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Mites as vector of *Tulip Virus X* in stored tulip bulbs

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Abstract: *Tulip virus X* (TVX) is a *Potexvirus* causing economic losses in tulip. Potexviruses are generally transmitted by mechanical contact and, indeed, several mechanical transmission pathways for TVX have been identified during tulip bulb production. However, TVX transmission does also seem to occur during bulb storage. Since mechanical transmission is excluded in this period, a biological vector should be involved. The eriophyoid mite *Aceria tulipae*, the acarid storage mite *Tyrophagus putrescentiae*, and the acarid bulb mite *Rhizoglyphus echinopus* are the main arthropod pests of stored tulip bulbs. Therefore, we studied their putative role in transmission of TVX during tulip bulb storage. We show that mites of each of these species can carry TVX with them after feeding on TVX-infected bulbs. In addition, some of the healthy bulbs acquired an infection with TVX when inoculated with mites. Although the current setup of the experiments does not confirm which species of mite transmit TVX, we have strong indications that mites are involved in transmission. Additional research with larger numbers of independent replicates is required to further prove the vector status of each species of mite, the efficiency, and mode of virus transmission. If our results will be confirmed, this would be the first case reporting a *Potexvirus* to be transmitted by mites, and the first case of an association between acarid mites and a plant pathogenic virus. Consequently, TVX control in tulip bulb production should include an adequate control strategy of both eriophyoid and acarid mites.

Key words: Acarid mite, *Aceria tulipae*, eriophyoid mite, plant pathogen, *Potexvirus*, *Rhizoglyphus echinopus*, *Tulip virus X*, *Tyrophagus putrescentiae*, virus transmission

Introduction

Tulip Virus X

Tulip virus X (TVX) is a member of the genus *Potexvirus* (Alphaflexiviridae) and was first described by Mowat (1982). The virus causes yield reduction and decreased plant quality because of prominent leaf chlorosis and/or necrosis and flower break in red, purple and pink coloured cultivars. Its natural host range is restricted to *Tulipa* spp. In general, Potexviruses are transmitted by mechanical contact (ICTVdB – The Universal Virus Database, 2006). Indeed, several pathways for mechanical transmission of TVX during tulip bulb and flower production have since been identified. Nowadays, control measures prevent this way of transmission successfully.

In The Netherlands, bulbs are stored at temperatures of 20-23°C in storage buildings upon harvesting of tulip bulbs in June and July until replanting of bulbs in the soil in November or December. Based on tracking and tracing information of virus-infected and virus-free batches of tulips, there was a strong indication that TVX transmission can occur during bulb storage. However, the bulbs are hardly handled in this period so mechanical transmission during bulb storage is unlikely. Therefore, a biological vector was suggested to be responsible for transmission during bulb storage.

Mites (Acariformes), especially eriophyoid species, are well-known vectors of plant-pathogenic viruses (Oldfield, 1970; Oldfield and Proeseler, 1996; Nault, 1997; De Lillo and Skoracka, 2010). Several species of mite occur frequently with stored tulip bulbs. Therefore, the relationship between these mite species and plant viruses is of interest.

Relationships between mites and plant pathogens

The dry bulb mite, *Aceria tulipae* Keifer (Eriophyidae) (Fig. 2), is a phytophagous mite associated with species of *Tulipa*, *Allium* and *Ornithogalum* (Keifer, 1952; Halliday and Knihinicki, 2004). It is a pest during tulip and *Allium* bulb production. These mites are minute in size (adults only measure 200µm in length) and are sensitive to desiccation (Lindquist *et al.*, 1996). Hence, they are closely associated with their host plants and harbour a cryptic lifestyle, which makes them hard to detect for growers. Infection of tulip bulbs with *A. tulipae* causes a range of morphological and chemical changes in bulbs and flowers and in severe cases, bulbs do not even produce any roots or above-ground plant structures (Conijn *et al*., 1996; Van Aartrijk, 2000; Aratchige *et al*., 2004; Lesna *et al*., 2004). As a result, bulbs and flowers might not be marketable. *Aceria tulipae* is a vector of several viruses. It transmits the Allexiviruses *Garlic mite-borne mosaic virus* (Garlic virus C), *Garlic mite-borne filamentous virus*, *Shallot mite-borne latent virus* and *Onion mite-borne latent virus* (Van Dijk *et al*., 1991; Yamashita *et al*., 1996, Kang *et al*., 2007). However, no eriophyoid species of mite is reported to be associated with transmission of Potexviruses.

