains to be elucidated which alternative hosts are present when onions are not grown.

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Figures

Fig. 1. Details of onion leaves from an IYSV-infested field in Zevenhuizen. The multiple small spots represent thrips feeding damage.

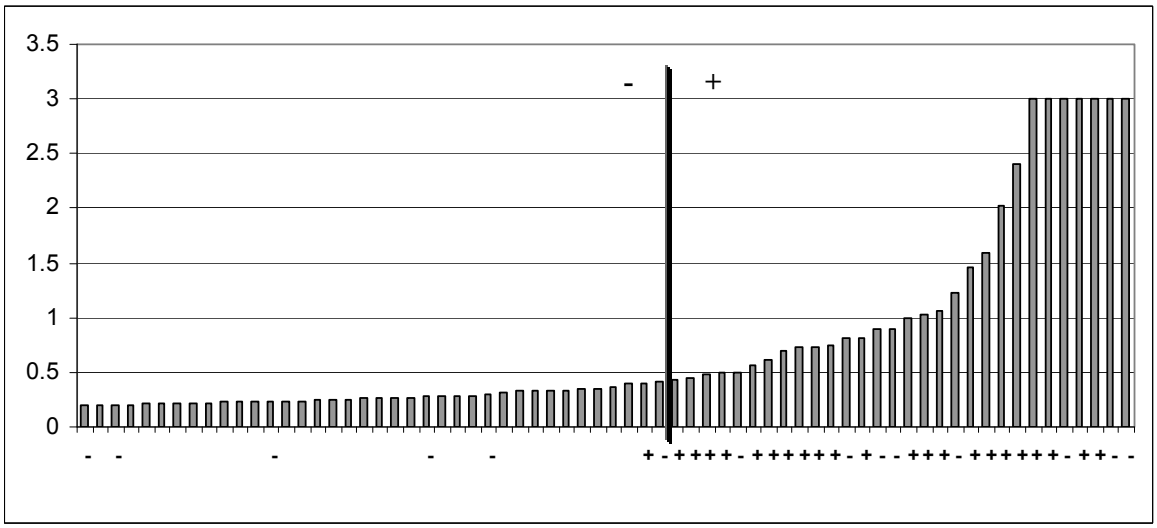

Fig. 2. DAS-ELISA values of extracts prepared from small samples collected from onion leaves from the Zevenhuizen field. The y-axis shows the ELISA values of the samples, which are arranged according to increasing readings. The plus and minus signs below of the x-axis indicate the RT-PCR scores of selected samples. The vertical line in the middle of the graph shows the threshold for infected samples based on ELISA value.

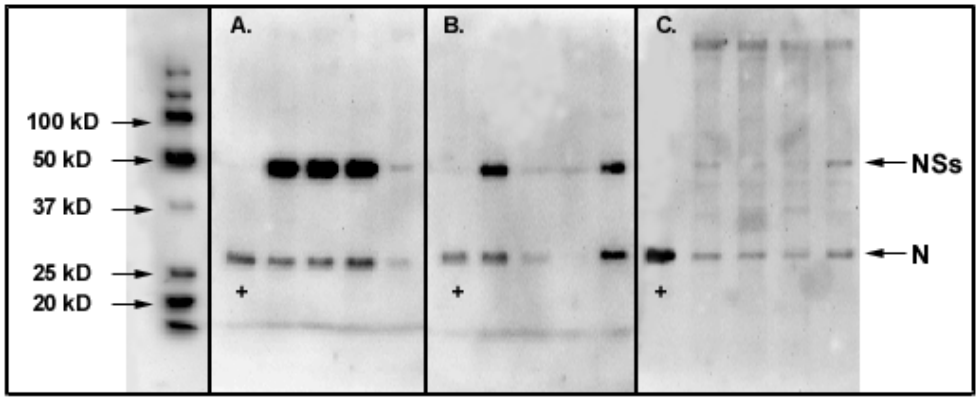

Fig. 3. Western blot detection of the N and NSs proteins of IYSV in individual larvae (A), pupae (B) and adults (C) of *T. tabaci*. The plus signs indicates positive controls (50 ng purified N-protein).

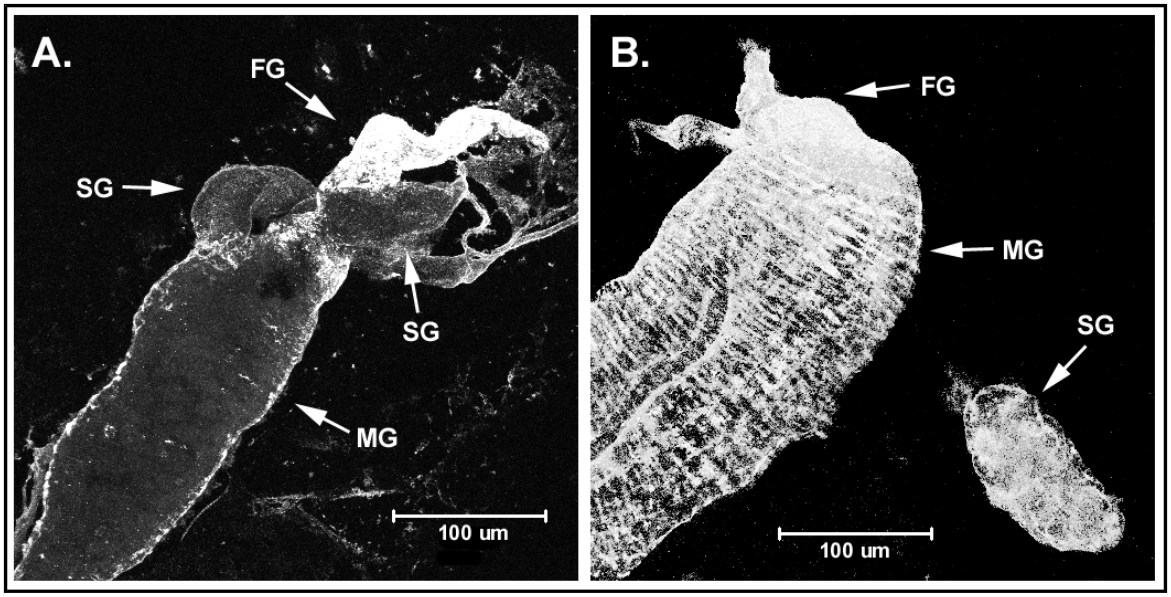

Fig. 4. Viral antigen detection in the intestinal tract and salivary glands of adult thrips that acquired IYSV as 0-4 h old larvae. A) Tissue with only infected foregut; B) Infected fore- and midgut and one salivary gland. FG: foregut, MG: midgut, SG: salivary gland.