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IDENTIFICATION OF AGENTS OF VIRAL AND PHYTOPLASMAL DISEASES AFFECTING GLADIOLI (*GLADIOLUS* L.)

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Gladiolus plants exhibiting symptoms characteristic of viral and phytoplasmal were collected at the Botanical Gardens of Vilnius University and the Experimental Station of Field Floriculture. Tobacco necrosis and tobacco ringspot viruses were isolated from diseased plants exhibiting symptoms characterized by chlorotic and necrotic spots and stripes on leaves. The viruses were identified by the methods of test-plants, electron microscopy and DAS–ELISA. Symptoms of general plant yellowing and stunting, proliferation of shoots were associated with infection by a phytoplasma. Phytoplasmal 16S rRNA gene sequences were amplified in polymerase chain reactions (PCRs) primed by phytoplasma universal primer pairs P1/P7 and R16F2n/R16R2. Restriction fragment length polymorphism (RFLP) analysis of the 1.2 kbp rDNA product, subjected to single enzyme digestions with ten restriction endonucleases, revealed that the *Gladiolus* plants were infected by a phytoplasma (named *Gladiolus* proliferation, GlPr) belonging to group 16SrI (aster yellows phytoplasma group) subgroup I–A (tomato big bud phytoplasma subgroup).

Key words: *Gladiolus*, tobacco necrosis virus, tobacco ringspot virus, 16SrI–A phytoplasma

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Introduction

The genus *Gladiolus* L. belongs to the family *Iridaceae* Juss. It includes more than 150 species, most of to Africa and a few originating from the Mediterranean area, Asia and Southern Europe. Cultivars of gladiolus exhibit a great diversity of color, size, shape, flowering time, bulbing and dormancy behavior. This variability arose from a complex of crosses among several diploid and polyploidy botanical species (Stein 1995). Gladiolus is a popular garden plant and commercial flower crop and has been grown in Lithuania for many years. Local breeders have created a great number Lithuanian gladiolus cultivars and hybrids. Due to vegetative propagation and worldwide commercial trade, gladioli are affected by a large number of viruses. Gladiolus is a highly susceptible crop that may suffer considerable losses if control measures are not taken. Two main viruses, cucumber mosaic virus (CMV) and bean yellow mosaic virus (BYMV) are the most prevalent in commercial gladioli. CMV presents major production problems (Stein 1995). Other viruses reported from different countries are: tomato ringspot virus from the USA (Bozarth & Corbett 1957), tomato black ring virus from Poland (Kamińska 1978), tomato spotted wilt virus from Australia (Lee et al. 1979), tobacco mosaic, tomato ringspot and tobacco ringspot viruses from Japan (Fukumoto et al. 1982), arabis mosaic, strawberry latent ringspot, tobacco ringspot and tobacco streak viruses from Italy (Bellardi & Marani 1982; Bellardi

et al. 1986; 1987), tobacco rattle virus (TRV) from Holland, Israel, Egypt, Poland (Stein 1995). CMV, BYMV and TRV have been isolated and identified from commercial and Lithuanian cultivars of gladioli in Lithuania (Navalinskienė & Samuitienė 2001; 2004).

Aster yellows disease of gladiolus was first described in the USA (Magie et al. 1952). Later diseases of gladiolus associated with phytoplasmas have been reported from Italy (Bertacini et al. 1994), Poland (Kamińska et al. 1999), Belgium and France (Marcone et al. 2000). Using the methods of molecular biology it was established that the causal agent was phytoplasma of the aster yellows phytoplasma group. During surveys of ornamental plants in prior decades, gladiolus plants showing symptoms of stunting, general yellowing, and flower virescence were frequently observed in different locations in Lithuania (Makutėnaitė–Navalinskienė 1981). Phytoplasmas were first detected by studying the ultra-thin sections with electron microscopy (EM) (Staniulis 1988). EM was used to establish the ethiology of disease, but not to identify the causal agents or estimate the biodiversity of phytoplasmas. Recently molecular biology methods have been introduced to detect, identify and differentiate phytoplasmas associated with diseases of plants in Lithuania. Phytoplasmas infecting cereals, vegetables, legumes, forest trees and ornamental plants have been identified and classified on the basis of 16s rRNA gene sequence analyses (Jomantiene et al. 2000; Valiūnas 2003; Samuitienė et al. 2007; Urbanavičienė et al. 2007).

The objective of the present study was to determine the possible association of both virus and phytoplasma with diseases in gladiolus and to increase knowledge on the biodiversity of these two classes of disease agents in Lithuania.

