ISSN 1392-3714

Identification of viral and phytoplasmal agents responsible for diseases affecting plants of *Thalictrum* L. in Lithuania

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Abstract

Thalictrum plants exhibiting symptoms characteristic of viral and phytoplasmal diseases were collected at botanical gardens and different floriculture farms in Lithuania. Viral disease was expressed by leaf curling, pinpoint light green spotting and malformation. The causal agent of viral disease, *Tomato ringspot nepovirus* was isolated and identified by the methods of test-plants and transmission electron microscopy. Phytoplasmal symptoms included general stunting of plants, proliferation and flower distortion. Amplification of phytoplasmal 16SrRNA gene sequence in polymerase chain reactions (PCRs) containing phytoplasma universal primer pairs P1/P7, R16F2n/R16R2 and template DNA extracted from diseased plants, confirmed that the plants were infected by phytoplasma. The 1,2 kbp rDNA product was subjected to single enzyme digestions with 9 restriction endonucleases. RFLP analysis revealed that the *Thalictrum* plants were infected by a phytoplasma belonging to group 16SrI (aster yellows phytoplasma group), subgroup L. This work was the first investigation of viral and phytoplasmal diseases of *Thalictrum* plants and identification of their causal agents. *Thalictrum* is the new host-plant of *Tomato ringspot nepovirus* (ToRSV) and subgroup 16Sr I–L of phytoplasmas.

Key words: EM, identification, PCR, phytoplasma, RFLP, test-plants, *Thalictrum*, virus.

INTRODUCTION

During last years great attention has been paid to the development of field floriculture in Lithuania. For small land property farmers field floriculture is perspective as family business. The farmers grow seedlings of perennial ornamental plants not only for Lithuanian domestic market, but also for neighboring countries. Like other segments of agriculture this sector is threatened by plant diseases. The quality and quantity of ornamental plants is affected by viral and phytoplasmal diseases. Since viral and phytoplasmal infections are systemic in diseased plants propagation by division may contribute to geographical spread of pathogens. The scientific workers of Plant Virus Laboratory of Institute of Botany carry out regular survey controlling the phytosanitary state of ornamental plants grown at Botanical gardens of Vilnius, Kaunas Vytautas Magnus, Klaipėda, Šiauliai Universities, Experimental Station of Field Floriculture and according to opportunities and requests in other floriculture farms and cities' parterres in order to help the growers to control plant diseases and also to collect plant samples for investigation.

The genus *Thalictrum* L. originating from Europe belongs to *Ranunculaceae* Juss. family. Over 300 species make up this genus of perennials known for their fluffy, showy flowers, slender upstanding stems and finely divided leaves. Blooming in spring and summer, the flowers have no petals, but instead have 4 or 5 sepals and conspicuous stamen tufts [Cheifets et al., 2006]. Species *Thalictrum lucidum, T. simplex* grow in natural meadows in Lithuania. *T. aquilegifolia, T. flavum* become popular ornamental plants grown as garden plant in our country. There are no data concerning *Thalictrum* viral or phytoplasmal diseases in literature.

The objective of this study was to determine possible association of virus and phytoplasma with diseases in *Thalictrum* and to identify the causal agents increasing knowledge on their biodiversity.

MATERIALS AND METHODS

Plant samples. The plant material for investigation was collected at Botanical gardens of Kaunas Vytautas Magnus University, Experimental Station of Field Floriculture, Vilnius city's and private parterres. The experimental work was carried out at the Plant Virus Laboratory of the Institute of Botany.

Virus identification. Virus has been identified by test-plant method [Brunt et al., 1996; Stace-Smith, 1984], transmission electron microscopy negative staining technique [Robinson et al., 1987]. The inocula for mechanical inoculation were prepared by homogenizing of infected plants with 0,1 M phosphate buffer (pH 7,0) containing as virus–stabilizing additives 0,2 % 2-mercaptoethanol or 0,01 M sodium diethyldithiocarbamate. The used test-plants are listed in Table 1.

Phytoplasmal DNA extraction and amplification in the polymerase chain reaction (PCR). Phytoplasma was detected in PCR. Nucleic acid for use as a template in PCR was extracted from the frozen tissues using a Genomic DNA Purification Kit (MBI *Fermentas*, Vilnius) according to the manufacturer's instructions. Ribosomal (r) DNA was amplified in nested PCRs using primer pair P1/P7 [Deng and Hiruki, 1991] and phytoplasma-specific primer pair R16F2n/R16R2 [Gundersen and Lee, 1996] as described by Jomantiene et al. [1998a].

