DETECTION OF CROWN GALL AGENTS ON UKRAINIAN VINEYARDS BY PCR

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Introduction. Crown gall of grape is one of the most serious diseases in commercial nurseries of many grape-growing countries. On young vineyards of susceptible cultivars up to 75% of plants may die from galls surrounding trunks and interfering the normal water and nutrients supply (Negrul, 1951, Burr, 1999). Crown gall agents (*Rhizobium vitis* and *R. radiobacter* (*Agrobacterium vitis*, *A. tumefaciens*)) survive in grapevine xylem and are transmitted by vegetative propagation (Lehoczky, 1968). In many cases infected plants remain symptomless for a long time, allowing to select planting material from infected vineyards (Burr, 1984). Use of such planting material results in further spread of pathogens and consequent losses in viticulture.

At present time none of the methods of complete pathogenic rhizobia eradication from grape are known. Appropriate control of crown gall of grape is needed for every stage of viticulture.

The first stage means a selection of pathogen-free plants for vegetative propagation. This stage includes indexing and certification of propagation material. Symptomless character of infection makes impossible selection of plants only by visual observation. Highly specific and rapid diagnostics methods are necessary to ensure healthy planting material selection. The following detection methods have commonly been employed for plant pathogenic bacteria: isolation on selective or semi-selective media, biochemical tests, pathogenicity tests on test-plants, serological methods and fatty acid analysis. The most reliable methods are those based on DNA composition. Such bacterial identification is less variable and can be interpreted more precisely. Polymerase chain reaction (PCR) remains the most useful and rapid method for pathogenic microorganism diagnostics including agents of plant diseases (Manulis, 2002).

Aim. The aim of our research was to reveal symptomless plants infected with crown gall agents by the help of PCR, and to select pathogen-free soil plots.

Materials and Methods. Rhizobia were isolated from grape wooden cuttings by Lehoczky method (1968). Roy and Sasser medium was used for isolation (Roy, 1983). Bleeding saps as well as extracts from tumors were also plated on this medium to increase the microbe's biomass (Szegedi, 2002). From soil rhizobia were isolated by a modificated method (Krimi, 2002; Limanska, 2003). PCR was performed by the method of Haas J. (1995) with our modifications (Milkus, 2005).

Results. In a period from 2003 to 2009 year, the collaborators of Microbiology and Virology Chair of Odesa National University carried out the diagnostics of crown gall on vineyards and plots intended for vineyards of Odesa, Cherson, Mykolaiv regions and Crimea.

Use of PCR methods allowed to investigate a lot of planting material and vineyards in short terms. For the first time in the Ukraine the crown gall agents were detected in wooden shoots, roots, galls, bleeding sap and soil by PCR based technique that was optimized in our research.

Before 2003, the diagnostics of crown gall of grape in Ukraine was based mainly on test-plants inoculation. This method needs a lot of time (minimum 2-3 days) and according to our observation, does not allow to reveal all pathogenic strains. By inoculation of three plants (grapevine, tomato, carrot) with pathogenic strains isolated from the South Ukraine vineyards only 70,0 % of strains contained an *ipt* gene could be detected.

We used the *ipt*- and *virD*$_2$-primers recommended by Haas J. et al. (1995) and Manulis S. (2002). PCR with these pairs of primers allows detecting pathogenic strains with high specifity. The use of PCR with *ipt* and *virD*$_2$ primers was examined in production of clean planting material of asters and roses and showed excellent results (Manulis, 2002).

Two ways in DNA-diagnostics of crown gall agents exist. The first means initial isolation of bacterial cultures on semi-selective media and testing of isolated strains in PCR (BIO-PCR) (Manulis, 2002, Haas, 1995). The second way means isolation of total DNA and use of such DNA sample for amplification of certain sequences (Eastwell, 1995; Krimi, 2002). Both ways have their advantages and disadvantages and can be used in disease-free propagation material indexing. We used the BIO-PCR method.

Our optimizations included firstly the period of bacteria incubation on the semiselective medium. We incubated not less than 7 days as it was recommended earlier by investigators who proved that on Roy and Sasser medium colonies of *R. radiobacter* grow more slowly than *R.vitis* colonies (Burr, 1987).

The concentrations of PCR mix components, the temperatures of primers annealing and the number of PCR cycles were adjusted to obtain the most specific results.

The total DNA of all bacteria grown on one plate was used for analysis.

PCR analyses allowed us to reveal that the amount of grapevines infected in latent stage was 1,6 – 6,2 times higher than the amount of plants with crown gall symptoms.

It was shown that the crown gall of grape is widely spread on the South part of the Ukraine. Our investigations conducted during 2002 – 2008 years revealed that in some vineyards up to 70 % of some scion and rootstock cultivars were infected with pathogenic *Rhizobium vitis* and *R. radiobacter*. The percentage of diseased grapevines depended on cultivar susceptibility and the origin of planting material.

We revealed that both regular planting material and clones including imported planting material were infected with crown gall agents.

Our last investigation, carried out in 2008 on vineyards of Odesa region revealed that 50-years plants of native cultivars with own roots were almost not infected comparing with grafted ones of highly popular cultivars widely used in grapegrowing.

Vineyards with high number of infected grapevines reveal high number of soil samples contaminated with pathogenic *Rhizobium vitis* and *R. radiobacter*.

We identified vineyards free from crown gall agent and recommended them for grapevine planting material production. Plots where soil didn't contain tumorigenic *Rhizobium vitis* and *R. radiobacter* were recommended as planting sites for vineyards.

Conclusion. The diagnostics of crown gall of grape should be carried out by modern molecular biology methods such as PCR. Applying the PCR diagnostics, we could test a lot of planting material and big areas of vineyards in short terms. We highly recommend the introduction of PCR-based techniques in testing plant material for Ukrainian agriculture.

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