The storage mite *Tyrophagus putrescentiae* Schrankand the bulb mites *Rhizoglyphus echinopus* (Fumouze and Robin) and *R. robini* Claparèdeare soil-inhabiting, polyphagous, cosmopolitan, species of Acaridae usually feed on micro-organisms and decaying plant material (Fig. 2). They are frequently reported from tulips and other bulbous plants (Diaz *et al*., 2000; Van Aartrijk, 2000; Fan and Zhang, 2003; Bayram and Çobanağlu, 2006, Rojas and Klimov, 2007; Ho, 2008). On tulips, they are found on bulbs infected with micro-organisms, such as the fungus *Fusarium oxysporum* f. sp. *tulipae*, on which they develop well (De Munk, 1972; Czajkowska and Conijn, 1992; Czajkowska, 2002). However, these acarid mites may also feed from young, soft plant tissues, which in tulips results in decreased growth rates (Muller and Hollinger, 1980; Czajkowska and Conijn, 1992; Czajkowska, 2002). The incidence of bud necrosis is also associated with the presence of these acarid mites. It is thought that the mites, by feeding on the developing sprout inside the bulb, facilitate the consequent development of micro-organisms, which the mites carry with them, on the damaged plant tissue (De Munk, 1972). In contrast, to our knowledge, acarid species are not known as vector of plant-pathogenic viruses.

Transmission of TVX by mites?

In the late nineties, Asjes and Blom-Barnhoorn (1998) observed an increase in TVX infection rate when batches of tulip bulbs were stored in the presence of *A. tulipae*. In contrast, the infection rate was unaltered when batches were stored without *A. tulipae.* Therefore, they suggested this species of mite to be a vector of TVX. Here, we report on recent experimental data further investigating the potential of *A. tulipae* to transmit TVX from bulb to bulb, as well as transmission by two other species of mite, *R. echinopus* and *T. putrescentiae*.

Material and methods

Transmission of TVX in stored bulbs

We conducted a transmission experiment with stored bulbs in 2007. Figure 2A presents a schematic of the experimental setup. *Rhizoglyphus echinopus* and *Tyrophagus* spp. had been collected from tulips and populations were maintained in the laboratory on bother and yeast, respectively. A population of *A. tulipae* was kept on cultivar Yokohama. In all the populations maintained, the species aimed for was dominant, but slight contamination with mites of other species cannot be excluded. The cultivars Pink Diamond and Renown were used for the transmission experiment. During tulip production, a leaf sample of each plant was tested for the presence of TVX by Enzyme-Linked Immunosorbent Assays (ELISA) using in-house generated TVX-antiserum. ELISA was performed according to Clark and Adams (1977). The bulbs of these plants were used in the transmission experiment. Although the bulbs appeared not infected by mites, the absence of mites cannot completely be ensured. Early September, groups of ten TVX-infected bulbs were inoculated with hundreds of mites of one of the three species bred, or remained not inoculated as control. Each treatment was replicated four times. Each of the replicates was packed in a closed plastic bag and kept at 20°C. Ten days later, bulbs were transferred into an open tube, and for each replicate 25 TVX-free bulbs of cv. Renown and 25 TVX-free bulbs of cv. Pink Diamond were added to investigate virus transmission. The tubes were stored in racks in such a way that they did not contact each other in a storage room at 20°C. Two weeks later, bulbs treated with *R. echinopus* or *Tyrophagus* spp. were re-inoculated because few mites were found back on the bulbs. All test bulbs were planted outdoors by the end of November. In May of the next year, a leaf sample of each plant was tested for the presence of TVX as described previously. Because of the low numbers of TVX-positive bulbs, the results were not analysed statistically.

Transmission of TVX under laboratory conditions

In order to exclude the unwanted influence of natural mite infections, we performed transmission experiments with pure-breeding mite populations and individual bulbs in the laboratory in 2009. We used two TVX-infected cultivars as a source of TVX: Pink Diamond (65% of bulbs with TVX) and Blue Herron (79% of bulbs with TVX). Cultivar Blue Herron was infested with *A. tulipae* and therefore, a part of this batch was kept separately to maintain a population of this species of mite. The remaining of the batch was successfully treated with pirimifos-methyl as to exterminate the mites. The acarid species *R. echinopus* and *T. putrescentiae* were individually isolated from tulip bulbs cv. Flaming Parrot. Of each of the species a pure-breeding laboratory population was then maintained on a diet of yeast. Their identity was confirmed by morphological identification by two individual experts. In addition, we sequenced the second internal spacer (ITS2) of nuclear ribosomal DNA, which is species-specific within the family of Acaridae (Noge *et al*., 2005; Yang *et al*., 2011).