Restriction fragment length polymorphism (RFLP) analysis of amplified phytoplasma DNA. Products from the nested PCR primed by R16F2n/R16R2 were analyzed by single enzyme digestion according to manufacturer's instructions with restriction endonucleases: *Alu*I, *Mse*I, *Kpn*I, *Hha*I, *Hpa*II, *Hae*III, *Rsa*I, *Hinf*I, *Taq*I (MBI Fermentas). RFLP profiles of digested DNA were analyzed by electrophoresis through 5 % polyacrilamide gel, staining with ethidium bromide, and visualization using an UV transilluminator. The RFLP profiles were compared with previously published data [Jomantiene et al., 1998 a, b; Lee et al., 1998, 2004; Marcone et al., 2000).

RESULTS

Virus identification. Infected *T. aquilegifolia* plants bearing symptoms of leaf malformation, curling, pinpoint light green mosaic were collected in the parterre of Park Verkiai (Fig. 1).

Fig. 1. Symptoms of ToRSV in naturally infected *Thalictrum aquilegifolia* leaf

1 pav. ToRSV sukelti požymiai natūraliai užsikrėtusio *Thalictum aquilegifolia* lape

T. flavum bearing symptoms of vein clearing at the Experimental Station of Field Floriculture, and *T. minus* expressing symptoms of inconspicuous ringspot in private garden in Vilnius district. Electron microscopy investigation of negatively stained dip preparations from naturally infected *Thalictrum* plants revealed the presence of isometric particles, 28 nm in diameter. The test-plants were inoculated by mechanical sap inoculation. Virus, isolated from all collected plants induced local and systemic reaction in the test-plants (Table 1). Test-plants *Nicotiana glutinosa, N. rustica* and *Physalis floridana* showed only local reaction. *Nicotiana rustica, Celosia argentea, Gomphrena globosa* and *Chenopodium quinoa* developed the most conspicuous reactions.

Table 1. The reactions of test-plants, inoculated with *Tomato ringspot nepovirus* (ToRSV) isolated from *Thalictrum* sp.

1 lentelė. Augalų-indikatorių reakcija, užkrėtus iš vingirių išskirtu pomidorų žiediškosios dėmėtligės virusu

Test plant	Symptoms
Amaranthus caudatus L.	L: NLL; S: Sp, LeDis
A. paniculatus L.	L:NSp; S: Sp, LeDis
Celosia argentea L.	L: DBrRi; S: VCl, LeDis, BrSp, Ln
Chenopodium amaranticolor Coste et Reyn	L: ClLL; S: VStu, TR, NT
C. murale L.	L: ClSp; S: VCl, Mo, N, LeDis
C. quinoa Willd.	L: ClSp, N, Dis; S: Cl, N, LeDis, ApN
C. urbicum L.	L: ClSp S: VCl, LeDis
Cucumis sativus L.	L: N or ClLL; S: Mo
Gomphrena globosa L.	L: NSp; S: Dis, Mo
Lycopersicon esculentum Mill.	L: NSp; S: Mo, N
Nicotiana glutinosa L.	L: NSp
N. rustica L.	L: NLn, RiSp
N. tabacum L.	L: NSp; S: NSp, Str
Petunia hybrida Vilm.	L: GNRi; S: LeDis, ClSp, NSp
Physalis floridana Rybd.	L: NLL, LeRu
Tetragonia expansa Murr.	L: DifClSp; S: LeDis, ClDot

Abbreviations / sutrumpinimai: L – local reaction / vietinė reakcija; LL – local lesions / vietiniai pažeidimai; Cl – chlorotic, chloruosis / chlorotiškas, chlorozė; S – systemic reaction / sisteminė reakcija; V – vein / gysla; Stu – stunting / žemaūgiškumas; Ap – apical / augimo kūgelis; T – top / viršūnė; N – necrotic, necrosis / nekrotiškas, nekrozė; Dot – dots / taškeliai; Le – leaves / lapai; Dis –distortion / deformacija; Sp – spots / dėmelės; D – dark / tamsus; Br – brown / rudas; G – gray / pilkas, Ri – rings / žiedai; RiSp – ringsot / žiediškos dėmės; Ln – line / linijos; Mo – mottling / margumas; R – rolling / užsisukimas; Dif – diffusive / difuzinis; Str – streaks / dryželiai; Ru – rugosity / garbanotumas.

