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BACTERIAL AND PHYTOPLASMA DISEASES

Transmission and detection of toria [*Brassica rapa* L. subsp. *dichotoma* (Roxb.)] phyllody phytoplasma and identification of a potential vector

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Abstract Phyllody disease associated with 16SrIX phytoplasma was observed in the range of 4.1–11% in 10 different lines of toria [*Brassica rapa* L. subsp. *dichotoma* (Roxb.)] in experimental fields of the Indian Agricultural Research Institute, New Delhi, India during 2008 and 2009. The toria phyllody (TP) phytoplasma was detected in all the symptomatic and 13.3% of asymptomatic toria plants by nested PCR. The phytoplasma was detected in midrib, flower part, siliquae, stem, and root of infected plants as well as seeds. TP was transmitted by grafting and by dodder to toria and nine other rapeseed/mustard species as confirmed by nested PCR. However, symptoms of phytoplasma infection were induced only in toria, yellow sarson [*Brassica rapa* L. subsp. *trilocularis* (Roxb.)], brown sarson [*Brassica rapa* L. subsp. *sarson* (Prain)], rapeseed (*B. napus* subsp. *oleifera*), and rocket or taramira (*Eruca sativa*) but not in mustard (*B. juncea*), black mustard (*B. nigra*), Ethiopian mustard (*B. carinata*), *B. tournefortii* and white mustard (*Sinapis alba*). Transmission of TP phytoplasma to periwinkle (*Catharanthus roseus*) was successful only through dodder, but no transmission to tomato (*Lycopersicon esculentum*) or brinjal (*Solanum melongena*) was found. TP phytoplasma was detected in *Laodelpax striatellus*, an abundant planthopper in toria fields, which indicates that this planthopper may be a potential vector for TP phytoplasma.

Keywords Phytoplasma · Rapeseed mustard · Small brown planthopper · Toria · Transmission

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Introduction

Rapeseed and mustard are important oilseed crops in India, but the productivity is much lower (0.96 t/ha) than the world average (1.72 t/ha). A range of fungal, bacterial, viral and phytoplasma diseases often attack these crops at all stages of their development (Anonymous 1999; Bailey et al. 2003; Bhowmik 2003; Cui et al. 2009). Phyllody symptoms in toria [*Brassica rapa* L. subsp. *dichotoma* (Roxb.)] have been observed in India since 1958, and the disease causes reduced seed yield and oil content in infected plants (Bhowmik 2003; Bindra and Bakhetia 1967; Kaushik et al. 1978). The phytoplasma associated with it was identified recently as 16SrIX pigeon pea witches'-broom phytoplasma group (Azadvar et al. 2009). Symptoms generally include phyllody (development of floral parts into leafy structures), virescence (development of green flowers and the loss of normal pigments), witches'-broom, extensive malformation of floral part, formation of bladder-like siliquae and flower sterility (Azadvar et al. 2009; Bhowmik 2003). Based on characteristic symptoms, the phytoplasma infecting toria has been named toria phyllody (TP) phytoplasma, which we will use in this paper.

In the present study on the incidence of TP disease under field conditions, we screened different plant parts including seeds of toria for phytoplasmas and tested the transmission

of TP phytoplasma to different species of rapeseed mustard and other potential hosts such as periwinkle, tomato and brinjal. Insects in the toria field were also trapped and tested for the phytoplasma.

Materials and methods

Incidence of TP disease

A field survey for occurrence of TP disease in toria plants was conducted during 2008 and 2009 in an experimental field of the Indian Agricultural Research Institute, New Delhi, India. Seeds of 10 toria lines were sown on 16 September. TP incidence in each toria line (each having 90–110 plants) was recorded by counting the plants with typical TP symptoms at the end of flowering season in the second week of December. Three symptomatic and three asymptomatic toria plants from each line were collected randomly and used to screen for TP phytoplasma DNA using nested PCR. Healthy toria plants grown in a greenhouse were used as negative control.