Materials and methods

The plant material was collected in the Botanical Gardens of Vilnius University and the Experimental Station of Field Floriculture. The experimental work was carried out at the Plant Virus Laboratory of the Institute of Botany. Viruses have been identified by the methods of test–plants (Kassanis 1970; Stace–Smith 1985; Brunt et al. 1996; Dijkstra & de Jager 1998), electron microscopy (EM) (Robinson et al. 1987; Dijkstra & de Jager 1998) and DAS–ELISA (Clark & Adams 1977).

The test–plants were inoculated in early stages of growth by mechanical sap transmission, applying carborundum as an abrasive. The inocula were prepared by homogenizing infected plant tissue with 0.1 M phosphate buffer (pH 7.2) containing 1 % polyvinylpyrrolidon, 1 % nicotine acid, 0.2 % sodium diethyldithiocarbamate trihydrate, 1 % polyethylenglycol 6000 as virus–stabilizing additives. The test-plants used for virus identification are listed in Table 1.

Virus particles were examined in leaf dip preparations negatively stained with 3 % uranyl acetate electron microscopically using a transmission electron microscope JEM–100S, at the instrumental magnification of 25000 (Robinson et al. 1987; Dijkstra & de Jager 1998).

DAS–ELISA was carried out using commercial kit from DSMZ Plant Virus Collection, Germany, according to standard procedure. IgGs and alkaline phosphatase conjugates specific to tobacco necrosis and tobacco ringspot viruses were used at a dilution of 1/1000. 50 mg of samples were triturated in 1 ml of sample buffer. 0.1 % solution of p–nitrophenylphosphate was used as substrate. Optical density (OD) of reactions was measured photometrically at 405 nm after 90 min incubation with substrate (Labsystems Multiskan RC).

Phytoplasma was detected in polymerase chain reactions (PCRs). Nucleic acid was extracted for use as template in PCR from the frozen tissue using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) in accordance with the manufacturer's instructions. In a nested PCR, phytoplasmal rDNA was initially primed by primer pair P1/P7 (Deng & Hiruki 1991). The amplified

DNA product was diluted 1 : 50 with sterile water and used as template in the second (nested) PCR primed by primer pair R16F2n/R16R2 (Gundersen & Lee 1996). All PCRs were carried out for 35 cycles using the following parameters: 1 min (3 min for the first cycle) denaturation at 94 ^{0} C, annealing for 2 min at 55 ^{0} C, and primer extension for 3 min (10 min in final cycle) at 72 ^{0} C in Perkin Elmer PCR buffer, 0.25 mM dNTP, 0.4 μM of each primer, and 1 unit of recombinant *Taq* polymerase per 50μl of reaction mixture. Resulting PCR products were analyzed employing electrophoresis through 1 % agarose gel, stained with ethidium bromide, and DNA bands were visualized using an UV transilluminator. DNA fragment size standard was PhiX174 RFI DNA *Hae*III digest (MBI Fermentas). Products from nested PCR primed by R16F2n/R16R2 were analyzed by single enzyme digestion, according to manufacturer's instruction with 10 different restriction endonucleases: *Alu*I, *Mse*I, *Rsa*I, *Hpa*II, *Hae*III, *Hinf*I, *Sau3A*I, *Hha*I, *Kpn*I, and *Taq*I (MBI Fermentas). The restriction fragment length polymorphism (RFLP) of digested DNA was analyzed by electrophoresis through 5 % polyacrilamide gel, stained with ethidium bromide, and visualized using an UV transilluminator. RFLP patterns were compared with previously published (Jomantiene et al. 1998a, b; Lee et al. 1998; 2004; Marcone et al. 2000).

Results and discussion

As was stated in the introduction, previously three viruses have been isolated from gladioli and identified as BYMV, CMV and TRV (Navalinskienė & Samuitienė 2001; 2004). In this work we present data of investigations which revealed two more viruses affecting gladioli in our country: tobacco necrosis virus and tobacco ringspot virus.

Tobacco necrosis virus (TNV). The leaves of infected gladioli developed light green dotted stripes and spots, and brown necrotic pinpoint spots appeared in places. Later in season the symptoms became severe, with the necrotic spots blended and leaves getting dry (Fig. 1). TNV was identified on the basis of test–plant reaction data (table 1), morphology of particles and positive reaction in DAS–ELISA. The virus induced specific, mostly local necrotic spots on test-plants (Figs. 2, 3). EM revealed isometric particles 26 nm in diameter (Fig. 4).

TNV is a type member of *Necrovirus* genus. This virus has a wide natural host range including many species of ornamentals. *Gladiolus* as host plant of TNV has not been described previously. The virus is transmitted naturally externally on zoospores of the fungus *Olpidium brassicae* and experimentally by mechanical inoculation usually causing local necrotic lesions and rarely infecting test–plants systemically. It is not transmitted by seeds and pollen. Virus particles are often found *in vivo* as crystal–like aggregates (Kassanis 1970; Brunt et al. 1996).

