PHYTOPLASMA DETECTION IN TISSUE CULTURE OF *GLADIOLUS* PLANTS GROWN UNDER VARIOUS CONDITIONS

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ABSTRACT

To test whether phytoplasmas are sensitive to temperature, phytoplasma affected micropropagated gladiolus plants were grown under varying conditions of media content and temperature, in the presence or absence of light. PCR analysis indicated that phytoplasma detection was more successful in plants grown at low temperatures. Plants kept from one to three months at reduced temperature tended to have higher titre of phytoplasma than the plants maintained in stable 20°C high temperature. The best detection was in plants grown on medium containing kinetin+NAA and in the presence of light. In those plants phytoplasmas were detected in direct PCR after one month of culture.

KEY WORDS: *Gladiolus*, micropropagation, phytoplasma, PCR, detection.

INTRODUCTION

Phytoplasmas are nonculturable, wall-less procaryotes of the class Mollicutes, that cause destructive diseases of many plant species (McCoy et al. 1989). Confirmation of phytoplasma etiology of plant disease is based on the restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) techniques. However, the low titre and seasonal fluctuation of phytoplasmas associated with aster yellows type diseases of many plants, make their detection and identification very difficult. Micropropagation of infected plants allows the continuous maintenance of phytoplasmas (Cousin et al. 1990; Bertaccini et al. 1992; Lansac et al. 1995) and provides a valuable source of material for experimental work.

Phytoplasma aster yellows (AY) was readily detectable by electron microscopy and PCR techniques in gladiolus plants grown in field conditions (Kamińska et al. 1999). However, we experienced serious difficulties in phytoplasma detection, using PCR method, in case of micropropagated gladiolus shoots after two years of maintenance in tissue culture at the continuous temperature of 20°C. The present study was undertaken to examine the effect of light and reduced temperature on phytoplasma PCR detection in *Gladiolus* cultured in vitro.

MATERIALS AND METHODS

Isolation of initial explants

Gladiolus X hybridus L. plants naturally infected with aster yellows phytoplasma and showing symptoms (Kamińska et al.

1999) were obtained from a lot of cv. 'Wars'. The main and axillary shoots 3 to 5 cm long were isolated from mother corms and surface sterilized in 2% chloramine T solution for 20 minutes with continuous stirring and then rinsed twice (2 × 10 min) in sterile distilled water. The explants were sterilized again for a few seconds in 0.1% HgCl₂ and rinsed in sterile distilled water. Finally, they were blotted dry on sterile paper and transferred onto the medium. The basic nutrient medium consisted of the Murashige and Skoog (1962) macro- and micro-salts (except Fe) at full strength, NaFeEDTA 40.4 mg · l⁻¹, sucrose 30 g · l⁻¹, BAP 0.2 mg · l⁻¹ and agar 7 g · l⁻¹. The pH of the medium was adjusted to 5.7 before adding the agar. Tissue cultures were maintained in the growth chamber at 20°C under artificial illumination with a 16-h photoperiod. Light was provided by white fluorescent lamps (Philips TLD 36W/15 – 88 μmol · m⁻² · s⁻¹).

Micropropagation of phytoplasma affected gladiolus plants

Micropropagated shoots that developed from initial explants were obtained eight weeks after isolation. Shoots were subcultured to fresh medium at intervals of 6 to 8 weeks for 2 years. The explants were grown on the Murashige and Skoog (1962) basic medium with mixture: BAP 0.5 mg · l⁻¹ + NAA 0.2 mg · l⁻¹; kinetin 0.5 mg · l⁻¹ + NAA 0.2 mg · l⁻¹ or on the medium without growth regulators (control). The growing shoots were maintained in stable temperature of 20°C.

The experiment designed to examine the effect of maintaining of in vitro tissue culture at reduced temperature was performed on diseased gladiolus shoots propagated in vitro for two years. The shoots were transferred on the fresh MS medium without growth regulators and on the medium containing BAP 0.5 mg · l⁻¹ + NAA 0.2 mg · l⁻¹ or kinetin 0.5 mg · l⁻¹ + NAA 0.2 mg · l⁻¹. Next, the shoots were maintained at the reduced temperature of 4°C (dark) or 13°C (dark, light) for 3 months. The culture of shoots growing at the temperature of

List of abbreviations:

BAP – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; kinetin – 6-furfurylaminopurine

20°C (dark, light) for 3 months was used as a control. Light was provided by fluorescent lamps for 16 hours. Photon fluence rates of photosynthetically active radiation were 24–30 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Narva LT 36 W/010 – daylight).

