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Identification of agents associated with viral and phytoplasmal diseases in common hyacinth (*Hyacinthus orientalis* L.)

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Plants of common hyacinth (*Hyacinthus orientalis* L.) exhibiting symptoms characteristic of viral and phytoplasmal diseases were collected in Botanical Garden of Vilnius University and Experimental Station of Field Floriculture. The causal agents of viral diseases were isolated and identified as *Hyacinth mosaic potyvirus* (HyMV), *Tobacco rattle tobravirus* (TRV), *Arabis mosaic nepovirus* (ArMV) and *Tobacco necrosis necrovirus* (TNV). The viruses were identified by the methods of test–plant, electron microscopy, DAS–ELISA. The causal agent of phytoplasmal disease was detected in polymerase chain reactions (PCRs) containing the phytoplasma universal primer pair R16F2n/R16R2 and template DNA extracted from diseased plants. The 1.2 kbp 16S rDNA product was subjected to single enzyme digestions with 10 different restriction endonucleases. Restriction fragment length polymorphism (RFLP) analysis revealed that hyacinths were infected by a phytoplasma belonging to the 16SrI group (aster yellows phytoplasma group) subgroup I–A (tomato big bud phytoplasma subgroup).

Key words: hyacints, identification, virus, phytoplasma, PCR, RFLP

INTRODUCTION

The genus *Hyacinthus* L., consisting of three species, belongs to the family of the *Hyacinthaceae* Batsch ex Borkh. It is indigenous to south-eastern Europe and Asia Minor. Only the species *H. orientalis* is of horticultural importance.

The main viruses affecting hyacints are *Hyacinth mosaic potyvirus* (HyMV) and *Tobacco rattle tobravirus* (TRV) [1]. HyMV is not transmissible to test plants in leaf extracts because of inhibitory substances [2]. HyMV is serologically related to *Ornithogalum mosaic potyvirus*, *Freesia mosaic potyvirus, Iris severe mosaic potyvirus,* and *Scilla mosaic potyvirus* [1, 3]. TRV has a very large natural host range, including many ornamental species [4]. Viruses occasionally detected in hyacints are *Arabis mosaic nepovirus* (ArMV), *Cucumber mosaic cucumovirus* (CMV), *Tobacco ringspot nepovirus* (TRSV) [1, 5].

A yellows disease in hyacinths, caused by phytoplasma, has been described in Netherlands in 1974 [6]. Inability to isolate and cultivate phytoplasmas in artificial mediam hindered the progress of their investigation, and for a long time these pathogens were investigated only basing on biological properties (symptoms on host plant, vector specificity) and by electron microscopy. In the literature, data are beginning to mount from molecular investigations of phytoplasmas. On the basis of the analysis of 16S rDNA, phytoplasmas were identified and classified into at least 15 groups and 40 subgroups [7–9]. Investigations of phytoplasmas in Lithuania revealed natural phytoplasma infections in ornamental, legume, vegetable, cereal crops, orchard and forest tree plants. Results from analysis of rRNA operon sequences (rDNA) revealed a broad biodiversity among these phytoplasmas. Phylogenetic analyses indicated that phytoplasmas affecting plants in Lithuania include some that have been previously known and others that represent the new phytoplasma lineages [10–20]. Most symptomatic plants, including ornamentals grown in Lithuania, were infected by phytoplasmas belonging to 16Sr-I (aster yellows, AY) group. This finding indicates that insect vectors spread in our country carry phytoplasmas of the 16SrI group. The AY phytoplasma group comprises AY phytoplasma and numerous related phytoplasmas worldwide, representing the most diverse and widespread phytoplasma group [8].

MATERIALS AND METHODS

The plant material was collected in Botanical Garden of Vilnius University and 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-plant [3, 4, 21, 22], electron microscopy (EM) [23], DAS–ELISA [24]. The inocula for mechanical inoculation were prepared by homogenizing 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 following test-plants were used: *Amaranthus caudatus* L., *A. paniculatus* L., *Atriplex hortensis* L., *Celosia argentea* L., *Chenopodium amaranticolor* Coste et Reyn, *C. ambrosioides* L., *C. foetidum* L., *C. hybridum* L., *C. quinoa* Willd., *C. urbicum* L., *Cucumis sativus* L., *Galinsoga parviflora* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Nicandra physalodes* (L.) Gaertn., *Nicotiana glutinosa* L., *N. rustica* L., *N. tabacum* L., *Petunia hybrida* Vilm., *Phaseolus vulgaris* L., *Tetragonia expansa* Murr., *Vicia faba* L.

