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Development and evaluation of different complex media for phytoplasma isolation and growth

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ABSTRACT

The focus of this research was the development and evaluation of different complex liquid and solid media for the isolation and growth of phytoplasma strains infecting grapevine plants. Previously reported media supporting phytoplasma isolation are commercial and not easy to modify in order to improve performance and selectivity towards obtaining pure cultures of '*Candidatus* Phytoplasma' species. Three media (Piv®, CB and MB) were therefore evaluated for phytoplasma isolation and colony formation under microaerophilic growing conditions, using grapevine canes from plants showing yellows symptoms, and infected by "flavescence dorée", "bois noir" and aster yellows phytoplasmas as sources. The newly developed methodology was applied for two years at three sample collection times. Broad applicability and a good repeatability in supporting phytoplasma colony formation were obtained in Pivs® and CBs media. While the MB medium did not support phytoplasma isolation and growth, the CB media support a phytoplasma growth comparable to the one obtained in the previously reported media. This medium has the advantage of a formulation that allow its modification to implement specificity towards selective phytoplasma growth. Moreover preliminary trials on serial dilutions and tetracycline addition confirmed some phytoplasma growth behaviours.

1. Introduction

Phytoplasma cultivation in artificial media was achieved using as a source micropropagated phytoplasma infected periwinkle shoots (Contaldo et al., 2012) and confirmed by isolation of five additional phytoplasmas strains (Contaldo et al., 2013), all from a collection established more than twenty years ago (Bertaccini et al., 1992). In order to implement and apply on large scale and on naturally diseased plant materials the phytoplasma cultivation, there is however still the need of media supporting growth from different plants sources and having flexible and modifiable composition.

Phytoplasmas are spread by sap feeding hemipteran insect vectors, and by exchanges of infected planting material mainly from nurseries. The grapevine (*Vitis vinifera* L.) was selected as field source for phytoplasma isolation considering its economic importance among all agricultural relevant species, since it is affected by a number of phytoplasma diseases (grapevine yellows) such "bois noir" (BN), "flavescence dorée" (FD) and aster yellows (AY) (Bertaccini et al., 2014). The production of colonies for the more widespread or relevant grapevine phytoplasmas will allow the production of specific, handily and cheap detection tools based on viable microorganism presence, and will implement a focused screening of grapevine susceptibility to these phytoplasma diseases. Complete methodology of phytoplasma cultivation from naturally infected grapevine plants is here reported to provide a robust and complete protocol; parts of the procedure were shortly published elsewhere (Contaldo et al., 2014, 2015).

2. Materials and methods

2.1. Phytoplasma sources

Isolation trials were carried out from leaves of three 15-year-old plants of grapevine cultivar Glera showing typical yellows symptoms, as well as from one asymptomatic grapevine plant of the same age and variety. The isolation was carried out in June/July during the two growing seasons 2014 and 2015, and was repeated in duplicate three times per each plant infected by FD and BN phytoplasmas. The same trials were carried out only in 2015 for one AY infected grapevine plant. The plants were tested for phytoplasma presence by nested-PCR/RFLP and sequencing analyses as reported (Prince et al., 1993; Lee et al., 1998; Martini et al., 1999) before each isolation trial.

2.2. Media

Three liquid media were prepared under sterile conditions for the phytoplasma isolation: PivL®, pH 7.3 ± 0.2 (Contaldo et al., 2012); TSB (Oxoid, UK; CM1065) pH 7.3 ± 0.2, an highly nutritious, general

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purpose medium containing essentially tryptone and soya peptone, and supporting the growth of a wide range of bacteria, and Mycoplasma Broth Base (Oxoid, UK; CM0403) pH 7.8 ± 0.2, a basic medium containing bacteriological peptone and lab-Lemco powder used in isolation and cultivation of mycoplasmas. The last two media after 20 min sterilization at 121 °C in autoclave, were enriched with a new developed supplement (S) containing 20 ml of sterile horse serum (Oxoid, SR0035), 25 μg/ml of ampicillin (Sigma, A9393), and 50 μg/ml of nystatin (Sigma, N6261) both 0.22 μm filter sterilized, 10 ml of autoclaved yeast extract (25% w/v), and 0.005% of phenol red for each 80 ml of medium. These latter two media are named CBl (TSB as base) and MBl (Mycoplasma Broth Base) respectively.

2.3. Phytoplasma isolation and growing conditions

From each grapevine leaf sample 9 midribs 3 to 5 cm long were surface sterilized for 1 min in 1% NaClO, rinsed in deionized, distilled, sterile water (DDSW), and dried on sterile filter paper disks under sterile hood. After the ends discarding, three midribs were moistened in 5 ml of each of the three media and sliced with sterile scalpels. The slurry was then transferred to a 8 ml Monovette urine tubes (Sigma Aldrich) and incubated at 25 ± 1 °C under normal atmospheric conditions. Uninoculated tubes (UT) and tubes inoculated with midribs from healthy grapevine (HG) were also maintained under the same conditions. The isolation procedure was repeated twice for each sample in each medium at each isolation time.