Phytoplasma detection and identification

The presence of phytoplasma in randomly tested micropropagated gladiolus shoots on different media was determined by polymerase chain reaction after 1, 2 and 3 months of maintaining at reduced temperature. The shoots grown in stable temperature (20°C) were tested only after 3 months. In addition, leaf samples from the naturally infected lily 'Casablanca' with aster yellows phytoplasma (Kamińska and Korbin, 1999) were included for comparison.

Polymerase chain reaction. Total nucleic acid was extracted twice from five fresh plant samples (1 g), according to Ahrens and Seemüller (1992). PCR was performed using 20 ng of obtained DNA template in 25 ml of reaction mix containing 150 μM dNTP, 0.5 U of Taq-polymerase and 5 pM of each primer.

Two pairs of primers specific to 16S rDNA conserved phytoplasma region were applied in these studies – R16F2/R2 in direct and R16(I)R1/F1 in nested PCR (Lee et al. 1994, 1995). Thirty five amplification cycles were conducted in Eppendorf thermal cycler 5330. PCR products were subjected to electrophoresis through a 1% agarose gel followed by staining in ethidium bromide. DNA bands were visualized using a UV transilluminator.

Restriction enzyme analyses (RFLP). PCR product (~10 μl) was digested with *MseI* endonuclease (Gibco) according to the manufacturer procedure at 37°C for 4 h. The restriction products were separated by electrophoresis through 5% polyacrylamide gel, stained with ethidium bromide and then visualized under UV light.

RESULTS AND DISCUSSION

Healthy and phytoplasma aster yellows affected *Gladiolus* plants were propagated in vitro for two years. On the contrary to the healthy plants the diseased gladioli showed leaf chlorosis and abnormal proliferation of the shoots (Fig. 1) known as 'germ fins' symptoms (Bertaccini et al. 1992). However, in spite of the distinct morphological symptoms the unfavorable effect of the continuous 20°C high temperature was evident and after two years of micropropagation of affected plants we were unable to state the presence of phytoplasma by PCR examination.

Fig. 1. *Gladiolus* healthy and phytoplasma-affected (at right) shoots micropropagated on MS medium without plant hormones during 20 months at continuous temperature of 20°C.

Our first experiment was undertaken to address the hypothesis that phytoplasma detection might be promoted by growing plants in reduced temperature. Its results, presented here in the table 1 and figures 2–3, demonstrate that indeed the detection of phytoplasma aster yellows using PCR technique in micropropagated gladiolus plants depends upon culture conditions, mainly on temperature, but also on the type of medium and on the presence or absence of light. An interaction of these factors was also demonstrated.

Phytoplasma detection

After the amplification with the universal primer pair R16F2/R2 (direct PCR) products with expected length ~1.2 kb were obtained only from the samples collected from in vitro plants kept for 2–3 month at 13°C under light on the medium containing kinetin+NAA. No evidence of phytoplasmas was obtained for the other tested plants kept under different conditions, the lily 'Casablanca' and water control. However, if tested PCR with the specific primer pair R16(I)F1/R1 was applied, a fragment of the size of 1.1 kb was detected in some of the micropropagated shoots of *Gladiolus* depending on the medium content and on light and temperature conditions (Table 1; Fig. 2). The same effect was in case of the lily 'Casablanca', but not in the case of water.

RFLP analysis of nested-PCR products with restriction enzyme *MseI* indicated that all the tested samples showed the restriction profile (Fig. 3) similar to those of the AAY strain of phytoplasma in the lily 'Casablanca' (Kamińska and Korbin, 1999) and gladiolus (Kamińska et al. 1999).

TABLE 1. Phytoplasma detection by PCR in *Gladiolus* plants micropropagated on different medium kept at 4°C and 13°C in the presence or absence of light.

Months of culture	Medium								
	Control (without cytokinin)			BAP + NAA			Kinetin + NAA		
	4°C		13°C	4°C		13°C	4°C		13°C
	Dark	Dark	Light	Dark	Dark	Light	Dark	Dark	Light
1	–	–	–	+	+	–	–	–	+
2	–	+	+	+	+	+	–	–	+
3	+	+	+	+	–	+	+	+	+
Control – 3 months at 20°C;	dark light			–			– +		

– PCR negative; + PCR positive

Fig. 2. Electrophoresis of nested PCR products of phytoplasma 16S rRNA sequences from micropropagated gladiolus shoots kept for one month at 4°C on the medium: without hormones (1), containing kinetin+NAA (2), with BAP+NAA (3) and from the shoots kept for one month at 13°C in darkness on medium without hormones (4), with BAP+NAA (5) and with kinetin+NAA (6); water (7). M – molecular marker (1 kb ladder, Promega). Nested PCR was performed using R16R2/F2 followed by amplification with the primer pair R16(I)F1/R1.