Local reaction in *Nicotiana rustica* was expressed by necrotic sinuous lines or rings on inoculated leaves (Fig. 2).

Fig. 2. Local symptoms of ToRSV in inoculated *Nicotiana rustica* leaf
2 pav. ToRSV sukelti vietiniai požymiai *Nicotiana rustica* lape

Inoculated leaves of *Celosia argentea* showed dark brown ringspot, which later extended along veins and became necrotic. Systemic reaction was expressed by malformation of leaves, vein clearing, small brown spots located mainly on the base of leaf lamina (Fig. 3).

Fig. 3. Systemic symptoms induced by ToRSV in *Celosia argentea* leaves
3 pav. ToRSV sukelti sisteminiai požymiai *Celosia argentea* lapuose

Gomphrena globosa develops local necrotic spots. Systemic reaction was expressed by mild leaf distortion and light green mottling on leaves. Inoculated *Chenopodium quinoa* leaves showed chlorotic local spots, necrosis, leaf distortion; systemic reaction was expressed by distortion of young leaves, and chlorotic spots on the base of leaf lamina. Tip of plant was distorted and turned downwards (Fig. 4).

Fig. 4. Local and systemic symptoms induced by ToRSV in *Chenopodium quinoa*

4 pav. ToRSV sukelti vietiniai ir sisteminiai požymiai *Chenopodium quinoa* augaluose

Electron microscopy investigation of negatively stained dip preparations from infected test-plants revealed the presence of isometric particles, 28 nm in diameter (Fig. 5).

On the basis of the results of test-plant reactions, morphology of virus particles also according to literature [Brunt et al., 1996; Stace-Smith, 1984] we concluded, that *Thalictrum aquilegifolium, T. flavum* and *T. minus* plants were infected by *Tomato ringspot nepovirus* (ToRSV).

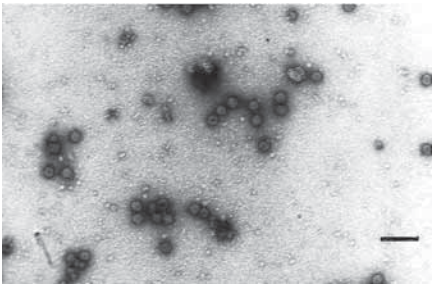

Fig. 5. Electron micrograph of negatively stained ToRSV particles (bar represents 100 nm)

5 pav. Negatyviai kontrastuotų ToRSV dalelių elektronomikrografija (brūkšnys – 100 nm)

Molecular detection and classification of phytoplasma and RFLP analysis of 16S rDNA. Nucleic acid for use as a template in PCR was extracted from *Thalictrum* sp. plants collected at the Botanical Garden of Kaunas Vytautas Magnus University. Diseased plants showed symptoms of general stunting, proliferation and reduced size of flowers (Fig. 6).

Fig. 6. Symptoms induced by phytoplasma in *Thalictrum* sp. (on the right – raceme of healthy plant)

6 pav. Fitoplazmų sukelti požymiai *Thalictrum sp.* (dešinėje – sveiko augalo žiedynas)

Amplification of phytoplasmal 16SrRNA gene sequence in polymerase chain reactions (PCRs) containing template DNA extracted from diseased plants and phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2 yielded phytoplasma characteristic 1,8 kbp and 1,2 kbp 16S rDNA PCR products indicating that the diseased plants were infected by phytoplasma (data not shown). The phytoplasma was named *Thalictrum* proliferation (ThPr). The 1,2 kbp rDNA product was subjected to single enzyme digestions with 9 different restriction endonucleases. RFLP analysis revealed that the *Thalictrum* plants were infected by a phytoplasma belonging to group 16SrI (aster yellows phytoplasma group), subgroup 16SrI–L (Fig. 7). We interpreted this pattern to indicate the presence of two sequence-heterogeneous 16S rRNA genes in ThPr phytoplasma.