When colour change from orange-red to orange-yellow occurred, 100 μl of broth cultures were inoculated onto plates containing 8 ml of the corresponding solid media: PivS®; TSB agar (30 g/l) and Mycoplasma agar (Mycoplasma broth base 25.5 g/l). The latter two media after addition with NaCl 20 g/l, agar No. 3 12 g/l (Oxoid, LP0013), autoclaving, then addition with 0.22 μm filter sterilized ampicillin and nystatin 50 μg/ml, were named CBs and MBs respectively and contain no serum nor yeast extract. The incubation was carried out in a 2.5 l anaerobic jar (Oxoid, AG0025) in a microaerophilic atmosphere using CampyGen sachets (Oxoid, CN0025). The isolation trials were repeated under the same conditions 3 times each year, and the same plant sources were sampled and used for duplicate isolation trials. Additional CBs plates were prepared adding 25 μg/ml of tetracycline as control for growth inhibition. Insemination of *Acholeplasma laidlawii* strain PG8 (ATCC # 1039) from a plate containing pure culture kindly provided by Dr. M. Kube (Thünen Institute of Forest Genetics, Waldsieversdorf, Germany) was also carried out on CBs plates without tetracycline.

2.4. Phytoplasma culture purification

After 1 to 1.5 days from the agar insemination distinct single colonies were picked and transferred into the corresponding fresh liquid media, for subsequent purification steps, following a slightly modified procedure (ICSB, 1979). After colour change each broth culture was 0.8 μm filtered using syringe filter non pyrogenic hydrophilic (Sartorius Stedim, Biotech, Germany) and 100 μl were again plated; this procedure was repeated three times per selected colony of each phytoplasma strain in each medium when there was colony formation. After the above procedure one colony was picked, transferred in broth medium and plated after 2 days of incubation using 10^{-3} and 10^{-4} dilutions. The procedure was repeated 2 time for each of the phytoplasma isolate.

The two and five days old colonies were photographed on the plates and also under optical bifocal microscope at 40× magnification. Single colonies were then collected, dissolved in 100 μl DDSW and subjected to nucleic acid extraction by DNeasy Plant Minikit (Qiagen, Germany). The agar surface between colonies was also collected and extracted separately as negative control for each of the media that allowed colony growth.

2.5. Phytoplasma molecular identification from colonies

Phytoplasma detection and identification was carried out from colonies by specific nested PCR/RFLP assays on 16S rRNA gene and on stamp gene (only for BN phytoplasmas) (Fabre et al., 2011). One microliter of extracted nucleic acid was employed as template using B5/P7 primers followed by nested amplification with M1/B6 primers (Gibb et al., 1995; Padovan et al., 1995) for FD phytoplasmas, and using R16F2n/R2 (Gundersen and Lee, 1996), followed by nested PCR with R16(I)F1/R1 primers (Lee et al., 1994) for BN and aster yellows phytoplasmas. Reference phytoplasma strains employed were AY1 (aster yellows, ribosomal group 16SrI-B), CH-1 ("stolbur" from grapevine, ribosomal group 16SrXII-A), EY (elm yellows, ribosomal subgroup 16SrV-A), FD-AS ("flavescence dorée", ribosomal group 16SrV-C). Strain FD-92 of "flavescence dorée" from grapevine (ribosomal subgroup 16SrV-D) kindly provided by E. Boudon-Padieu (INRA, Dijon, France) was also employed. In each PCR and nested-PCR experiment two negative control samples represented by DDSW and media not inseminated were used. Each 25 μl PCR reaction mix contained 12.5 μl of 2× Red PCR Master Mix (Rovalab, Germany), and 0.4 mM of each primer. Nested-PCR assays were carried out using 1 μl of a 1:30 dilution of amplicons from direct PCR as template. Cycling condition was as reported for the 16Sr RNA and stamp genes amplification respectively (Schaff et al., 1992; Fabre et al., 2011). Six microliters of PCR products was separated in 1% agarose gel, stained with ethidium bromide and visualized with UV transilluminator. Identification of detected phytoplasmas was done using RFLP analyses with *Tru1*I and *Taq*I (Fermentas, Vilnius, Lithuania) restriction enzymes. RFLP products were separated in a 6.7% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator. Direct sequencing of 10 selected PCR products per phytoplasma strain isolates was also performed; sequences were assembled using the Staden program package (Staden et al., 2000), aligned using Clustal X (Thompson et al., 1997), and deposited in GenBank.

3. Results

3.1. Phytoplasma sources

The symptomatic grapevine plants employed for isolation and cultivation trials resulted infected by BN, 16SrXII-A (BN-Yan), FD, 16SrV-C and -D (Martini et al., 1999) (FD-05M and FD-7Bariv, respectively) and AY, 16SrI-B (AY-3Bar) phytoplasmas in single infection (data not shown). The asymptomatic plant resulted negative in all the molecular tests carried out.

3.2. Phytoplasma isolation and purification from cultures

The isolation trials resulted in broth colour changes at different times after insemination and differed among the phytoplasma detected in the source plant materials. The two repetitions of isolation were substantially consistent in each isolation time, and in each medium tested in both years for FD and BN phytoplasmas; for the AY phytoplasma only one year of isolation was carried out, however results were also highly consistent (Table 1). Uninoculated tubes did not show colour changes, while tubes inoculated with healthy grapevine midribs sometimes gave rapid acid colour changes as result of other endophytes growth.

Transfer of liquid medium aliquots from FD, BN and AY inoculated tubes into agar media PivS® and CBs produced colonies after 1–2 days from insemination (Table 1). In particular, FD-05M and FD-7bariv growths were visible after 1 day as small spherical, granular colonies (<0.01 mm) that were fully visible after 2 days (Fig. 1A), while BN-Yan and AY-3Bar phytoplasmas were producing spherical colonies visible to naked eye (>0.1 mm diameter) 2–3 days after inoculation (Figs. 1B and 2A and B). The growth supported by MBs was clearly