Phytoplasma was detected in polymerase chain reactions (PCRs). Nucleic acid to be use of as a template in PCR was extracted from a frozen tissue using the Genomic DNA Purification Kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instruction. In a nested PCR, phytoplasmal rDNA was initially primed by the primer pair P1/P7 [25]. The amplified DNA product was diluted 1 : 50 with sterile water and used as template in the second (nested) PCR primed by the primer pair R16F2n/R16R2 [26]. All PCRs were carried out for 35 cycles using the following parameters: 1 min (3 min for the first cycle) denaturation at 94 °C, annealing for 2 min at 55 °C, and primer extension for 3 min (10 min in final cycle) at 72 °C in Perkin Elmer PCR buffer, 0.25 mM dNTP, 0.4 μM of each primer, and 1 unit of recombinant *Taq* polymerase per 50 μl of the reaction mixture. The resulting PCR products were analysed employing electrophoresis through 1% agarose gel stained with ethidium bromide, and DNA bands were visualized using an UV transilluminator. The DNA fragment size standard was PhiX174 RFI DNA *Hae*III digest (Fermentas).

Products from nested PCR primed by R16F2n/R16R2 were analysed 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 (Fermentas). The restriction fragment length polymorphism (RFLP) of digested DNA was analysed by electrophoresis through 5% polyacrilamide gel stained with ethidium bromide and visualized using an UV transilluminator. RFLP patterns were compared with previously published ones [7–9, 27, 28].

RESULTS AND DISCUSSION

***Hyacinth mosaic potyvirus* (HyMV)**. Infected plants show a mottle on leaves, mainly on the basal parts, ranging in color from pale green to bright yellow. The mottle is stripe-like or consists of many spots and ring patterns. Flower stocks of some cultivars show stripe and ring patterns, occasionally flower breaking occurs. HyMV causes the diminishing of flowers and a reduction in bulb yield. The vegetation period of infected plants one or two weeks shorter. HyMV was detected with other viruses affecting hyacinths (mixed viral infection) (Fig. 1 A).

In agreement with reports in literature [1], our attempts to transmit HyMV to test-plants by mechanical inoculation were also unsuccessful, HyMV was identified based on the morphology of particles established by EM. Particles are filamentous, flexuous, 740–750 nm in length (Fig. 2 A).

HyMV is widespread in hyacinths. The other natural hosts are *Muscari* sp., belonging also to family *Hyacinthaceae*. The virus is a member of the genus *Potyvirus.* It is transmitted in a non-persistent manner by the aphids *Myzus persicae, Macrosiphum solanifolii* and is not transmitted by seed and pollen. All descendants after vegetative propagation from a diseased bulb are also HyMV-infected [1, 3].

***Tobacco rattle tobravirus* (TRV)**. Leaves and stalks show pale green to yellow spots and stripes. These symptoms may turn to brown or grey necrotic stripes later in season. In some cases, leaves tear and bend along stripes. Transparent and in the later stage yellow or brown spots become visible on the bulb scales. Necrosis typical of TRV infection can be seen in cross-sections of infected bulbs.

Fig. 1. Symptoms on naturally infected hyacinths: A – induced by mixed viral infection B – caused by phytoplasma