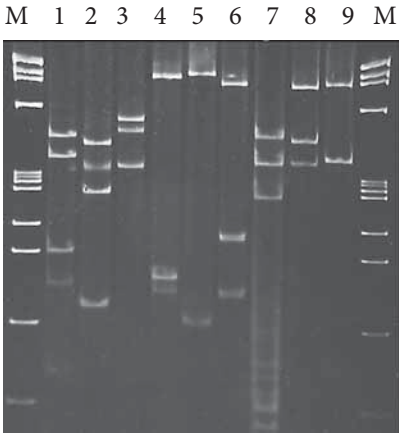

Fig. 7. RFLP analysis of ThPr (16SrI–L subgroup) phytoplasma 16S rDNA, amplified in PCR. Lanes M, DNA marker PhiX174 DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72; 1 – *Alu*I; 2 – *Mse*I; 3 – *Kpn*I; 4 – *Hha*I; 5 – *Hae*III; 6 – *Hpa*II; 7 – *Rsa*I; 8 – *Hinf*I; 9 – *Taq*I

7 pav. ThPr (16SrI–L pogrupio) fitoplazmos 16S rDNR RFLP analizė. Takeliai M – DNR dydžio standartas PhiX174 DNA HaeIII digest, fragmentų dydžiai (bp) nuo viršaus į apačią: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72; 1 – AluI; 2 – MseI; 3 – KpnI; 4 – HhaI; 5 – HaeIII; 6 – HpaII; 7 – RsaI; 8 – HinfI; 9 – TaqI

DISCUSSION

This work was the first investigation of viral and phytoplasmal diseases of *Thalictrum* plants and identification of their causal agents. The results provide information about biodiversity, variability and distribution of plant pathogens in Lithuania. The work also revealed the new host-plants of ToRSV and subgroup 16Sr I–L of phytoplasmas.

ToRSV is a type member of nepovirus group and causes economically important diseases in a range of crops. ToRSV has been added to the EPPO list and is considered as a quarantine pest. The virus has isometric particles about 28 nm in diameter, sedimenting

as three components and containing RNA as a bipartite genome. It is readily transmissible by inoculation of sap and has a wide host range, including both woody and herbaceous plants. It is transmitted by the nematode *Xiphinema* spp. Seed transmission has been reported in several crops. Most infected plants show distinctive symptoms as a shock reaction; chronically infected plants usually exhibit no obvious symptoms but show a general decline in productivity [Brunt et al., 1996; Stace-Smith, 1984]. The virus occurs in nature mostly in perennial crops. Ornamental hosts have been found naturally infected by ToRSV including: *Anemone* L., *Gladiolus* L., *Hydrangea* L., *Iris* L., *Narcissus* L., *Pelargonium* L'Her., *Petunia* Juss. [Loebenstein et al., 1995]. Our investigation has enlarged the range of ToRSV host-plants [Navalinskienė ir Samuitienė, 2006]. Wide distribution of ToRSV can be explained by efficient action of virus vectors – nematodes a high soil infestation by them. Methods of controlling virus diseases consist of growing and propagation selected health planting material, inspection plants during vegetation for symptoms presence and elimination of affected plants. The losses by nematode transmitted viruses can be reduced by soil fumigation with fumigant nematicides to control nematodes. Diseases problems can be sometimes minimized by employing crop rotations that diminish nematode populations. Virus free stocks of valuable cultivars can be produced by treatment and meristem culture (Murant, 1981).

Subgroup 16SrI-L phytoplasma strains have previously been found in *Primula, Aster* in Germany, *Cynodon* in Tailand [Marcone et al., 2000; Bertaccini, 2007], *Hyacinthus orientalis* [Alminaite et al., 2001], *Gladiolus* sp., *Brassica napus* [Valiūnas, 2003], *Grosheimia macrocephala* [Samuitienė ir kt., 2005], *Avena sativa, Lolium multiflorum* in Lithuania [Urbanavičienė et al., 2007]. However, subgroup 16SrI–L phytoplasma not been reported in the America or in Australia, in spite of considerable research on phytoplasma diseases in these regions, raising the possibility that subgroup 16SrI-L phytoplasma strains were introduced into Europe from Asia, or the reverse.

