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Research Note

Importance of the vegetative stage for phytoplasma detection in yellow-diseased grapevines

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Summary: The influence of the vegetative stage on the detection of phytoplasmas (=MLOs) in different grapevine tissues during six phenological stages is reported. From the beginning of the annual growth cycle of the plants it is possible to detect phytoplasmas in infected vines using purified DNA from leaves and shoots in slot blot hybridization. The hybridization signal is stronger in later stages reaching the highest intensity with leaf tissue at berry ripening. The detection is possible with cortical scrapings from mature canes. This opens an interesting possibility for massive screening of propagative material for certification purposes.

Key words: phytoplasmas, Vitis vinifera, yellows disease, diagnosis, vegetative stage.

Introduction: A phloem-limited wall-less prokaryote (i. e. phytoplasma, formerly called mycoplasma-like organism, MLO, see TULLY 1993) with sequence homology to the DNA of the European aster yellow (EAY) phytoplasma cluster, is known to be associated with a grapevine yellows (GY) disease reported from Latium (Central Italy) in 1992 (BARBA and DEL SERRONE 1993). This disease spreads naturally in vineyards but the vector has not yet been identified (DEL SERRONE and BARBA 1995).

A method was developed for detecting the putative causal agent of GY in crude grapevine sap (MINUCCI *et al.* 1994 b; DEL SERRONE *et al.* 1995). This method, however, did not always afford positive results. To explain these failures it was hypothesized that symptom appearence was not necessarily indicative of a phytoplasmas concentration in the vines high enough to make detection possible (DEL SERRONE *et al.* 1995).

Therefore, in the present study, the influence of the vegetative stage on the detection of phytoplasmas in grapevine tissues was investigated in order to determine the best phenological period for collecting samples from different parts of the plant and to compare different methods for sample preparation.

Materials and methods: Ten yellows-diseased plants of cv. Chardonnay, known to be affected by phytoplasmas in previous molecular hybridization tests with a radiolabeled DNA probe (EAY 352) cloned from fragments of EAY phytoplasma genome at I.F.A. (Istituto di Fitovirologia Applicata, C.N.R., Turin, Italy), were chosen (DEL SERRONE *et al.* 1995).

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The following six vegetative stages were considered:

I leaf unfolding
II inflorescences visible
III inflorescences separated
IV berry set
V veraison
VI berry ripening

Leaves, shoots or canes collected at each stage were rinsed, surface sterilized (1 % sodium hypochlorite), and stored at -80 °C. Sampling was for two consecutive growing seasons.

Phytoplasmas were detected using either purified total DNA (i) or crude sap (ii) obtained as follows:

i) Preparation of purified DNA: tissues (1 g) were ground in 14 ml of 0.1 M phosphate buffer, 30 mM ascorbic acid, 10 % (w/v) sucrose, 0.15 % (w/v) bovine serum albumine, 2 % (w/v) polyvinylpyrrolidone (MW 40,000), clarified at 3,500 rpm for 25 min at 4 °C. The supernatant was then centrifuged at 13,500 rpm for 25 min at 4 °C. The pellet was resuspended in 2 ml of DOYLE and DOYLE (1990) extraction buffer preheated at 60 °C, lysed at 60 °C for 30 min and then extracted with 1 vol of chloroform:isoamyl alcohol (24:1). The DNA was pelleted from the aqueous phase by centrifugation at 14,000 rpm for 30 min, after addition of 0.67 vol of isopropyl alcohol. The pellet was washed with 70 % (v/v) ethanol and resuspended in distilled water.

ii) Crude sap: tissues (0.5 g) were ground in 4 ml of buffer containing 2 % (w/v) CTAB (hexadecyltrimethyl-ammonium bromide), 1.4 M NaCl, 1 % (w/v) SDS, 0.5 % (v/v) β-mercaptoethanol, 20 mM EDTA in 100 mM Tris-HCl, pH 8. Extracts were then incubated at 70 °C for 15 min, clarified with 1 vol of chloroform:isoamyl alcohol (24:1) and boiled in 0.2 M NaOH for 10 min. After neutralization in 0.2 M Tris-HCl pH 8, extracts were clarified by centrifugation for 15 min at 13,000 rpm.