Tobacco ringspot virus (TRSV). The leaves of infected gladioli showed chlorotic diffuse spots. The leaves of some varieties showed light grey spots. Symptoms could temporarily disappear during hot periods. TRSV was identified on the basis of test–plant reaction data, morphology of particles (isometric particles 28 nm in diameter) and positive reaction in DAS–ELISA. The virus induced specific local and systemic reactions in inoculated test-plants (Table 1; Figs. 5, 6).

TRSV is a member of *Nepovirus* genus. The virus is transmitted by nematodes, and also non-specifically by insects and mites, transmitted by mechanical inoculation; by seeds and pollens; not transmitted by contact between plants. Virions isometric, not enveloped, 25–29 nm in diameter (Stace-Smith 1985; Brunt et al. 1996). Natural spread of the virus in gladioli has been reported in North America there the vector *Xiphinema americana* occurs and was probably disseminated to other countries in infected planting material (Stein 1995). TRSV was also reported from Australia (Randles & Francki 1965, Japan (Fukumoto et al. 1982), Iran (Kaniran & Izadpanah 1982), Italy (Bellardi & Marani 1985).

Phytoplasma subgroup 16SrI-A. The diseased gladioli exhibited symptoms of general yellowing

Table 1. Test-plants reaction to inoculation with viruses isolated from gladiolus

Test-plant	Tobacco necrosis virus	Tobacco ringspot virus (TRSV)
Amaranthus caudatus L.	L:NRi	
A. paniculatus L.	L:NRi	
Atriplex hortensis L.	L: GNLL	
Celosia argentea L.	L: ReNRi	
Chenopodium amaranticolor Coste et Reyn	L: BrGrRi,NSp	L:ClLL
C. ambrosioides L.	L:NLL	
C. hybridum L.	L:NLL	
C. quinoa Willd.	L:NRiSp	L: ClSp,N
Cucumis sativus L.	L:NLL	L: YSp,N; S: Mo,DisT
Datura stramonium L.		L: DifRiSp; S: Cl,NV
Gomphrena globosa L.	L:NSp	L: SmGLL,N
Nicandra physalodes (L.) Gaertn.		L: NV,DBrRi; S:ClV,DBrN,DNSp
Nicotiana debney Domin.		L: DifClSp; S: ClMo,NSp
N. glutinosa L.	L; BlNRiSp	L: DNRi; S: RiSp,NV
N. rustica L.	L:NSp	
N. tabacum L. ‚Samsun‘	L:NRiSp	L: NRiSp; S: NV, RiSp
Petunia hybrida Vilm.	L: BlNSp	L: DifClSp
Phaseolus vulgaris L.	L: BrNRi	L: NSp; S: RiSp
Tetragonia expansa Murr.	L: GNLL	L: Sp,ClRiSp,N

Abbreviations: Bl – black; Br – brown; C – clearing; Cl – chlorotic, chlorosis; D – dark; Dot – dots; Dif – diffuse; Dis – distortion; Et – etching; Fl – flowers; G – grey; Gr – green; L – local reaction; Le – leaves; Li – light; LL – local lesions; Mo – mottling; N – necrotic, necrosis Pat – pattern; Re- red; Ri –rings; S – systemic reaction; Sm – small; Sp – spots; Str – streaks; Stu – stunting; T – leaf tip; V – vein; Y – yellow.

and stunting, proliferation of shoots (Fig. 7). 16S rDNA was amplified in nested PCR primed by primer R16F2n/R16R2, confirming that the plants were infected by phytoplasma (data not shown). The phytoplasma was termed gladiolus proliferation phytoplasma (GlPr). The RFLP patterns of GlPr phytoplasma 16S rDNA were similar of 16S rDNA from phytoplasmas classified in group 16SrI (group I, aster yellows phytoplasma group) (Fig. 8). The *Mse*I RFLP pattern distinguished GlPr phytoplasma from other I group phytoplasmas, except for tomato big bud (BB) phytoplasma (member of subgroup I-A) and aster yellows (AY1) I-B phytoplasma subgroup.