The present study expands basic data on phytoplasma infections in ornamental plant species in Baltic region. Results from this

study and related work indicate that a wide diversity of group 16SrI phytoplasma strains infect ornamental plants in Lithuania [Alminaite et al., 2001; Samuitienė ir kt., 2005; Samuitienė et al., 2007; Valiunas et al., 2000; Valiūnas, 2003]. It has also become apparent that these phytoplasmas have broad pathogenic potential, since they infect an extensive range of ornamental plant species and families. As the quality and quantity of diverse ornamental plants is adversely affected by the diseases attributed to these phytoplasmas, they could have a significant impact of the growth of the ornamental plant industry in the Baltic region.

CONCLUSIONS

1. The causal agent of *Thalictrum* diseases expressed by leaf malformation, curling, pinpoint light green mosaic, vein clearing and inconspicuous ringspot of that was isolated and identified as *Tomato ringspot nepovirus* by the methods of test-plant inocula-tions and electron microscopy.

2. *Thalictrum* proliferation disease is associated with a phytoplasma belonging to the phytoplasma group 16SrI (aster yellows), subgroup 16SrI–L.

3. This work was the first investigation of viral and phytoplasmal diseases of *Thalictrum* plants and identification of their causal agents. *Thalictrum* sp. is the new host-plant of *Tomato ringspot nepovirus* and subgroup 16Sr I–L of phytoplasmas.

REFERENCES

ALMINAITE A., VALIUNAS D., NAVALINSKIENE M., STANIULIS J., JOMANTIENE R. 2001. *Hyacinthus orientalis* is the host plant for a new phytoplasma exhibiting ribosomal interperon sequence heterogeneity // Biologija, Vol. 4: 37–39.

BERTACCINI A. 2007. Phytoplasmas: diversity, taxonomy, and epidemiology // Frontiers in Bioscence, Vol. 12, No 1: 673–689.

BRUNT A. A., CRABTREE K., DALWITZ M. J., GIBBS A. J., WATSON L. (Editors) 1996. Viruses of Plants. Descriptions and Lists from VIDE Database. Cambridge, p. 1484.

CHEIFETZ A., DOUBLE C., BARNARD L., IMWOLD D. (Editors) 2004. Annuals and perennials. Australia, p. 1008.

DENG S., HIRUKI C. 1991. Genetic relatedness between two non-culturable mycoplasmalike organisms reveales by nucleic acid hybridization and polymerase chain reaction // Phytopathology, Vol. 81: 1475–1479.

GUNDERSEN D. E., LEE I. M. 1996. Ultrasensitive detection of phytoplasmas by nested PCR assays using two universal primers // Phytopathologia Mediterranea, Vol. 35: 144–151.

JOMANTIENE R., DAVIS R. E., DALLY E. L., MAAS J. L. 1998a. The distinct morphology of *Fragaria multiplicata* is due to phytoplasma // Horticultural Science, Vol. 33: 1069–1072.

JOMANTIENE R., DAVIS R. E., MAAS J. L., DALLY E. L., 1998b. Classification of new phytoplasmas associated with diseases of stawberry in Florida, based on analysis of 16 rRNA and ribosomal protein gene operon sequences // International Journal of Systemic Bacteriology, Vol. 48: 269–277.

LEE I. M., GUNDERSEN-RINDAL D. E., DAVIS R. E., BARTOSZYK I. M. 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16rRNA and ribosomal protein gene sequences // International Journal of Systematic Bacteriology, Vol. 48: 1153–1169.

LEE I. M., GUNDERSEN–RINDAL D. E., DAVIS R. E., BOTTNER K. D., MARCONE C., SEEMÜLLER E. 2004. "Candidatus Phytoplasma asteris" a novel phytoplasma taxon associated with aster yellows and related diseases. // International Journal of Systemic and Evolutionary Microbioly, Vol. 54: 1–12.

LOEBENSTEIN G., LAWSON R.H., BRUNT A. A. (eds.) 1995. Virus and virus-like disease of bulb and flower crops. Chichester, p. 876.

MARCONE C., LEE I. M., DAVIS R.E., RAGOZZINO A., SEEMÜLLER E. 2000. Classification of aster yellows – group phytoplasmas base on combined analyses of RNA and *tuf* gene sequences // International Journal of Systemic and Evolutionary Microbiology, Vol. 50: 1703–1713.

