MICROPROPAGATION OF PHYTOPLASMA-AFFECTED *LIMONIUM SINUATUM* MILL. PLANTS

ELEONORA GABRYSZEWSKA[1], MARIA KAMIŃSKA[1], MAŁGORZATA KORBIN[1], ANNA RUDZIŃSKA-LANGWALD[2]

[1]Research Institute of Pomology and Floriculture
Pomologiczna 18, 96-100 Skierniewice

[2]Agriculture University
Rakowiecka 26/30, 02-528 Warszawa

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ABSTRACT

Healthy and AY-affected plants of *L.sinuatum* have been propagated in vitro for 12 months on the media with and without cytokinins. In the contrary to the healthy plants the phytoplasma affected statice showed abnormal proliferation of the axillary shoots, shortening of the internodes, smaller leaves and severe chlorosis. On the medium without cytokinins, diseased plants proliferated and formed 7.0 axillary shoots per explant but the healthy ones only formed 2.3 shoots; however, the fresh weight of them was similar. On the media with cytokinins, the multiplication rate and fresh weight of healthy shoots greatly increased, but of the diseased plants were on the same laevel or decreased. During tissue culture phytoplasma could be detected in symptomatic plants by PCR as well as electron microscope however, the phytoplasmas showed the symptoms of degeneration.

KEY WORDS: *Limonium*, electron microscopy, micropropagation, PCR, phytoplasma.

INTRODUCTION

Aster yellows (AY) type diseases are caused by several genetically slightly different types of phytoplasmas, formally called mycoplasma-like organisms (MLOs). So far, using polymerase chain reaction (PCR) technic for detection and identification, several AY phytoplasmas have been found in many herbaceous as well as some woody plants producing typical symptoms like witches' broom, flower distortion and virescence (Seemüller et al. 1998).

Knowledge about AY has been limited by our inability to isolate them in pure culture. For scientific purposes, phytoplasmas must be maintained in living hosts. In the nature, they are maintained through a cycle of transmission involving plant hosts and vectors. In the laboratory, *Catharanthus roseus* is a host commonly used for maintenance of collection of several phytoplasmas by grafting. In cases where transmission by grafting has not been achieved, other technics like tissue culture of infected plants can be used to keep the collection of a pathogen.

Micropropagation of phytoplasma-diseased plant material was first introduced by Dosba et al. (1986) and Lansac et al. (1995) to maintain apple proliferation phytoplasma. This procedure was also applied by Cousin et al. (1990), Davies and Clark (1994) and by Bertaccini et al. (1992) to keep the collection of phytoplasma strains in several plant species. On the other hand, tissue culture has been successfully used for the elimination of phytoplasma by tip culture (Sears and Klomparens 1989), stem culture (Dai et al. 1997) or callus culture (Mollers and Sarkar 1989). Micropropagation of annual statice (*Limonium sinuatum* Mill.) plant has already been adapted to produce the plant material throughout the year (Harazy et al. 1985; Gabryszewska and Podwyszyńska 1992).

The objective of the present work was to study the presence of AY related phytoplasmas in micropropagated natural hosts of *L. sinuatum*. Also the influence of cytokinins (BAP, 2iP, kinetin) on the growth and development in vitro of phytoplasma-diseased and healthy plants of *L. sinuatum* was investigated.

MATERIAL AND METHODS

Plant material

Plants for this study were derived from naturally phytoplasma infected *Limonium sinuatum* Mill. collected in the field. The diseased statice showed severe stunting, chlorosis, shoot proliferation and flower malformations. The presence of phytoplasmas in these plants was demonstrated by electron microscopy and PCR examination (Kamińska et al. 1999).

List of abbreviations:
BAP – 6-benzylaminopurine; 2iP – 6-(τ,τ – dimethylallyamino) purine; MS – Murashige and Skoog basal medium.

TABLE 1. The growth and development of diseased and healthy *Limonium sinuatum* Mill. shoots on the media with and without cytokinins.