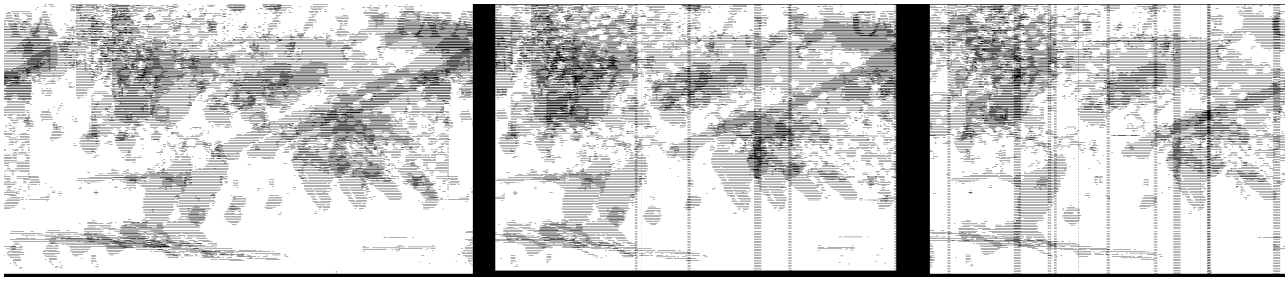

Fig.2. Particles of viruses: A – HyMV B – TRV C – TNV. Bars represent 100 nm

TRV was identified by the methods of test-plants and EM. Data of test-plant reactions are presented in Table. The most specific necrotic local lesions were expressed on *Gomphrena globosa*, *Nicotiana tabacum* (Fig. 3). EM investigation of negatively stained dip preparations prepared from naturally infected hyacinths and from inoculated test-plants revealed rod-shaped particles of two modal lengths, 55–115 nm (short particles) and 200 nm (long particles). Such particle morphology is characteristic of TRV (Fig. 2 B). TRV has a very wide natural host range, including several flowerbulb crops. The virus particles are detected in the cytoplasm of hosts. They are transmitted by *Trichodorus* and *Paratrichodorus* nematodes. The virus is also seed-borne in some host species. [3, 4].

***Arabis mosaic nepovirus* (ArMV).** Leaves of infected plants show chlorotic or pale brown necrotic stripes and spots of various shape. Leaves are distorted.

ArMV was identified on the base of test-plant reaction data (Table), particle morphology and positive reaction in DAS–ELISA. The test-plants showed local and systemic reactions (Fig. 4). EM revealed isometric particles 25–27 nm in diameter.

ArMV has a wide natural host range, including many ornamental species. Virus particles occur in the cytoplasm, tubules, and in plasmodesmata. Infected cells also contain cytoplasmic inclusion bodies. ArMV spreads due to vegetative plant propagation and is transmitted by nematodes. The principal natural vector is *Xiphinema diversicaudatum*. The virus is reported to be seed-borne in 20 species of 14 families [3, 21].

Table. **Test-plant reactions to inoculation of viruses isolated from common hyacinth**

Test-plant	*Tobacco rattle tobravirus* (TRV)	*Arabis mosaic nepovirus* (ArMV)	*Tobacco necrosis necrovirus* (TNV)
Amaranthus caudatus		L: LLN;S: Cl and NSp,LeDis	L: NRi
A. paniculatus	L:BrLL		
Atriplex hortensis	L:LL	L:LL; S: NDot,VC, LeDis, TDis	
Celosia argentea		L:LL; S: LeMo, LeDis	
Chenopodium amaranticolor	L:NLL	L:LYDot; S: ClMo with N, LeDis	L: BrGrRi,NSp
C. ambrosioides	L:NLL	L: SmBrLL; S: LeMo, TDis	
C. foetidum	L:LL		
C. hybridum		L: LL; S: LeNDot, LeDis	
C. quinoa	L:Cl and NLL	L: ClLL; S: YDot, LeDis	L: NRiSp
C. urbicum	L:LL		
Cucumis sativus	L:Cl or NLL	L: ClSp; S: LeMo, TDis	L:NLL
Galinsoga parviflora	L:NStr,Ri		
Gomphrena globosa	L:NSp		L: NSp
Lycopersicon esculentum		S: LiGrMo	
Nicandra physalodes	L:NLL		L: ClNSp,NEt
Nicotiana glutinosa	L:GNRi;S:NSp,NRi,Stu		L: BlNRiSp
N. rustica		L: GRiSp; S: YSp, NRi	
N. tabacum	L:NSp;S:NRiPat,N, Dis	L: SmNRi; S: Cl,NRi	L: RiSp
Petunia hybrida			L: BlNSp
Phaseolus vulgaris		L: ClSp,NDot; S: MrbPat, NFl	L: BrNRi
Tetragonia expansa			L:NSp
Vicia faba			L: NLL

