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Attempts to eliminate phytoplasmas from grapevine clones by tissue culture techniques

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Abstract

Individual lines of *Vitis vinifera* Barbera and Chardonnay were obtained by *in vitro* culture of axillary buds collected from phytoplasma-affected mother plants. The molecular assays did not detect the "flavescence dorée" (FD) phytoplasma in the 66 lines tested after 6-9 months of culture, while the "bois noir"-associated phytoplasma was detected in 22 out of 50 lines. Despite the observed inefficiency of the FD phytoplasma in infecting micropropagated grapevine plants, additional strategies for phytoplasma elimination are advisable. Preliminary assays on the sensitivity of grapevine explants to oxytetracycline showed that axillary bud cultures were severely damaged by 100 $mg \cdot l^{-1}$ oxytetracycline in the culture medium, while 50 $mg \cdot l^{-1}$ did not inhibit the growth of plantlets with young roots.

Key words: *Vitis vinifera*, grapevine yellows, "flavescence dorée", "bois noir", micropropagation, antibiotics.

Introduction

Grapevine yellows (GY) are dangerous diseases of grapevines, associated with phytoplasmas and known worldwide. The most important GY in Italy and Europe are "flavescence dorée" (FD) and "bois noir" (BN). Recently FD has spread in many viticultural areas of North Italy causing severe damages and strong concerns among grapegrowers and phytopathological services.

Significant advances have been made in molecular diagnosis, etiology and epidemiology of GY in the last years (Boudon-Padieu, 2005). FD and BN diffusion in the vineyards is mainly due to the insect vectors, but the infected plant propagation in the nurseries may contribute to worsen the problem. Strategies to reduce GY disease dissemination are based mainly on control of vectors through insecticide sprayings, uprooting of infected plants and monitoring the sanitary status of planting material. Hot water treatment has been proposed to cure dormant woody plant material from phytoplasmas and recent experiences confirmed its reliability for grapevine (Mannini and Marzachì, 2007). While tissue culture techniques are routinely used for virus eradication, few reports have been published on their potentiality in phytoplasma elimination (Dai *et al.*, 1997; Parmessur *et al.*, 2002; Chalak *et al.*, 2005). In this work we report the results of a) micropropagation from GY-diseased grapevine plants, and b) preliminary assays on the sensitivity of grapevine explants to the antibiotic oxytetracycline.

Materials and methods

In summer 2004 and 2005, whole shoots were collected in vineyards in North Piedmont (Italy) from grapevine plants of the cultivars Barbera and Chardonnay. The mother plants showed GY symptoms and were tested for FD with positive results; in some of the mother plants the phytoplasma associated with BN was also detected. Apical and axillary buds were isolated, surface-sterilized and cultivated on a modified Murashige and Skoog (1962) medium with half strength mineral salts, full strength vitamins, 20 $g \cdot l^{-1}$ sucrose, 8 g^{-1} agar, 2.5 μM benzyl-amino-purine (BAP medium). The position of the bud explant on the original shoot, and of the shoot on the cane or spur or trunk was recorded. Buds were taken in May and July. In 2005, a second medium with the same basal composition but without plant growth regulators was also tested (PGR-free medium). All the cultures were kept at 24 °C, 16 h photoperiod and 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. Shoots sprouting from the cultivated buds were rooted and micropropagated by repeatedly sub-culturing apical cuttings (3-4 cm long) on the PGR-free medium, thus giving rise to individual lines.

The presence of GY phytoplasmas was assayed by nested PCR and RFLP (Marzachì and Boarino, 2002) in mother plants and in micropropagated lines.

The sensitivity of grapevine *in vitro* cultures to tetracycline was assayed on buds and plants of the cultivar Barbera. In a first test, axillary buds were cultivated on the BAP medium, with or without 100 $mg \cdot l^{-1}$ oxytetracycline hydrochloride (Duchefa Biochemie, NL) added to the medium after autoclaving. In a second experiment, grapevine plantlets with very young roots (1 to 5 mm) were transferred to the PGR-free medium with or without 50 $mg \cdot l^{-1}$ oxytetracycline.