Plantlets*	Cytokinins mg l^{-1}	Number per plantlets		Fresh weight of shoots per plantlet [g]	Maximum length of leaf [mm]	Maximum width of leaf [mm]
		axillary shoots	leaves			
Healthy	Control**	2.3	9.2	2.0	59.3	12.3
	Kinetin 2	10.0	5.3	2.5	53.0	10.5
	BAP 0.5 + 2iP 2	9.8	6.7	3.8	69.0	9.5
Diseased	Control	7.0	6.3	2.0	55.7	7.5
	Kinetin 2	6.9	6.0	2.3	36.5	8.3
	BAP 0.5 + 2iP 2	4.8	7.6	3.0	46.5	8.7

* Six plantlets were used per each treatment
** Murashige and Skoog (1962) medium without cytokinins

Tissue culture

Plants symptomatic and testing positive as well as asymptomatic and testing negative were used for tissue culture. Axillary buds were isolated in the autumn of 1996. Initial explants were surface-sterilized by soaking for 15 min in solution containing 3% chloramine T, subsequently rinsed with $HgCl_2$ – 0.1% solution and then three times with sterile distilled water. Shoot cultures were initiated on Murashige and Skoog (1962) medium with BAP – 0.5 mg l^{-1}. After several subcultures on this medium, during one year, shoots were cultured on the MS media containing kinetin 2 mg l^{-1} or BAP 0.5 mg l^{-1} + 2iP 4 mg l^{-1} and on the medium without growth regulators. Cultures were incubated in a growth chamber at 20-21°C under 16 h light/8 h dark cycles. After 8 weeks of culturing of healthy and diseased plants, the following parameters were determined: the number of axillary shoots, the number of leaves, the maximum length and width of the leaf blade (mm) and the fresh weight of shoots (g).

Detection of phytoplasma

Micropropagated shoots that developed from diseased and symptomless plants in tissue culture were randomly tested for the presence of phytoplasma by electron microscopy and PCR. The tests were performed after several subcultures of plants on medium with BAP and on shoots kept 8 weeks on medium with and without cytokinins.

Electron microscopy examination. The segments of tissues collected from the base of leaf were cut immediately into small pieces by hand dissection and fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer pH 7.2 for 4h. Postfixation in 1% osmium tetraoxide for 2h at 4°C was applied. The material was dehydrated in concentration gradients of ethanol (10-70%), acetone (0-100%) and embedded in Epon 812. The ultrathin sections on copper grids were stained with uranyl acetate and lead citrate. The grids were examined with JEM 100C electron microscope in Laboratory of Electron Microscopy of the Warsaw Agricultural University.

PCR. DNA extract from diseased and healthy plants was prepared according to Ahrens and Seemüller (1992) and used in PCR. Two universal primers rU3/fU5 (Lorenz et al. 1995), which amplify a fragment of 16S rRNA gene from phytoplasmas, but not from healthy plants were chosen in this reaction. Amplification was carried out in 50 µl volume containing: 5 µl of DNA-template, 0.5 unit of Taq-DNA polymerase (Promega), 125 µM of the four dNTP and 0.5-1.0 µM of each primers. The mixture was subjected to 25 cycles of the following condition in Mastercycler 5330 (Eppendorf): 1 min at 95°C, 1 min at 55°C, 1 min at 72°C (10 minutes in the last cycle). PCR products were analyzed by electrophoresis through 1% agarose gel followed by staining with ethidium bromide. DNA bands were visualized using UV-transilluminator (UV-Biotech).

RESULTS

Healthy and AY-affected plants of *L.sinuatum* have been propagated in vitro for 12 months on the medium with BAP. In the contrary to the healthy plants the phytoplasma affected statice showed abnormal proliferation of the axillary shoots, shortening of the internodes, smaller leaves and severe chlorosis.