To investigate whether mites can transport TVX virus particles, we performed a molecular analysis with TVX-specific primers on mites after inoculation of TVX-infected bulb tissue. Figure 2B depicts the setup of this experiment. Mites of all three species were allowed to feed on TVX-infested bulb tissue for at least ten days. Then, a group of 15 mites per species was gently collected by a brush in 100µl lysis buffer. Two samples of TVX-infected bulb tissue were taken as a positive control, and water was used as a negative control. RNA extraction was performed using RNeasy (Plant) mini kit of Qiagen (Qiagen Benelux, Venlo, The Netherlands) following the manufacturer's protocol. First strand synthesis was performed at 42°C for 1 hour, with 200ng TVX reverse primer (TVX-Rev) and 200 units M-MLV reverse transcriptase (Promega, Madison, USA). Two µl first strand cDNA solution was

added to 23µl amplification mixture containing 12.5µl PCR Mastermix (Promega, Madison, USA), and 100ng of each primer (TVX-For: 5'- ACG CCA AGC TTA TCT GGA AC -3'and TVX-Rev: 5'- CCC ACC AGA CTT TCA CTG GT -3'). PCR reaction was done by incubating at 94°C for 2 min, followed by 40 cycles, each consisting of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C. PCR products were examined by electrophoresis in 1% agarose gels. The expected amplicon is 453 bp long.

We then used the same populations of mites to test whether mites can transmit the virus to healthy bulbs (Fig. 2C). In November and early December, bulbs of the uninfected cultivar Ben van Zanten (0% of bulbs with TVX) were carved slightly at the top to facilitate accessibility for the mites, and were then individually placed in a cup. To each cup, one of the species of mite was added as follows: Mites of *R. echinopus* and *T. putrescentiae* had been allowed to feed on slices of mite-free, TVX-infected bulbs for two days. The mite-populated slices from three bulbs (with 10-50 mites each) were then added to a cup. We ensured that the carved surface of the healthy bulb was not contacted in order to prevent mechanical transmission of TVX. Five to ten mites of *A. tulipae* were individually transferred from TVX-infested bulbs onto the healthy bulbs by a brush. As a control, three slices of TVX-infected bulbs without mites were added to a cup. After the last transmission, the single Ben van Zanten bulbs were grouped according to the mite species they had been infected with and stored following regular storing conditions. Two weeks later they were all planted outdoors. As an extra control, a batch of 100 untreated Ben van Zanten bulbs that had been stored under regular storing conditions was planted onto the same field.

In May 2010 we tested infection of these plants with TVX by ELISA on a sampled leaf per plant, according to the standard testing procedure previously described. The numbers of bulbs planted and tested are given in Table 2. The low numbers of infected plants did not allow any statistical analyses.

Results

Transmission of TVX in stored bulbs

Figure 2 summarizes the results of all experiments performed. Numbers of bulbs planted, plants tested, and plants in which TVX was detected, are presented in Table 1, pooled for replicates. Many bulbs planted did not give rise to a plant because of infection with the fungus *Penicillium*. No transmission of TVX was found in the control treatment or bulbs inoculated with *R. echinopus*. TVX was detected in six bulbs inoculated with *A. tulipae* from two of the four replicates and in two bulbs from two different replicates with *Tyrophagus* spp.

Transmission of TVX under laboratory conditions

The nucleotide sequence of the ITS2 of nuclear ribosomal DNA of *T. putrescentiae* isolated from tulip bulbs was determined and deposited in Genbank (HQ681248 en HQ681249). Comparative analysis of the obtained ITS2 sequences by BLAST showed 82-100% sequence homology with those of other specimens of *T. putrescentiae* sequences *(*Yang et al., 2011; and unpublished results) and 85-86% with *T. neiswanderi* (Noge et al.. 2005).

TVX was detected by PCR analyses in *A. tulipae*, *R. echinopus* and *T. putrescentiae* after feeding on TVX-infested bulbs (Fig. 1).

Table 1. Transmission of TVX in bulbs of two cultivars stored in groups by three species of mite. Cultivar: “R = Renown”, “PD” = Pink Diamond.

