Isolation of Diverse Phytoplasmas from Symptomatic Grapevine Samples

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***Abstract*—Phytoplasmas from different ribosomal groups were isolated from grapevine samples in which "flavescence dorée" or "bois noir" phytoplasmas had been identified. The results indicate that the developed medium is not phytoplasma specific and supports the growth of phytoplasmas which cannot be detected by other molecular methods and are very likely present in a very low titre in the endobiome of the plants. The applied method discriminates the presence of bacterial contamination already in the liquid phase, and the colony purification technique allows excluding the contamination.**

***Key words*—"bois noir", "flavescence dorée", grapevine yellows, molecular identification, phytoplasmas, isolation**

I. INTRODUCTION

Phytoplasmas are phloem-restricted bacteria from the class Mollicutes transmitted by plant sap-feeding insect species. They were long reported as unculturable in cell-free media and thus very well characterized only from the genetic perspective. However, phytoplasma cultivation was recently achieved using micropropagated periwinkle plants as a source of phytoplasma-infected shoots and also naturally phytoplasma-infected grapevine plants using a complex medium (Contaldo et al. 2012, 2016). In the frame of the INGRAPA project the isolation of phytoplasmas from naturally infected grapevine plants was carried out evaluating also the activity of some antibiotics.

II. MATERIAL AND METHODS

Symptomatic field collected grapevine samples were tested on-site by LAMP for the presence of "bois noir" and "flavescence dorée" phytoplasmas (Kogovšek et al. 2015, 2017) Leaves from samples positive to "flavescence dorée" or "bois noir" phytoplasmas (Table 1) were used for isolation. Approximately 5 cm midribs were surface sterilized, rinsed in deionized distilled sterile water (DDSW) under a sterile laminar hood and after drying soaked in 5 mL of CBl orange liquid medium (Contaldo et al. 2016), chopped into smaller pieces with sterile scalpels and transferred into 8 mL sterile tubes, incubated at 25±1°C. Non-inoculated tubes were subjected to the same conditions. After the medium acidification colour change, aliquots of 100 µL of medium per sample were inoculated onto plates containing 8 mL of CBs solid medium (Contaldo et al. 2016), incubated at 25±1°C under microaerophilic atmosphere. Vancomycin and tetracyclin at the concentration of 25 ug/ul were also added to the plates to verify their effectiveness on phytoplasma and bacteria presence. Phytoplasma single colonies were transferred into new tubes containing 2 mL of fresh CBl liquid medium. Colony purification was performed by filtration through 0.8 µM membrane filters prior 10-3/10-4 serial dilutions in CBl liquid medium. Both the undiluted filtrate and filtrate dilutions were cultured onto solid CBs plates, and incubated at 25±1°C. The process was repeated for the grown single colonies and the procedure, including filtration, was repeated three times. The plates containing colonies were assessed and photographed under an optical bifocal microscope at 40X magnification (Nikon, Japan).

Table 1 Results of phytoplasma isolation from field collected grapevine samples.

Sample	Phytoplasma identification from grapevine plant samples	Phytoplasma identification from cultures
		mixed bacterial population
		16SrI (3 plates)
TVS01	16SrV-C	16SrXII
		16SrV
		16SrIII
		16SrV
TVS02	16SrV-C	16SrI
TVK03	16SrV-C	16SrXII
TVMF02	16SrXII-A	mixed bacterial population

Single colonies were suspended in 100 µL of DDSW, and subjected to nucleic acid extraction (DNeasy Plant Minikit, Qiagen). Phytoplasma detection and identification were carried out by specific PCR/RFLP assays on the 16S rRNA gene sequence. One µL of total extracted DNA was used as template for PCR with primers 758f (=M1)/B6 followed by nested amplification with primers M1/1232r (=M2) (Gibb et al. 1995; Padovan et al. 1995). For each PCR test, DDSW and non-inoculated media were used as negative controls. Each 25 µL of PCR master mix contained 12.5 µL of 2X Red PCR Master Mix (Rovalab, Germany), and 0.4 mM of each primer. Nested-PCR assays were performed

using 1 µL of a 30-fold dilution of the direct PCR amplicons as template. RFLP on the M1/M2 amplicons was performed with the restriction endonuclease *Tru1*I. PCR and RFLP products were visualized under a UV transilluminator.