Next, shoots of healthy and diseased plants were transferred on the media with kinetin, BAP + 2iP and on the medium without cytokinins. After subculture intervals of 8 weeks, phytoplasma-diseased plants still showed severe symptoms and differed from the healthy annual statice, regarding growth parameters like number of axillary shoots per plant, fresh weight of shoots, length, width and shape of leaves (Fig. 1; Tab. 1). On the medium without cytokinins, phytoplasma-diseased buds proliferated and formed 7.0 axillary shoots per explant but the healthy ones only formed 2.3 shoots; however, the fresh weight of them was similar (Tab. 1). On the media with cytokinins, the multiplication rate and fresh weight of healthy shoots greatly increased, respectively, from 2.3 to 9.8-10.0 and from 2.0 g to 2.5 g-3.8 g. In the contrary to the healthy tissue, the multiplication rate of diseased statice kept on the medium with regulators decreased or was on the level of those plants on control medium. In spite of this, the fresh weight of diseased plants kept in enriched medium was 15-

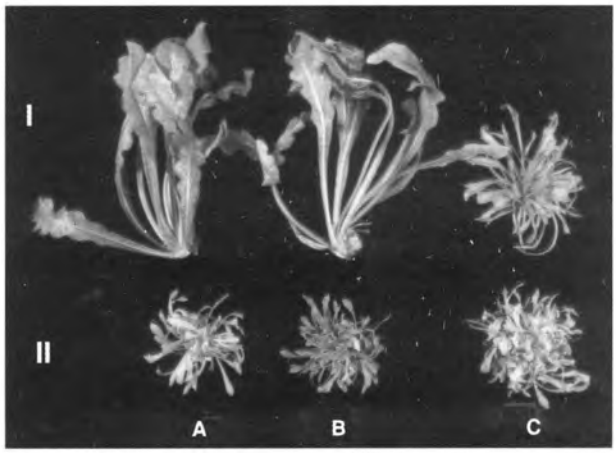
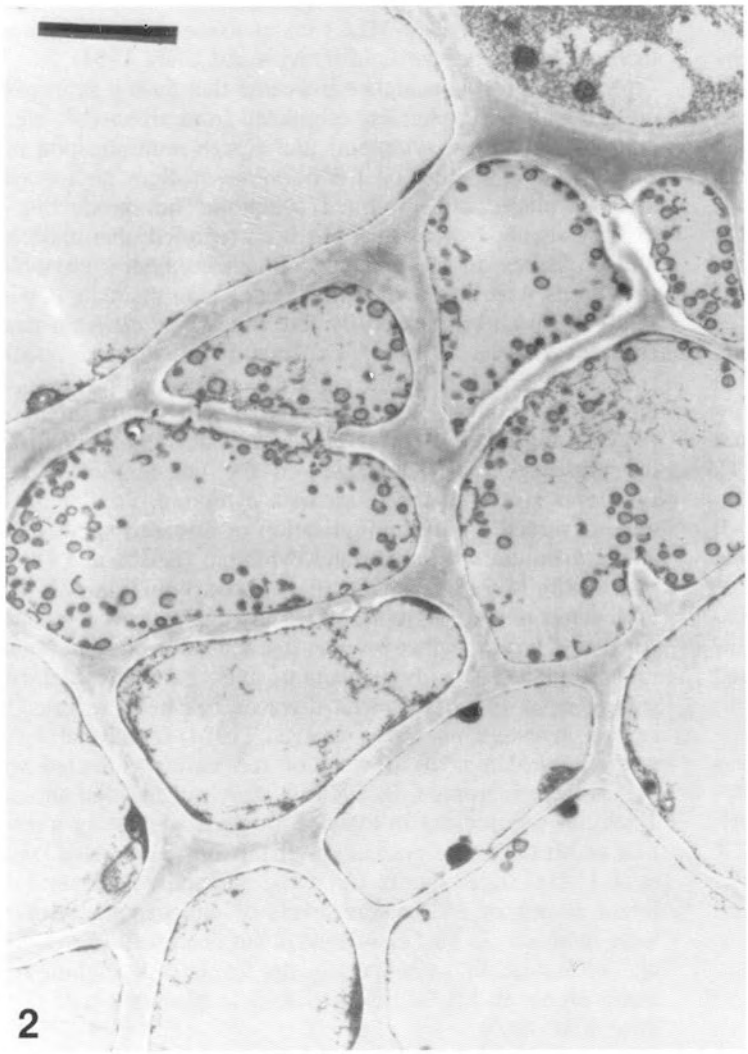
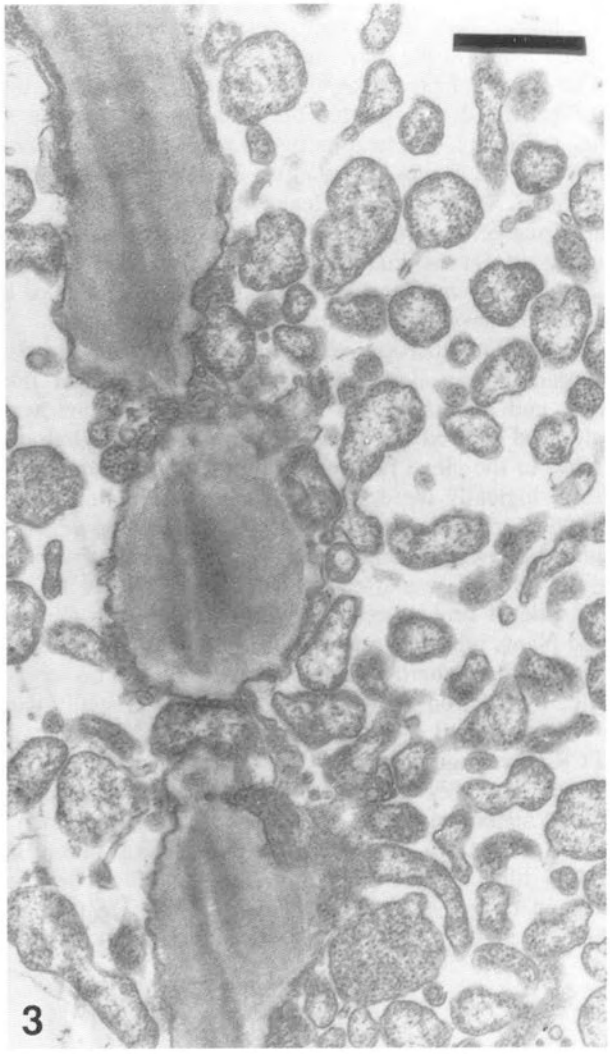
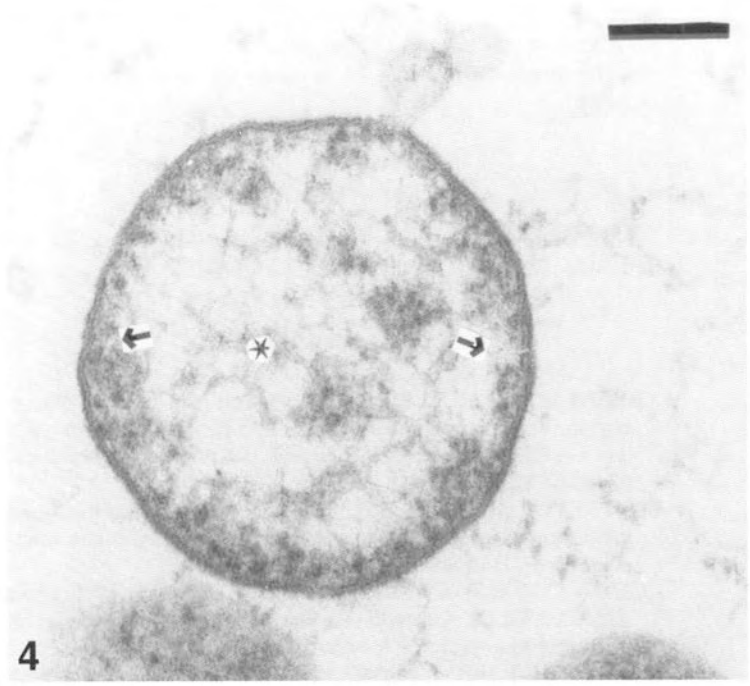
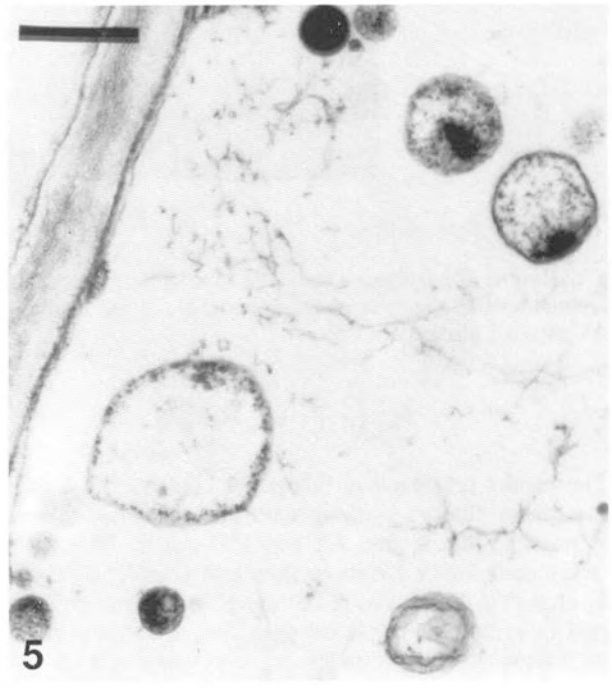