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

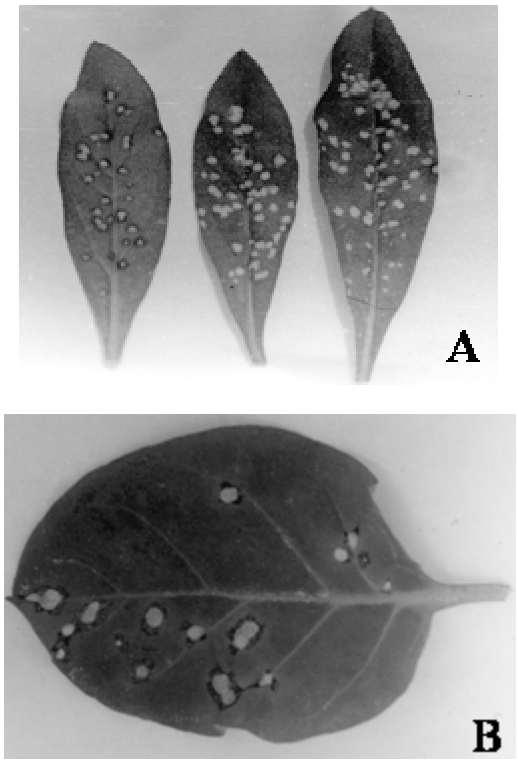

Fig. 3. Local necrotic lesions induced by TRV in test plants: A – *Gomphrena globosa* B – *Nicotiana tabacum*

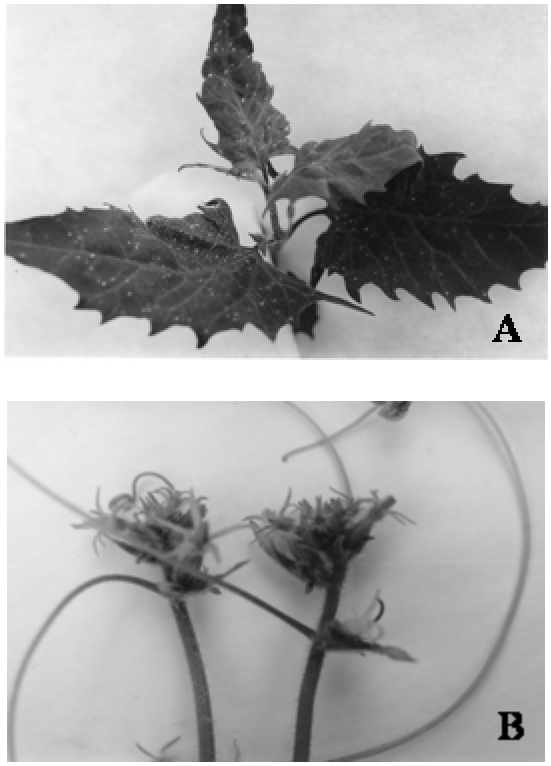

Fig. 4. Local and systemic reaction induced by ArMV in test plants: A – *Atriplex hortensis,* B – *Cucumis sativus*

Tobacco necrosis necrovirus **(TNV).** Leaves of infected hyacinths develop pale brown stripes and spots which later become necrotic. TNV infection causes distortion, narrowing of leaves. Severely infected plants do not flower.

TNV was identified on the basis of test-plant reaction data (Table), particle morphology and positive reaction in DAS-ELISA. The virus induced specific, mostly local, necrotic spots on test-plants. EM revealed isometric particles 26 nm in diameter (Fig. 2 C).

TNV is a type member of genus *Necrovirus*. Virus has a wide natural host range including many species of ornamentals. *H. orientalis* as a host plant of TNV has not yet been described. The virus is transmitted naturally externally on zoospores of the chytrid fungus *Olpidium brassicae* and experimentally by mechanical inoculation, usually causing local necrotic lesions and rarely infecting test-plants systemically. It is not transmitted by seed and pollen. Virus particles are often found *in vivo* as crystal-like aggregates [3, 22].