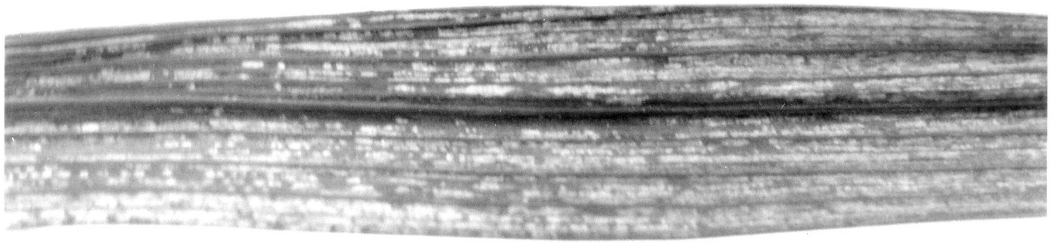

Fig. 1. Symptoms of tobacco necrosis virus in leaf of naturally infected *Gladiolus* plant

Fig. 2. Necrotic local lesions induced by tobacco necrosis virus in inoculated leaves of *Atriplex hortensis*

Fig. 3. Necrotic local lesions induced by tobacco necrosis virus in inoculated leaves of *Tetragonia expansa*

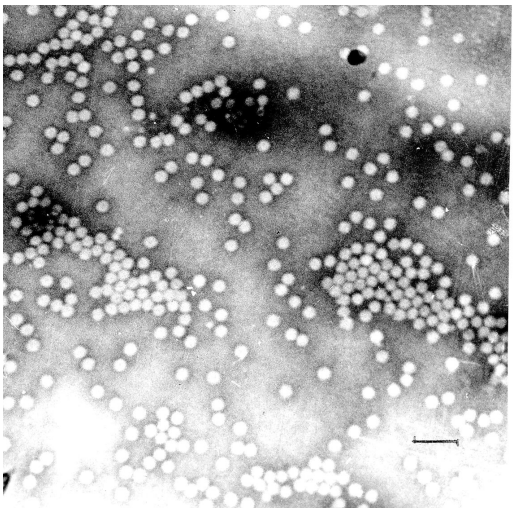

Fig. 4. Electron mikrograph of particles of tobacco necrosis virus, negatively stained with uranyl acetate. Bar represents 100 nm

Fig. 5. Necrotic local lesions induced by tobacco ringspot virus in inoculated leaves of *Chenopodium quinoa*

Fig. 6. Necrotic ringspots induced by tobacco ringspot virus in inoculated leaves of *Nicotiana tabacum* cv. Samsun

Fig. 7. Symptoms of phytoplasmal disease in *Gladiolus* plant

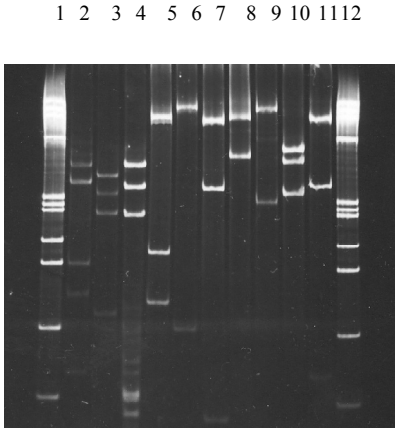

Fig. 8. RFLP analysis of 16S rDNA amplified in nested PCR primed by primer pair R16F2n/R16R2 from infected gladioli. Lanes 1, 12 – PhiX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. 2- *Alu*I, 3 - *Mse*I, 4 – *Rsa*I, 5 – *Hpa*II, 6 – *Hae*III, 7 – *Hinf*I, 8 – *Sau3A*I, 9 –*Hha*I, 10 – *Kpn*I, 11 – *Taq*I.

The *Hha*I RFLP pattern distinguished GlPr from AY1 phytoplasma. Basing on these analyses, the GlPr phytoplasma was tentatively classified in 16SrI group (aster yellows phytoplasma group) subgroup I–A. (tomato big bud phytoplasma subgroup). Subgroup I–A has been reported in diseased plant species: *Medicago sativa*, *Avena sativa* (Jomantiene et al. 2000; 2002), *Aconitum napellus*, *Limonium sinuatum*, *Daucus carota* (Valiūnas, 2003), *Bellis perennis, Callistephus chinensis, Dicentra formosa, Gaillardia pulchella, Geum coccineum, Helenium autumnale, Heleborus lividus, Hyacinthus orientalis, Lychnis chalcedonica, Pachysandra terminalis* (Samuitienė et al. 2007). Identification of subgroup I–A phytoplasmal infection in gladioli together with previous findings of subgroup I–A in other plant species emphasizes the broad host range of subgroup I–A phytoplasma strains in Lithuania.

Conclusions

1. Tobacco necrosis and tobacco ringspot viruses were isolated from diseased *Gladiolus* plants exhibiting symptoms characteristic for viral diseases and identified by the methods of test-plants, electron microscopy, and DAS–ELISA. Previously it was established that gladioli grown in Lithuania were affected by bean yellow mosaic, cucumber mosaic and tobacco rattle viruses.

2. The agent of phytoplasmal disease in *Gladiolus* expressed by symptoms of general plant yellowing and stunting, proliferation of shoots was identified as phytoplasma belonging to group 16SrI (aster yellows phytoplasma group) subgroup I–A (tomato big bud phytoplasma subgroup).

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