Transmission of TP phytoplasma

Test plants

Seeds of 10 different brassicaceous species (*B. rapa* L. subsp. *dichotoma* [toria, line T-9], *Brassica rapa* L. subsp. *trilocularis* (Roxb.) [yellow sarson, cv. Pusa Gold], *Brassica rapa* L. subsp. *sarson* (Prain) [brown sarson, line BSH-1], *B. nigra* line IC-247, *B. napus* line GSL-1, *B. juncea* cv. Pusa Jagannath, *B. carinata* cv. Pusa Aditya, *B. tournefortii*, *Eruca sativa* line T-27, *Sinapis alba*, *Solanum melongena* (brinjal, cv. Kokila), *Lycopersicun esculentum* (tomato, cv. Century-12), and *Catharanthus roseus* (periwinkle)) were planted on 13 November in sterile soil in 30-cm pots in an insect-free greenhouse. A minimum of three plants of each species were used in each experiment for graft/dodder transmission of TP phytoplasma from infected plants. Seed-grown, healthy *Brassica* species, tomato, brinjal and periwinkle plants were grown in the greenhouse and confirmed for absence of phytoplasma by nested PCR and were used as controls.

Naturally infected toria plants with typical TP symptoms were confirmed for presence of the phytoplasma by nested PCR and were used as source of inoculum for grafting and dodder.

Graft transmission

Graft transmission was carried out from TP phytoplasma-infected toria to healthy rapeseed mustard species and periwinkle. A small branch of TP phytoplasma-infected toria was side/top grafted to 1-month-old plants of 10 different *Brassica* species and periwinkle during the second week of December. In control, branch collected from healthy toria plant was used. All the graft-inoculated plants were observed for 2 months for symptom expression, and infection was confirmed by nested PCR and sequencing.

Dodder transmission

Seeds of dodder (*Cuscuta campestris*) were germinated on wet tissue and established on five healthy plants each of 1-month-old periwinkle and toria. The established dodder branches were transferred on naturally infected toria plants. The plants were covered with insect-proof nets. New branches of dodder that were established on TP phytoplasma-infected periwinkle were transferred onto seed-grown plants of all 10 brassicaceous species as well as tomato, brinjal and periwinkle. For the controls, the same species were inoculated with healthy dodder. Plants were kept in glasshouse to observe symptom appearance and phytoplasma confirmation by nested PCR detection and sequencing. In all the experiments, dodders were removed after 1 month.

Detection of TP phytoplasma in different plant parts of toria

One hundred milligrams of midrib, flower, siliquae, stem, and root were excised separately from infected toria plants collected from a field and tested for TP phytoplasma DNA using nested PCR. For detection of TP phytoplasma in seeds, 100 mg of seeds from each of five TP infected and five healthy toria plants were collected from the field and were tested for phytoplasma by nested PCR. The seeds from healthy toria (confirmed for absence of TP phytoplsma in nested PCR) and infected toria plants were segregated into normal and abnormal seeds, and nested PCR was used to test for the TP phytoplsma.

Identification of potential insect vector

Hoppers were captured weekly by 100 sweeps in 360-cm^2 plot for each sampling with a 30-cm net hoop (Munyaneza et al. 2008) from the early growth stage to flowering stage in a toria field during October 2009 from 1700 to 1900 hours. The samples were collected in plastic bags and brought to the laboratory. Hoppers were counted, identified taxonomically at the Division of Entomology, IARI, New Delhi, India and stored at −20°C. Thereafter, nested PCR and sequencing were used to detect TP phytoplasma in these insects.

Nucleic acid extraction

The total genomic DNA was isolated from 100 mg of each plant tissue from each symptomatic and asymptomatic plant collected from the field and from healthy toria grown in a greenhouse, using a DNeasy plant mini kit (Qiagen Gmbh, Hilden, Germany), according to the manufacturer's instruction. For DNA extraction from insects, 10 planthoppers for each batch were crushed in liquid nitrogen, and a DNeasy blood and tissue kit (Qiagen Gmbh) was used as per manufacturer's instruction with a modification at the initial stage of DNA extraction; a HiShredder (DS0010) Miniprep spin column (HiPur Insect DNA Miniprep Purification Spin Kit, Himedia, India) was used to remove insect debris after extraction of insect tissues in ATL buffer and before addition of proteinase K. *Hishimonus phycitis* leafhoppers, collected from a brinjal field and naturally infected with brinjal little leaf (BLL) phytoplasma, were used as a positive control.