MURANT A. F. 1981. Nepoviruses. In: Kurstak E. (Editor) Handbook of Plant Infection. Comparative diagnosis. Amsterdam, New York, p. 943.

NAVALINSKIENĖ M., SAMUITIENĖ M. 2006. Dekoratyvinių augalų virusinės ligos ir jų sukėlėjai Lietuvoje. Kaunas, p. 254.

ROBINSON D. G., EHLERS U., HERKEN R., HERMANN B., MAYER F., SCHÜRMAN F. W. 1987. Methods for Electron Microscopy. An introduction for the Biomedical Sciences. Berlin, p. 190.

SAMUITIENĖ M., NAVALINSKIENĖ M., LOSINSKA R. 2005. 16SrI-L pogrupio fitoplazmos identifikavimas didžiagraižės grosheimijos augaluose // XIII pasaulio lietuvių mokslo ir kūrybos simpoziumas. Tezių rinkinys: 333.

SAMUITIENĖ M., JOMANTIENĖ R., VALIŪNAS D., NAVALINSKIENĖ M., DAVIS R. E. 2007. Phytoplasma strains detected in ornamental plants in Lithuania // Bulletin of Insectology, Vol. 60, No 2: 137–138.

STACE-SMITH R. 1984. Tomato ringspot virus // CMI/AAB. Descriptions of Plant Viruses, No 290: 1–6.

URBANAVIČIENĖ L., JOMANTIENĖ R., VALIŪNAS D., DAVIS R. E. 2007. Molecular identification of 16SrI-A, 16SrI-B, 16SrI-C, and 16SrI-L subgroups of phytoplasmas in gramineous plants in Lithuania // Bulletin of Insectology, Vol. 60, No 2: 127–128.

VALIUNAS D., JOMANTIENE R., DAVIS R. E., SINDARAVICIENE I., ALMINAITE A., STANIULIS J. 2000. Molecular detection and characterization of phytoplasmas infecting vegetables, legumes, and ornamental plants in Lithuania // Transactions of the Estonian Agricultural University, Vol. 209: 220–223.

VALIŪNAS D. 2003. Identification of phytoplasmas in Lithuania and estimation of their biodiversity and molecular evolutionary relationships // Summary of doctoral thesis, Vilnius, p. 35.

VINGIRIUS (*THALICTRUM* SP.) PAŽEIDŽIANČIŲ VIRUSINIŲ IR FITOPLAZMINIŲ LIGŲ SUKĖLĖJŲ IDENTIFIKAVIMAS

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Santrauka

Vingirių (*Thalictrum* L.) augalai, turintys virusinėms ir fitoplazminėms ligoms būdingus požymius, buvo surinkti Lietuvos botanikos soduose, gėlininkystės ūkiuose, Vilniaus miesto ir privačiuose gėlynuose. Virusinės ligos pasireiškė lapų deformacija, garbanotumu, taškine žalsva mozaika ant lapų, gyslų pašviesėjimu, neryškiu žiediškuoju dėmėtumu. Virusinių ligų sukėlėjas, pomidorų žiediškosios dėmėtligės virusas (*Tomato ringspot nepovirus,* ToRSV), buvo išskirtas ir identifikuotas augalų indikatorių ir elektroninės mikroskopijos metodais. Fitoplazminės ligos požymiai pasireiškė augalų žemaūgiškumu, ūglių proliferacija, žiedų susmulkėjimu. Polimerazinėje grandininėje reakcijoje, atliktoje naudojant universalius fitoplazmų pradmenis P1/P7, R16F2n/R16R2 ir iš sergančių augalų išskirtą matricinę DNR, buvo pagausintas 16S rRNR geno sekos fragmentas, patvirtinantis augalų fitoplazminę infekciją. 1,2 kbp dydžio rDNR produktas buvo sukarpytas su 9 restrikcijos endonukleazėmis. Restrikcijos fragmentų ilgio polimorfizmo (RFLP) analizė parodė, kad augalai buvo užsikrėtę 16SrI (astrų geltos) grupės I–L pogrupio fitoplazma. Tai yra pirmas darbas apie vingirių virusinių ir fitoplazminių ligų sukėlėjų tyrimus. Vingiris yra naujas ToRSV ir 16SrI–L pogrupio fitoplazmos augalas šeimininkas.