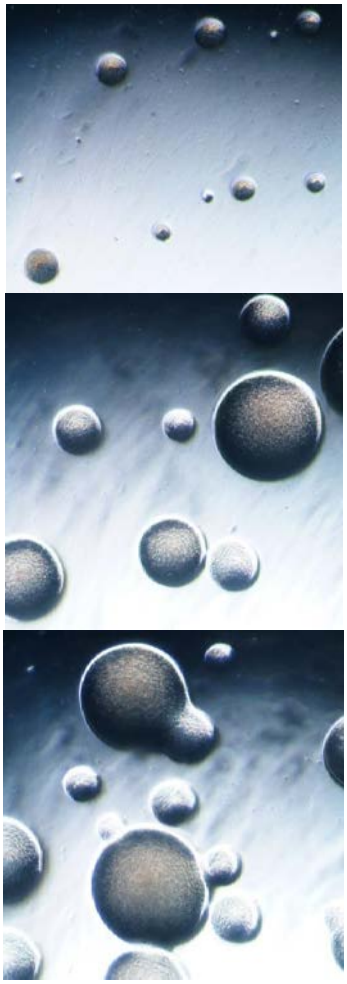

Figure 1. Phytoplasma colonies produced after isolation from grapevine yellows infected samples at different days of growth (magnification x 40).

III. RESULTS

The CBl liquid media changed colour from orange to yellow at different times after inoculation depending on the sample. Non-inoculated tubes did not show colour changes nor colony growth throughout the experiments. The plating from tubes, immediately after the medium acidification (color change), produced phytoplasma colonies 1-2 days post-insemination (Figure 1). Single colony purification method ruled out contamination with larger bacteria and allowed to prepare isolates for further analyses. Phytoplasma growth and colony production was observed for three of the four samples employed (Table 1). The sample TVMF02 “bois noir” infected resulted in a contaminated growth and was therefore discarded. The plates with medium containing vancomycin showed an increased bacterial growth while in the plates with tetracyclin no colony growth was observed (data not shown).

Phytoplasma DNA amplification was obtained in all the colonies obtained from the three samples infected with 16SrVC phytoplasmas when tested on plant samples (Table 1). In particular colonies obtained after isolation from two samples resulted to contain 16SrXII-A phytoplasmas (“stolbur”/”bois noir”) (samples TVK03 and TVS01). In sample TVS01 plates 16SrI, 16SrV and 16SrIII phytoplasmas were identified, while in sample TVS2 16SrI and 16SrV phytoplasmas were detected in the colonies. The not inoculated tubes did not show colony growth.

III. DISCUSSION

The plate isolation of diverse phytoplasmas from grapevine samples previously identified as infected by 16SrV-C phytoplasmas (“flavescence dorée” FD-C) indicated that the medium developed is not phytoplasma specific and supports the growth of phytoplasmas that are present in the endobiome of the plants without being LAMP detected. All the phytoplasmas identified are reported as present in grapevine affected by yellows disease in diverse areas of the world, therefore, they are not produced by some system contamination, also considering that the control resulted always negative indicating the correct processing. Moreover, it was possible to discriminate the presence of bacterial contamination already in the liquid phase, and the colony purification technique allows to further exclude contamination.

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REFERENCES

Contaldo, N., Bertaccini, A., Paltrinieri, S., Windsor, H. M., Windsor, D. G. 2012. Axenic culture of plant pathogenic phytoplasmas. *Phytopathologia Mediterranea*, 51(3), 607−617.

Contaldo, N., Satta, E., Zambon, Y., Paltrinieri, S., Bertaccini, A. (2016). Development and evaluation of different complex media for phytoplasma isolation and growth. *Journal of Microbiological Methods*, 127, 105–110.

Gibb, K. S., Padovan, A. C., Mogen, B. A. (1995). Studies on sweet potato little-leaf phytoplasmas detected in sweet potato and other plant species growing in Northern Australia. *Phytopathology*, 85, 169-174.

Kogovšek, P., Hodgetts, J., Hall, J., Prezelj, N., Nikolić, P., Mehle, N., Lenarčič, R., Rotter, A., Dickinson, M., Boonham, N., Dermastia, M., Ravnikar, M. (2015). LAMP assay and rapid sample preparation method for on-site detection of "flavescence dorée" phytoplasma in grapevine. *Plant Pathology*, 64(2), 286–296.

Kogovšek, P., Mehle, N., Pugelj, A., Jakomin, T., Schroers, H.-J., Ravnikar, M., Dermastia, M.(2017). Rapid loop-mediated isothermal amplification assays for grapevine yellows phytoplasmas on crude leaf-vein homogenate has the same performance as qPCR. *European Journal of Plant Pathology*, 148(1), 75–84.

Padovan, A. C., Gibb, K. S., Bertaccini, A., Vibio, M., Bonfiglioli, R. E., Magarey, P. A., Sears, B. B. (1995). Molecular detection of the Australian grapevine yellows phytoplasma and comparison with a grapevine yellows phytoplasma from Emilia-Romagna in Italy. *Australian Journal of Grape Wine Research*, 1, 25-31.