Amplification of phytoplasma DNA in nested PCR

DNA from either plants or insects was used as template in first round of amplification. Universal primer pairs P1/P7 (Schneider et al. 1995) and R16F2n/R16R2 (Gundersen and Lee 1996) were used in first and second round of PCR amplification, respectively, for amplification of 16S rDNA of phytoplasma. PCR amplification was performed in 50-μL reaction mixtures using 200 pmol of primer, 200 μM each of dNTPs, 2.5 unit of *Taq* DNA polymerase, 1 × reaction buffer (Fermentas, Vilnius, Lithuania), 1.5 mM of $MgCl_2$ and 5 μL of DNA template (200 ng) either from symptomatic or nonsymptomatic as well as healthy plants. The thermal conditions were as follows: denaturation at 94°C for 40 s (5 min for the first cycle), annealing at 55°C for 1 min and extension at 72°C for 2 min. The last cycle was extended for an additional 10 min at 72°C. Annealing temperature for nested PCR was 56°C. The PCR was carried out for 35 cycles in Bio-Rad thermal cycler (MyCycler).

Analysis of PCR products

Seven microliters of amplification products were analysed by electrophoresis in 1% agarose gel stained with ethidium bromide and visualized under UV light. Representative sample of PCR products in all the experiments were sequenced directly at Chromos Biotech (Bangalore, India). Sequences of PCR products were aligned, and sequence identities were compared with TP phytoplasma (accession GU111554) by sequence identity matrix after ClustalW multiple alignment using BioEdit software (Hall 1999). Blast analysis was also done with sequences archived in GenBank (http://ncbi.nlm.nih.gov/blast).

Results

Symptoms, disease incidence and detection of TP phytoplasma in different parts of infected toria

Malformed and bladder-like siliquae were first observed 1.5 months after sowing toria seeds in the experimental field. Plants that developed symptoms at an early growth stage failed to produce flowers and set siliquae. Typical symptoms of phytoplasma infection such as phyllody, viresence, proliferation of axillary shoot and malformed and bladder-like siliquae were observed in several TP diseased plants at the time of flowering (Fig. 1). In 2008, disease incidence 3 months after sowing among 10 different lines of toria ranged from 4.8 to 11%. In 2009, it varied between 4.1 and 10.5% (Table 1). Nested PCR assays with the phytoplasma universal primer pair P1/P7 followed by primers R16F2n/R16R2 gave an ca. 1.25 kb DNA fragment from all diseased toria samples, but no amplification product was obtained from healthy toria plants. Phytoplasma DNA was detected in the midrib, flower, siliquae, stem, root, and normal and abnormal seeds of toria plants with typical TP symptoms (data not shown). Phytoplasma DNA was detected in midribs of four of 30 asymptomatic toria plants (13.3%).

Graft transmission

TP phytoplasma was transmitted from TP phytoplasma-infected toria to healthy toria and other brassicaceous species through grafting. Typical phytoplasma disease symptoms such as phyllody, virescence, siliquae malformation, flower sterility and little leaf were induced on toria, yellow sarson and brown sarson, *B. napus* and *Eruca sativa* plants by 2 months after grafting (Fig. 2). Symptoms were induced in *E. sativa*, 2 weeks earlier and in toria and *B. napus* 1 week earlier than in other *Brassica* species. No symptoms were observed on *B. juncea*, *B. nigra*, *B. carinata*, *B. tournefortii* and *Sinapis alba*. TP phytoplasma was detected in all 10 grafted brassicaceous species, with or without phytoplasma symptoms, by nested PCR (Table 2; Fig. 3a).

Dodder transmission

TP phytoplasma was successfully transmitted from toria to toria and to periwinkle by dodder. Branches of dodder from TP phytoplasma infected periwinkle were established on different species of rapeseed mustard, periwinkle, brinjal and tomato. One month after removing the dodder, typical TP phytoplasma symptoms were observed on dodder-inoculated toria (line T-9), yellow sarson (line Pusa Gold) and brown sarson (line BSH-1), *B. napus*, *Eruca sativa* and

Fig. 1 Symptoms of toria phyllody disease on toria [*Brassica rapa* L. subsp. *dichotoma* (Roxb.)]. **a** flowering stage, **b** siliqua formation stage, **c** flower sterility, phyllody and virescence, **d, e** bladder-like siliquae and pod malformation. *H* healthy, *I* infected

Table 1 Toria phyllody disease incidence on 10 different toria lines in experimental field, IARI, New Delhi, India in 2008 and 2009

Toria line	Disease incidence (%)	
	2008	2009
ORT-17-6-16	11.0	8.6
JMT-08-13	8.8	10.3
JMT-8-11	6.9	10.5
TL-2025	9.6	7.2
TH-0301	8.3	6.4
PT-2006-8	5.6	8.5
TK-8-2	7.2	5.8
PT-303	5.0	7.5
TK-8-1	5.9	4.1
TL-15	4.8	4.7

periwinkle plants (Fig. 2). The symptoms were induced on periwinkle 3 weeks after dodder removal, 4 weeks after removal for *B. rapa* subsp. *dichotoma* and after 5 weeks for four other *Brassica* species. Although no symptoms were observed after dodder inoculation on *B. juncea*, *B. nigra*, *B. carinata*, *B. tournefortii*, *Sinapis alba*, brinjal or tomato, positive amplification signals were obtained from all the brassicas and periwinkle with or without symptoms of phytoplasma infection in nested PCR (Table 2; Fig. 3b).