Treatment	**cultivar**	**number of bulbs planted**	**number of plants tested**	**number of plants with TVX**
Control	R	100	23	0
	PD	100	5	0
A. tulipae	R	100	22	5
	PD	100	11	1
R. echinopus	R	100	28	0
	PD	100	31	0
Tyrophagus spp.	R	100	70	2
	PD	100	69	0

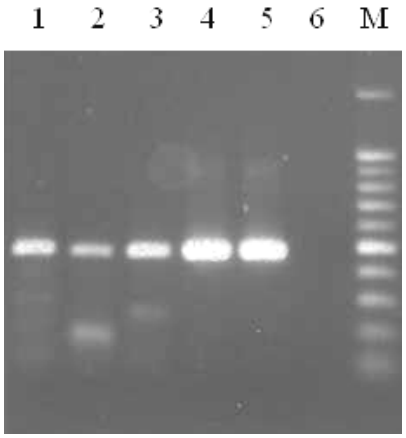
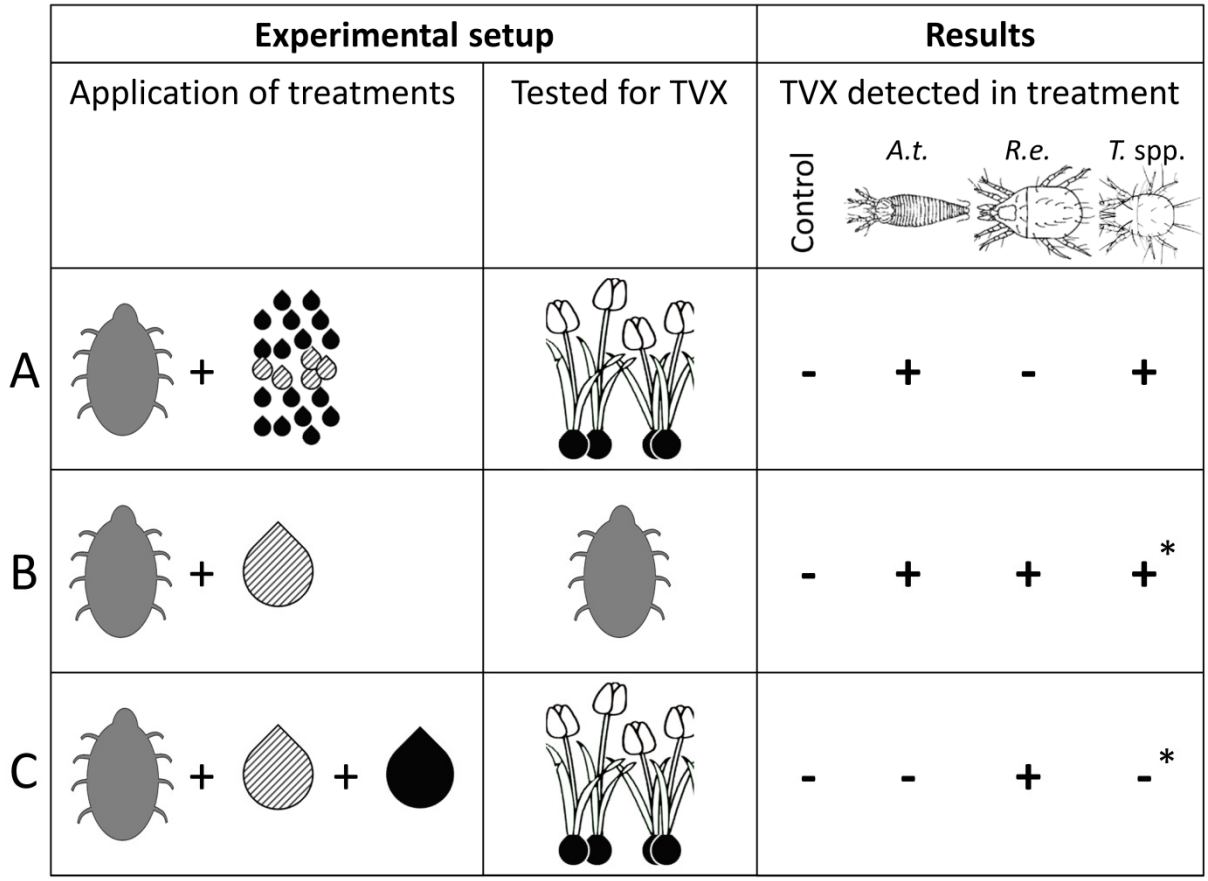


Figure 1. TVX from mites feeding on TVX-infected tulip bulbs. PCR products were obtained by amplification of cDNA synthesized from TVX RNA, extracted from different mite species after feeding on TVX-infected bulbs. Lane 1: *R. echinopus*; lane 2: *T. putrescentiae;* 3: *A. tulipae*; lane 4-5: TVX-infected bulb tissue (positive control); lane 6: water (negative control); M: 100bp ladder (Promega).