Results

Micropropagation allowed to obtain a large number of lines, each originated by a single bud. Six to nine months after culture initiation, the molecular assays did not detect the FD phytoplasma in the 66 tested lines. The BN-associated phytoplasma was detected in 22 out of 50 lines deriving from BN-affected mother plants (44% infection). A few lines positive to BN were tested again at least 8 months later and the phytoplasma was detected in 2 samples out of 7.

Table 1. Growth of grapevine axillary buds cultivated for one month on media with 0 (control) or 100 $mg \cdot l^{-1}$ oxytetracycline; 20 explants/treatment.

	Control	+ oxytetracycline (100 $mg \cdot l^{-1}$)
Sprouting buds (%)	100	35
Shoots/bud (average ± SE)	1.7 ± 0.7	1.1 ± 0.3
Shoot length (cm) (average ± SE)	1.8 ± 0.9	0.5 ± 0.4

Oxytetracycline (100 $mg \cdot l^{-1}$) reduced explant growth and proliferation, as reported in table 1. After one month on the oxytetracycline-containing medium, the explants were transferred onto an antibiotic-free medium but none of them resumed growth and they gradually became necrotic.

In the second experiment, grapevine plantlets with very young roots, transferred to a PGR-free medium containing 50 $mg \cdot l^{-1}$ oxytetracycline, did not show notable difference in growth compared to control plants.

Discussion

In the present work no FD-affected plant was obtained through micropropagation starting from infected vineyard-growing mother plants, irrespective of the position of the explant on the mother plant, the moment of culture initiation and the absence of PGR in the medium. On the contrary, BN phytoplasma was detected in nearly half the micropropagated lines, but its persistence in *in vitro* cultures is doubtful and must be verified with further studies. Previous experience on a smaller scale were made starting from FD-affected potted mother plants: a few micropropagated grapevine plants resulted still positive to FD assays after years of *in vitro* culture, although samples from the same line gave non-homogeneous results (unpublished data).

Despite considerable efforts, phytoplasmas have not been isolated in pure cultures on artificial media so far. Tissue culture techniques have been proposed both for maintaining the pathogen in living hosts, allowing the set up of strain collections in some plant species (Bertaccini *et al.*, 1992; Jarausch *et al.*, 1996), and for elimination of phytoplasma from diseased plants. Phytoplasmas were eradicated in high percentages from mulberry plants by axillary bud culture (Dai *et al.*, 1997), from almond varieties by shoot tip culture (Chalak *et al.*, 2005), and from sugarcane by indirect somatic embryogenesis (Parmessur *et al.*, 2002). Although Petrovic *et al.* (2000) used micropropagation to increase GY phytoplasma concentration in grapevine tissues and to improve its detection, our experience supports the hypothesis that FD phytoplasma does not infect efficiently the *in vitro* cultured grapevine plants, and that micropropagation itself can be considered for eradication attempts at least with the cultivars used in this study.

Nevertheless, the adoption of additional strategies for phytoplasma elimination during tissue culture is advisable. The addition of tetracyclines to the culture media allowed permanent phytoplasma elimination from *Pyrus* (Davies and Clark, 1994). Oxytetracycline (50 to 150 $mg \cdot l^{-1}$) completely inhibited the growth of almond axillary bud cultures (Chalak *et al.*, 2005). In our experience, addition of 100 $mg \cdot l^{-1}$ oxytetracycline to culture medium was detrimental to axillary bud cultures while 50 $mg \cdot l^{-1}$ did not inhibit plant growth when applied to plantlets with young roots. Further trial on GY-diseased material will give indication on the efficiency of antibiotic-containing media in phytoplasma eradication.

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