Potential insect vector

The planthopper populations captured by sweep netting in the toria field during October 2009 were identified as *Laodelphax striatellus* (Fallen), *Sogatella vibix* (Haupt) and *Stenocranus* sp. A total of 940 *L. striatellus* were netted, six and eight times more than for the other two species, respectively, during the period from germination to flowering (data not shown). Nested PCR detected TP phytoplasma in the body of *L. striatellus*, but not for the other two planthoppers (Fig. 3c).

In all experiments, the BLAST search revealed that the amplified PCR products shared 100% sequence identity with GenBank accession GU111554 of TP phytoplasma and belonged to 16SrIX pigeon pea witches'-broom group.

Discussion

The phyllody symptom in toria was observed as early as 1958 in India (Bindra and Bakhetia 1967), and the phytoplasma associated with the disease was characterized as 16SrIX phytoplasmas by Azadvar et al. (2009). Phytoplasma pathogens that infect rapeseed/canola in Canada, Czech, Italy and Greece (Bertaccini et al. 1998; Maliogka et al. 2009; Olivier et al. 2006; Wang and Hiruki 2001), identified as the 16SrI aster yellows group, cause stunting, leaf yellowing or purpling. None of these symptoms have been observed on toria in India, which is infected with the 16SrIX pigeon pea witches'-broom group phytoplasma (Azadvar et al. 2009). Ten-week-old stock (*Mathiola incana*), a garden flower plant of *Brassicaceae* family is affected by 16SrII-A group phytoplasmas in Italy (Davino et al. 2007). The percentage of canola plants with aster yellows symptoms ranged from traces to 12% with a provincial average of 2% in Canada in 2007, but the phytoplasma was detected in 10–30% of asymptomatic canola

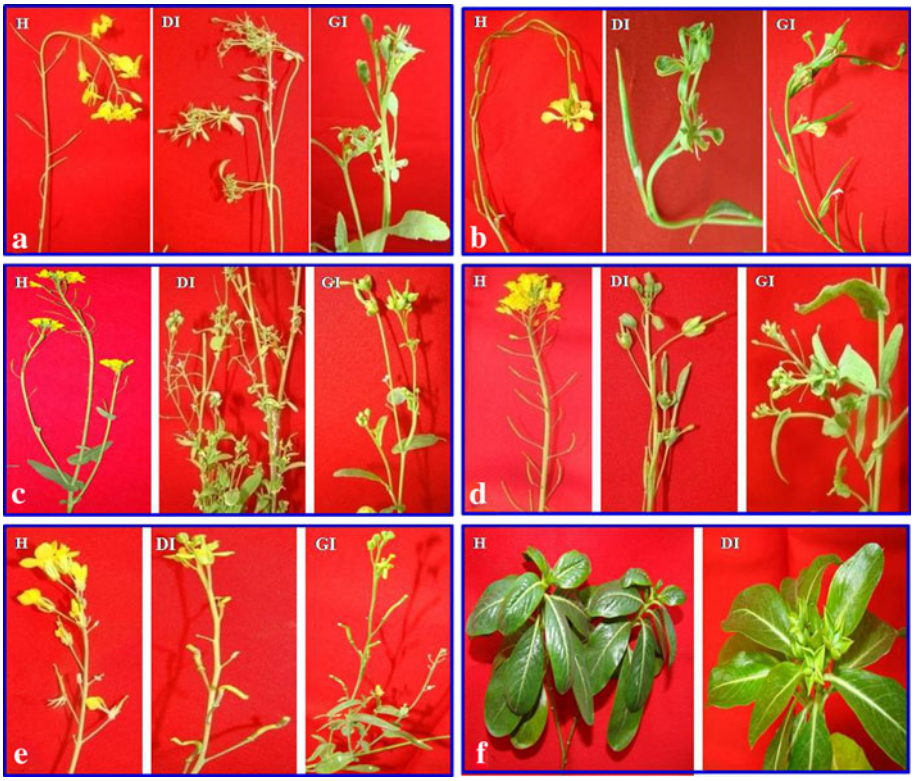

Fig. 2 Symptom appearance after inoculation by dodder or grafting in brassicaceous species and periwinkle plants. **a** *Brassica napus*, **b** *Eruca sativa*, **c** *B. rapa*. subsp. *dichotoma*, **d** *B. rapa* subsp. *sarson*, **e** *B. rapa* subsp. *trilocularis*, **f** periwinkle (*Catharanthus roseus*). *H* healthy, *DI* dodder inoculated, *GI* graft inoculated