Phytoplasma subgroup 16SrI-A. The diseased common hyacinth 'Orange Boven' plants exhibited symptoms of general yellowing and stunting, virescence of flowers (Fig. 1 B). 16S rDNA was amplified in nested PCR primed by the primer R16F2n/R16R2, confirming that the plants were infected by a phytoplasma (data not shown). The phytoplasma was termed hyacinth virescence phytoplasma (HyVir).

The RFLP patterns of HyVir phytoplasma 16S rDNA were similar of 16S rDNA from phytoplasmas classified in group 16SrI (group I, aster yellows phytoplasma group)

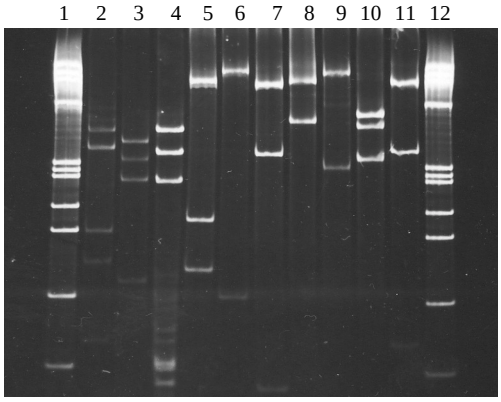

Fig. 5. RFLP analysis of 16S rDNA amplified in nested PCR primed by primer pair R16F2n/R16R2 from infected hyacinths. Lanes 1, 12 – PhiX174 RFI DNA *Hae*III digest, fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72. 2- *Alu*I, 3 - *Mse*I, 4 – *Rsa*I, 5 – *Hpa*II,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

(Fig. 5). The *Mse*I RFLP pattern distinguished the HyVir phytoplasma from other I group phytoplasmas, except for the tomato big bud (BB) (member of subgroup I-A) and aster yellows (AY1) phytoplasmas (member of subgroup I-B). The *Hha*I RFLP pattern distinguished HyVir from AY1 phytoplasma. Based on these analyses, the HyaVir phytoplasma was tentatively classified in subgroup I–A. The previous investigation of phytoplasmas

in hyacinths revealed a new member of 16SrI group whose genome exhibited a ribosomal interoperon sequence heterogeneity [11]. Valiūnas [18] reported the 16SrI-M phytoplasma subgroup isolated from hyacinths. Subgroup I–A has been reported in diseased plant species: *Medicago sativa* [12], *Bellis perennis* [16], *Avena sativa* [14], *Aconitum napellus*, *Limonium sinuatum*, *Daucus carota* [18]. Identification of subgroup I–A phytoplasmal infection in hyacinths 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.

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VIRUSINIŲ IR FITOPLAZMINIŲ RYTINIO HIACINTO (*HYACINTHUS ORIENTALIS* L.) LIGŲ SUKĖLĖJŲ IDENTIFIKAVIMAS

Santrauka

Rytinio hiacinto (*Hyacinthus orientalis* L.) su virusinės ir fitoplazminės infekcijos požymiais buvo rasta Vilniaus universiteto Botanikos sode ir Lauko gėlininkystės bandymų stotyje. Augalų indikatoriais, elektroninės mikroskopijos ir DAS–ELISA metodais buvo išskirti ir identifikuoti šie virusinių ligų sukėlėjai: hiacintų mozaikos (*Hyacinth mosaic potyvirus*), tabako garbanotosios dryžligės (*Tobacco rattle tobravirus*), vaistučio mozaikos (*Arabis mosaic nepovirus*) ir tabako nekrozės (*Tobacco necrosis necrovirus*) virusai. Hiacintų fitoplazmozės sukėlėjas buvo identifikuotas polimerazės grandininės reakcijos (PGR) metodu. Fitoplazmos 16S rRNR geno sekos pagausinimas lizdinės PGR metodu, naudojant universalią pradmenų porą R16F2n/R16R2 ir iš pažeistų hiacintų išskirtą matricinę DNR, patvirtino fitoplazminę infekciją. 1,2 kbp 16S rDNR produktas buvo sukarpytas su 10 skirtingų endonukleazių. Kirpinių ilgio įvairavimo analizė rodo, kad hiacintų fitoplazma priklauso 16SrI (astrų geltos) fitoplazmų grupei ir I-A (padidėjusių pomidorų pumpurų) fitoplazmų pogrupiui.