Table 2 presents the results of the transmission experiment. Not all bulbs produced a plant. Of those giving a plant, TVX was only found in one of them infected with *R. echinopus*. Not in any of the 100 untreated control bulbs from the regularly stored batch planted as extra control, TVX could be detected.

Table 2. Transmission of TVX in individual bulbs under laboratory conditions by three species of mite.

Treatment	Number of bulbs planted	Number of plants tested	Number of plants with TVX
Control	10	8	0
A. tulipae	7	6	0
R. echinopus	55	41	1
T. putrescentiae	56	52	0



* In these experiments, pure populations of *T. putrescentiae* were used.

Figure 2. Overview of the experimental setup and obtained results: transmission of TVX in stored bulbs (A), TVX in samples of mites (B), and transmission of TVX in bulbs under laboratory conditions (C). The first column depicts the method used to inoculate bulbs with mites of one of the three species of mite. Control treatments were not inoculated. Dashed bulbs represent TVX-infected specimens; black ones represent bulbs uninfected with TVX. The second column depicts the material tested for the presence of TVX after inoculation with mites. The third column presents the results. It indicates whether TVX was detected in each of the treatments: controls not inoculated with mites; inoculation with *A. tulipae* (*A.t.*), inoculation with *R. echinopus* (*R.e.*), or inoculation with *Tyrophagus* spp. *(T.* spp). Details can be found in the sections Material and methods and Results.

Discussion

Transmission of TVX

The results showed that TVX transmission occurred during storage of tulip bulbs and can be mediated by a biological vector. We obtained strong indications that mites are involved in transmission. The detection of TVX in *A. tulipae*, *R. echinopus*, and *T. putrescentiae* after colonization of TVX-infected bulb tissue indicate that all these mite species are able to carry TVX with them (Figure 1). The transmission experiments showed that healthy bulbs can obtain a TVX-infection after inoculation with mites in the presence of TVX-infected bulb tissue (Table 1 and 2). However, solely from these experiments we cannot confirm which species of mite transmit TVX. Firstly, the number of cases with successful transmission of TVX is too small. This might be explained by the early mortality of inoculated mites under our experimental conditions. Secondly, in the transmission experiment with stored bulbs (Fig. 2A), natural mite infection of the bulbs cannot be ruled out. Nevertheless, control treatments did not result in transmission of TVX, whereas inoculation with *A. tulipae* and *T. putrescentiae* did, suggesting that the latter two might be vectors. Additional research with larger numbers of independent replicates is required to further prove the vector status of each species of mite and the efficiency of transmission.

Transmission of viruses by mites and other arthropods

Transmission of a *Potexvirus* by a biological vector has only been reported for *Pepino mosaic virus*, which is transmitted by the oomycete *Olpidium virulentus* (Alfaro-Fernándezet *et al*., 2009), and Potato aucuba mosaic virus (PAMV), which is transmitted by the aphid *Myzus persicae* (Manoussopoulos, 2000). PAMV is not aphid-transmittable on its own, but is only transmitted by aphids that had previously been fed on a source of the Potyvirus potato virus Y. In both cases, the mode of virus transmission in non-persistent.

If our results are confirmed, this would, to our knowledge, be the first case reporting a Potexvirus to be transmitted by mites (Acariformes). So far, *A. tulipae* is only known to transmit Allexiviruses (Van Dijk *et al*., 1991; Yamashita *et al*., 1996; Kang *et al*., 2007), which, like Potexviruses, belong to the family of Alphaflexiviridae. The closely related eriophyoid mite *A. tosichella* Keifer is a vector of *Wheat strike mosaic virus* (WSMV), a Potyviridae (Gates, 1970; Kozlowski, 2000; Sánchez-Sánchez *et al*., 2001). In fruits, other eriophyoids are responsible for the transmission of *Black currant reversion virus* (a *Nepovirus*), *Peach mosaic virus* (a *Trichovirus*) and a not-assignable virus-like particle in fig (Thresh, 1964; Gispert *et al*., 1998; Serrano *et al*., 2004; ICTVdB – The Universal Virus Database, 2006). Acarid mites are not known to transmit viruses. In all cases, transmission by eriophyoid mites is persistent.