Table 2 Transmission of toria phyllody (TP) phytoplasma to oilseed brassicas by grafting and dodder transmission

Plants used for transmission	No. of plants with symptoms/no. of plants inoculated		Detection of TP phytoplasma in grafted and dodder transmitted plants by nested PCR
	Grafting[a]	Dodder[b]	
B. rapa L. subsp. *dichotoma* (toria, T-9)	3/4	3/3	+[c]
Brassica rapa L. subsp. *trilocularis* (Roxb.) (yellow sarson, Pusa Gold)	1/3	2/3	+[c]
B. rapa L. subsp. *sarson* (Prain) (brown sarson, BSH-1)	2/3	3/3	+[c]
Eruca sativa (T-27)	3/3	3/3	+
B. napus (GSL-1)	2/3	2/3	+[c]
B. nigra (IC-247)	0/3	0/3	+
B. tournefortii	0/3	0/3	+
B. carinata (Pusa Aditya)	0/3	0/3	+
B. juncea (Pusa Jagannath)	0/3	0/4	+
Sinapis alba	0/3	0/3	+
Catharanthus roseus (periwinkle)	0/3	6/6	+
Solanum melongena (Brinjal) (Kokila)	ND	0/3	−
Lycopersicum esculentum (Tomato) (Century-12)	ND	0/3	−

+ positive in PCR, − negative in PCR, *ND* not done

Inoculum source: [a]TP-infected toria was source of inoculum, [b]TP-infected periwinkle was source of inoculum

[c] Samples were collected from symptomatic plants

plants by PCR (Olivier and Galka 2008). The Iranian cabbage yellow caused by 16SrVI group of phytoplasma caused up to 50% disease incidence in cabbage fields (Salehi et al. 2007). Even though the level of phytoplasma-induced symptoms in our study was less than 11%, field incidence of the disease could be up to 22.6% in view of the fact that 13% of asymptomatic plants also carried the phytoplasma.