Both purified total DNA and crude sap extracts were spotted under vacuum onto a positively charged nylon membrane (Hybond N, Boehringer Mannheim) using a Minifold II apparatus (Schleicher and Schuell). After denaturation (NaOH 0.5 M) and neutralization (Tris-HCl 1 M pH 8) the membranes were hybridized with the EAY 352 DNA probe. Pre-hybridization and the hybridization conditions are previously described (MINUCCI *et al.* 1994 a; DEL SERRONE *et al.* 1995).

Four arbitrary classes of symptoms were established as follows:

– = absence of symptoms
+ = light symptoms
++ = strong symptoms
+++ = very strong symptoms

Likewise, 4 classes were also considered for the hybridization signal:

– = no signal
+ = light signal
++ = strong signal
+++ = very strong signal (as with periwinkle experimentally infected with EAY)

Total DNA and crude sap from healthy grapevine of the same variety and from healthy periwinkle were used as healthy (negative) controls and similar extracts from periwinkle experimentally infected with EAY-MLO as positive controls.

Results: The EAY 352 probe was able to detect phytoplasmas in vines, although to a variable extent, depending on the vegetative stage, the plant material and extraction methods.

As shown in the Table leaves and canes, collected at stage VI (berry ripening), proved to be the best sources for phytoplasma detection for the hybridization signal was always clear-cut and strong, regardless of the method used for preparing the sample.

Table

Intensity of symptoms and hybridization signals from samples collected at different phenological stages (for intensity of symptoms and hybridization signals and for phenological stages see "Materials and methods")

Phenological stage	Symptoms	DNA extracts		Crude sap	
		shoot/cane	leaf	shoot/cane	leaf
I	-	+	+	-	-
II	-	++	+	-	-
III	+	++	+	+	+
IV	++	++	++	++	++
V	++	++	++	++	++
VI	+++	++	+++	++	+++

The use of canes or leaves did not influence the sensitivity of detection from late spring (stage IV = berry set), to the end of summer (stage V = veraison).

It was not possible to detect phytoplasmas in crude sap from shoots and leaves collected in the first two phenological stages (early spring). At this time phytoplasmas were detected in purified DNA extracts only.

The vegetative stage played an important role not only for the intensity of the signal but also for the percentage of plants positive in hybridization. In fact, as shown in the Figure, only a low percentage of plant extracts hybridized with the EAY 352 probe in the stage of leaf unfolding. This proportion reached 100 % in the last sampling (stage VI).

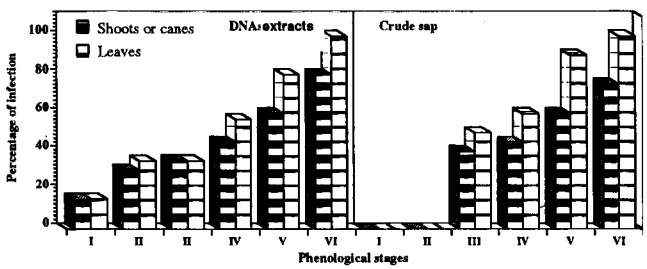

Figure: Percentage of vines in which phytoplasmas were detected at different phenological stages. Average of two sets of tests. (Stages I - VI see "Materials and methods").

In early spring, it was necessary to hybridize total DNA extracts to obtain positive results, even though in low percentage, whereas in August both purified DNA or crude sap reacted positively with the totality of tested plants.

The intensity of the signal and the percentage of positive reactions as a function of different phenological stages were similar during the two seasons. No positive reaction was observed with healthy controls.

Discussion: The results show that it is possible to detect phytoplasmas in infected grapevine tissues at different phenological stages. However, the signal obtained in the first two phenological stages only with purified DNA is too weak and not always detectable. It cannot be used for routine detection.

The hybridization signal was stronger at later stages, reaching the highest intensity with leaf tissue extracts at the stage of berry ripening. At this stage a clear differentiation between healthy control and infected samples was possible.

The use of crude sap is less time consuming for diagnosis. However the possibility of detecting phytoplasmas in cortical scrapings from mature canes is interesting. This allows winter screening for freedom from these pathogens in material propagated from vines taken up in certification schemes.

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