Fig. 1. *Limonium sinuatum* phytoplasma-affected (II) and healthy shoots (I) propagated on MS medium: A – without growth regulators; B – with kinetin 2 mg l^{-1}; C – with BAP 0.5 mg l^{-1} + 2iP 4 mg l^{-1}.









Cross sections of the stem of phytoplasma-affected *L. sinuatum* micropropagated in tissue culture (Figs 2-5):
Fig. 2. Group of sieve tubes with phytoplasmas. Magn. 8 000X, bar represents 2 mm.
Fig. 3. Sieve tube plate with pores and numerous phytoplasmas. Magn. 30 000X, bar represents 500 nm.
Fig. 4. A single phytoplasma with nucleoid in the middle (star) and thin layer of cytoplasm with ribosomes (arrows) surrounded by membrane. Magn. 135 000X, bar represents 100 nm.
Fig. 5. Vesicles in sieve element which may represent degenerated phytoplasmas.

50% higher than in a control medium but smaller than of healthy plants in the same medium.

The leaves of phytoplasma-infected shoots were yellowish, very narrowed and shorter than that of the healthy shoots (Fig. 1, Tab. 1). The healthy plants propagated on control medium had 9.2 leaves per plantlet while on the medium with cytokinins, the number of leaves was decreased to 58-75%. The diseased statice kept on control medium had smaller number of leaves that the healthy ones. The same plants maintained on medium with cytokinins had similar or higher number of leaves than plants on control medium.

Electron microscopy examination. Electron microscopy examination revealed that plantlets derived from diseased plants and maintained 12 months in vitro culture were infected and showed the presence of pleomorphic phytoplasmas limited to the sieve tube elements of the phloem (Figs 2-4). Morphologically these structures were resembling phytoplasmas occurring in field collected statice, however small rounded bodies predominated. The phytoplasmas were visible only in phytoplasma affected symptomatic shoots.

In spite of pronounced symptoms observed in micropropagated plants after 8 weeks culture in medium with cytokinins and without, we hardly found single phytoplasmas in diseased statice by electron microscopy examination. Usually were observed only small rounded cells (Fig. 5).

PCR. Using universal primers rU3/fU5 specific product was detected in tissues derived from originally infected and symptomatic *Limonium* plants, but not from the healthy once. Position of these DNA bands was similar to the position of PCR product obtained for gladiolus sample, which disease was determined as aster yellows phytoplasma (Kamińska et al. 1999a). Length of this product was about 880bp (Fig. 6).

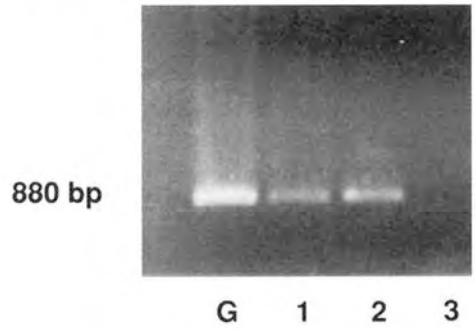


Fig. 6. Results of polymerase chain reaction with primers rU3/fU5 in the samples of *L. sinuatum* plants diseased (1, 2) and healthy (3); G – AY infected gladiolus.