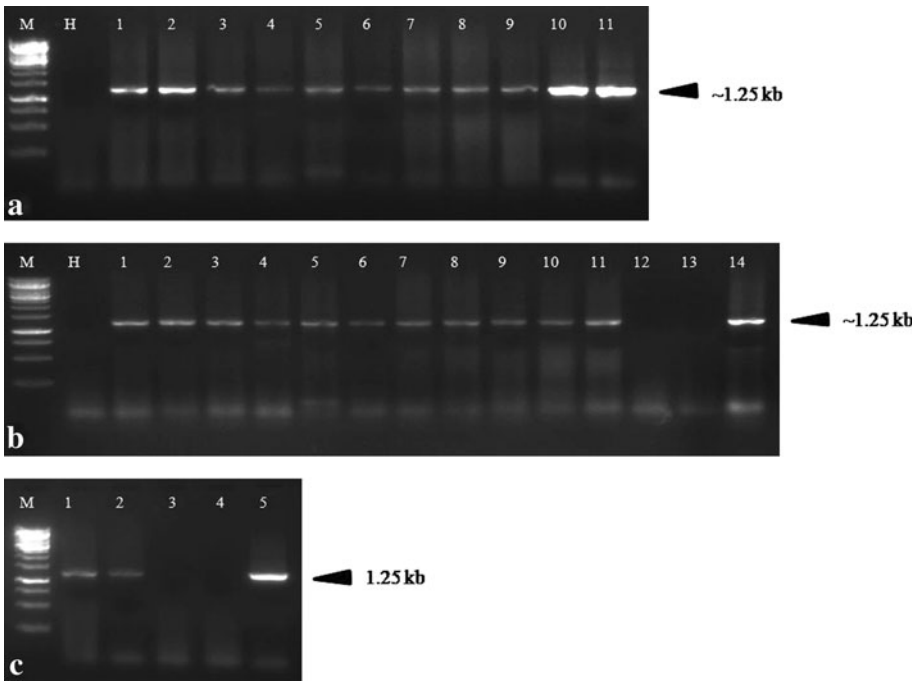

Fig. 3 Agarose gel electrophoresis of nested PCR products using universal primer pair P1/P7 followed by R16F2n/R16R2. **a** Different brassicas after grafting with toria phyllody (TP) phytoplasma-infected toria plant. *H* healthy *Brassica rapa* subsp. *dichotoma*, *1*, *B. rapa* subsp. *dichotoma*; *2*, *B. rapa* subsp. *sarson*; *3*, *B. rapa* subsp. *trilocularis*; *4*, *B. nigra*; *5*, *B. napus*, *6*, *B. juncea*; *7*, *B. carinata*; *8*, *B. tournefortii*; *9*, *Sinapis alba*; *10*, *Eruca sativa*; *11*, TP phytoplasma (positive control). **b** Different brassicas after inoculation by dodder infected with TP. *H* healthy toria, *1*, *B. rapa* subsp. *dichotoma*; *2*, *B. rapa* subsp. *sarson*; *3*, *B. rapa* subsp. *trilocularis*; *4*, *B. nigra*; *5*, *B. napus*; *6*, *B. juncea*; *7*, *B. carinata*; *8*, *B. tournefortii*; *9*, *Sinapis alba*; *10*, *Eruca sativa*; *11*, periwinkle; *12*, brinjal; *13*, tomato; *14*, TP phytoplasma (positive control). **c** TP phytoplasma in collected planthoppers from toria field. *1*, *Hishimonus phycitis* naturally infected by brinjal little leaf phytoplasma (positive control); *2*, *Laodelphax striatellus*; *3*, *Sogatella vibix*; *4*, *Stenocramus* sp.; *5*, TP phytoplasmas in toria (positive control)

Four major species of *Brassica*—*B. juncea*, *B. rapa*, *B. napus* and *B. carinata*, are grown widely in India. Our studies on dodder and graft transmission indicated that other *Brassica* species are also host of 16SrIX group phytoplasma. Symptoms were pronounced in *E. sativa* and three *B. rapa* cultivars (toria, brown sarson and yellow sarson). *Brassica juncea*, the most important commercial oilseed *Brassica* species did not develop any symptoms even though we could detect the presence of 16SrIX phytoplasmas after transmission by graft and dodder. Thus, the genes involved in symptom expression may not have been upregulated in the asymptomatic *Brassica* species (Nicolaisen and Horvath 2008). In fact, no phyllody symptoms were observed on *B. juncea* grown near toria fields, and these plants were negative for phytoplasmas in nested PCR (data not shown). The absence of phytoplasmas in these *B. juncea* may also be due to vector preference for the host and other factors such as phenology and age of the plant and the environment. Host range of the phytoplasma and the insect vector are both known to influence phytoplasma infection in plants (Bosco and D'Amelio 2010).

The insect vector is an important factor that can influence the host range of phytoplasmas in nature (Kakizawa et al. 2010), so identification of the vector is important. *Laodelphax striatellus* was the major planthopper in the toria field, and its detection with TP phytoplasmas in the present is the first record of it as a potential vector of 16SrIX phytoplasma. Earlier studies have associated *L. striatellus* as a potential vector of 16SrI, 16SrXII-A and 16SrIII phytoplasmas in *Myrtus communis* in Italy (Prota et al. 2007). Further experiments are required to determine the specific association of this vector with TP phytoplasma. None of the planthoppers feeding on toria plants were otherwise economically damaging.

It was interesting to detect TP phytoplasma in seeds of phytoplasma-infected toria plants; seed transmission of phytoplasmas is assumed to be unlikely because phloem sieve elements lack any direct connection to seeds, (Weintraub and Beanland 2006). Nevertheless, phytoplasmas have been detected in seeds, embryos and seedlings of infected parent plants of alfalfa, coconut and *Brassica* and in seed-grown progeny (Khan et al. 2002; Nipah et al. 2007; Olivier and Galka 2008; Olivier et al. 2008; Starzycki and Starzycka 2000). Reports on the association of aster yellows phytoplasma with canola and its transmission to the progeny plants (Olivier et al. 2008; Olivier and

Galka 2008) and the results of this study raise concern that seeds may be a means to spread of phytoplasmas in brassicas. This is the first time that 16SrIX phytoplasma has been detected in toria seeds, but more evidence is required to determine whether phytoplasmas in infected seeds can be translocated into the developing plant.

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