Fig. 3. Polyacrylamide gel electrophoresis of *Mse*I digests obtained by nested PCR from micropropagated shoots kept one month at 4°C on the medium containing BAP+NAA (1) and from the lilies affected with AAY strain of phytoplasma (2); M – molecular marker (ϕ X 174/*Hinf*I, Promega).

Effect of medium content and temperature

PCR analysis indicated that plants kept for 1-3 months in reduced temperature tended to have higher titre of detectable phytoplasmas than the plants before culture or plants maintained continuously at 20°C.

The detectability of phytoplasma in infected plants kept at the reduced temperatures of 4° and 13°C in darkness was the best for plants grown on the BAP+NAA medium. In plants micropropagated on the medium without cytokinins or with kinetin+NAA (kinetin has lower activity than BAP) the phytoplasma was not detectable before three months of culture at 4°C. At 13°C, the presence of phytoplasma was stated in the plants after two months of growing on the control medium but not on the medium with kinetin+NAA.

These results indicated that application of cytokinins in the medium increased the detectability of phytoplasma in the micropropagated gladiolus shoots. The involvement of growth regulators in pathogenesis of phytoplasma disease has been reported by Chen et al. (1991). It is thought that the symptoms of phytoplasma disease are mainly caused by a rise of cytokinin/auxin ratio in infected plants. Dai et al. (1997) has stated that the elimination of phytoplasma from the infected *Morus alba* culture may be related to the decrease of the cytokinin/auxin ratio. Cytokinin plays an important role in the distribution of nutrient materials (Bidwell 1979). The decrease in cytokinins can limit the nutrients required by the phytoplasma

and inhibit their reproduction; the resumption of normal growth of the stem segments can increase their resistance to the phytoplasma (Dai et al. 1997).

Effect of medium content and light

When plants were kept for 1-3 month at 13°C under light, the detectability of phytoplasma was further improved depending on the medium and on light-exposure time. After 2 months of exposure the phytoplasma was detected in all tested samples regardless the type of medium. The best detection was stated for the plants grown on the medium containing kinetin+NAA. In these plants phytoplasmas were detected in direct PCR after one month of culture.

When affected plants were micropropagated for three months at 20°C on different media under light, the phytoplasma was detected only in plants growing on the medium containing kinetin+NAA but not in the others.

These results suggest that the reduced photosynthetic activity connected with the reduced temperature during culture were responsible for the higher titre and detectability of phytoplasmas in *Gladiolus* shoots. In this aspect our results are in agreement with the results of Sears et al. (1997) who stated that nonphotosynthetic leaf tip cultures of *Oenothera* contained more phytoplasmas than did those from green tissues. However, we noticed that when the gladiolus plantlets were grown in the presence of light, the phytoplasma reached a higher titre than in the plantlets kept in darkness. It was especially true for the plants grown on the medium containing kinetin+NAA even at the continuous temperature of 20°C and in the presence of light.

Further research work may be useful to study the condition promoting phytoplasma detection in diseased plants.

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WYKRYWALNOŚĆ FITOPLAZMY W ROŚLINACH MIECZYKA UTRZYMYWANYCH W KULTURACH IN VITRO W RÓŻNYCH WARUNKACH

STRESZCZENIE

Rośliny mieczyka porażonego fitoplazmą żółtaczki astra utrzymywane w kulturach in vitro w stałej temperaturze 20°C były chlorotyczne i silnie proliferowały. Po dwóch latach trwania doświadczenia stwierdzono, że mimo występowania wymienionych objawów chorobowych, w roślinach chorych nie można było wykazać obecności fitoplazm, stosując technikę PCR. Celem zbadania, czy wykrywalność fitoplazm nie jest zależna od warunków prowadzenia kultur in vitro, mikropędy mieczyka utrzymywano na różnych pożywkach, w obniżonej temperaturze 4°C lub 13°C, na świetle i w ciemności.

Badania wykazały, że wykrywalność fitoplazm w kulturach in vitro mieczyka była promowana nie tylko przez utrzymywanie roślin w obniżonej temperaturze ale także przez pożywkę i obecność światła. Rośliny przetrzymywane 1-3 miesiące w obniżonej temperaturze miały wyższe miano fitoplazm niż utrzymywane w stałej temperaturze 20°C. Stosunkowo najwyższą wykrywalność fitoplazm stwierdzono w roślinach utrzymywanych na pożywce zawierającej kinetynę+NAA na świetle. W kulturach tych fitoplazmy były wykrywane przy pomocy bezpośredniego testu PCR już po jednym miesiącu, podczas gdy w innych warunkach – dopiero po zastosowaniu gniazdowego testu PCR.

SŁOWA KLUCZOWE: *Gladiolus*, mikrorozmnażanie, fitoplazma, PCR, wykrywanie.