DISCUSSION

The results presented in this paper confirmed the results of some earlier authors working with different plant species that it is possible to maintain AY phytoplasma in the micropropagated annual statice for more than one year. The PCR results indicated that during tissue culture phytoplasma could be detected in symptomatic plants. In addition, the results of electron microscopy examination were consistent with those of PCR and showed the presence of bodies suggesting degeneration process. According to Jacoli (1978) and Mollers and Sarkar (1989) during micropropagation of diseased plants the phytoplasma degenerate or fail to be transmitted to the newly formed cells. In the contrary, the *Pyrus* species visual assessment by UV light microscopy strongly indicated a much higher concentration of MLOs under tissue culture conditions than in plants from the field (Davies and Clark 1994).

Morphological appearance indicated that during stem tissue culture with BAP, plantlets originated from affected *L. sinuatum* showed severe symptoms and a high multiplication rate. During the subculture on the hormone-medium phytoplasma affected plants still exhibited symptoms but production of axillary shoots decreased. It has been reported, that upon culture of tissues on MS medium without hormones, phytoplasmas levels were maintained in *Populus alba* plantlets (Cousin et al. 1990) and increased by five times in *Paulownia* tissue culture (Wang et al. 1994). Explants of pear decline infected pear maintained in tissue culture were symptoms free, although the concentration of MLOs consistently increased (Davies and Clark 1994). Bertaccini et al. (1992) found that the presence of MLOs increased the proliferation rate of *Gladiolus* sp. on the medium with cytokinin. Similar results were observed during multiplication of diseased apple shoots on the medium with auxin and cytokinin (Dosba and Ducroquet 1986). More intensive axillary shoot proliferation in vitro of the mentioned phytoplasma-diseased plants was probably connected with a higher level of cytokinins or other hormones in these plants. The involvement of plant growth regulators in pathogenesis of phytoplasma diseases has been reported by several investigators. Vizarova et al. (1984) found that the levels of cytokinins in flowers of red currant infected with MLOs were increased. In addition, they noticed that an extra cytokinin was present in infected flowers, suggesting a direct role of MLOs in the production of this hormone. Also Davey et al. (1981) stated that in *C. roseus* infected with several different strains of MLOs, the levels of endogenous cytokinin were increased in virescent flowers but decreased in roots and mature leaves. In sweet potato, the levels of cytokinin were much higher in MLOs diseased than in healthy plants (Fang Ting et al. 1997).

In conclusion, AY-related phytoplasmas can be maintained on a natural host of *Limonium sinuatum* propagated in vitro for more than 1 year. Thus, micropropagation of infected plants provides a useful tool to study the host-pathogen relationship.

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MIKROROZMNAŻANIE ROŚLIN *LIMONIUM SINUATUM* MILL. PORAŻONYCH FITOPLAZMĄ

STRESZCZENIE

Rośliny *Limonium sinuatum* Mill., zdrowe i porażone fitoplazmą żółtaczki astra, rozmnażano in vitro przez 12 miesięcy na pożywce bez regulatorów wzrostu oraz z cytokininami. Zatrwian chory, w przeciwieństwie do zdrowego, wykazywał nadmierną proliferację pędów bocznych, skrócenie międzywęźli, zdrobnienie liści i silną chlorozę. Na pożywce bez cytokinin, rośliny porażone wytwarzały średnio 7,0 pędów na eksplantat, natomiast zdrowe tylko 2,3 ale ich świeża masa była podobna. Na pożywce z cytokininami, współczynnik namnażania i świeża masa pędów roślin zdrowych znacznie wzrosły zaś porażonych były na tym samym poziomie lub obniżyły się. W zatrwianie rozmnażanym w kulturach in vitro obecność fitoplazmy można było stwierdzić zarówno techniką PCR, jak i przy pomocy mikroskopu elektronowego. Badania elektronomikroskopowe wykazały też, że fitoplazmy występujące w rurkach sitowych miały symptomy degeneracji.

SŁOWA KLUCZOWE: fitoplazma, *Limonium*, mikrorozmnażanie, mikroskopia elektronowa, PCR.