Mode of transmission of TVX

Potexviruses are generally mechanically transmitted and the few Potexviruses associated with vectors are transmitted in a non-persistent mode (Alfaro-Fernández *et al*., 2009; Manousso-poulos, 2000). Our molecular analysis identified the presence of TVX in sampled mites of *A. tulipae*, *R. echinopus* and *T. putrescentiae* after feeding on TVX-infected bulbs (Fig. 2). This indicates that these species can carry TVX with them. However, our experiment does not reveal whether TVX is transported inside or on the outside of the mite bodies, or both, and how the virus survives in or on the vector.

The mouthparts of eriophyoid mites consist of stylets penetrating the epidermal plant cells and sucking up the contents, including virus particles. Virus particles have been found in the digestive track as well as in other body tissues (Nault, 1997; Serrano *et al*., 2004).

Generally, viruses with an eriophyoid vector are transmitted in a persistent manner, in which the virus can survive several weeks in the host and persists moulting to subsequent life stages of the mite.

The mouthparts of the acarid mites are not stylet-like; they rather 'scrape' or 'chew' organic material. Abdel-Sater and Eraky (2001) showed that *T. putrescentiae* and *R. robini* transfer fungal pathogens between bulbous plants, both through their digestive track and by attachment of the fungi to the outside of their body. Fungal spores have also been detected in the alimentary canal of these species of mite in other studies (Price, 1976; Okabe, 1999). However, it is not likely that these mite species are closely associated with the transmission of a particular virus, because they do not exclusively feed on plant tissue, and occur on a broad range of host plants.

Altogether, we hypothesize that TVX transmission by mites is non-persistent. We expect the mouth parts of the mite to become contaminated with virus particles upon feeding on a TVX-infected bulb. Subsequently, cells of a virus-free bulb are wounded by mite-feeding and can become infected with the virus particles remaining on the mouth parts. This hypothesized mechanism is analogous to the mechanism of non-persistent virus transmission by aphids (Nault, 1997; Ng and Falk, 2006), and can be regarded as a vector-mediated mode of mechanical transmission. Dedicated experiments are required to investigate the mode of action of virus transmission for each species of mite in more detail.

Management of TVX in tulips

TVX transmission by mites would have important implications for virus control in tulip bulb production. The species of mite investigated are the main arthropod pests in tulip storage. The mites probably come with the harvested tulips bulbs in small numbers. However, the warm period of bulb storage with temperatures above 20°C promote their development and reproduction (Sakurai *et al*., 1992; Courtin *et al*., 2000; Aspaly *et al*., 2007; Sánchez-Ramos and Castañera, 2001; 2005). Stored bulbs can harbour extremely high numbers of *A. tulipae* and species of *Rhizoglyphus* and *Tyrophagus* (Czajkowska and Conijn, 1992; and unpublished results). Besides, acarid mites are found in dust and organic material on walls and floors and of storage buildings (unpublished results).

Currently, control measures lack or do not consistently control mites effectively. *Aceria tulipae* is not always controlled satisfactory by the application of pirimifos-methyl, the main control method, nor by the alternative method, exposure to ultra low levels of oxygen. The hidden life-style of *A. tulipae* makes it hard to detect an infection and makes them unattainable for chemicals (Van Leeuwen *et al*., 2010). The acarid mite species are not killed by the current legal application of pirimifos-methyl in tulips. Moreover, strong variation in susceptibility to pirimifos-methyl exists between strains of *R. robini* and *T. putrescentiae* and resistance has been found (Stables, 1984; Kuwahara, 1986). Mites could thus play a significant role in dispersal of TVX. Therefore, TVX control in tulip bulb production includes an adequate mite control strategy.

Rhizoglyphus echinopus occurs in many species of bulbs and *T. putrescentiae* occurs in a broad range of products (Jeppson *et al*., 1975; Ho, 1980; Diaz *et al*., 2000). Hence, we believe it is important to investigate the relationship between these acarid mites and plant-pathogenic viruses in other plant species as well.

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