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PHYSIOLOGICAL PROFILE OF SOME PATHOGENIC BACTERIA ASSOCIATED WITH GRAPEVINE CROWN GALL

Oana-Alina BOIU-SICUIA[1, 2], Sorina DINU[2], Lavinia BARBU[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2]Research-Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd, District 1, Bucharest, Romania

Corresponding author email: sicuia_oana@yahoo.com

Abstract

The most devastating bacterial infection of grapevine is crown gall disease, especially for young vineyards and orchards. Considering these, a better understanding of the pathogen physiology will improve the diagnostic of the causal agent of tumours induction. Therefore, small tumours collected from young grapevine plants of Italian Riesling and Fetească neagră cultivars were analyzed in order to identify and characterize the causal pathogenic bacteria. Several bacteria were isolated from grapevine tumours of Miniş-Măderat vineyard. Only four isolates were selected (vv1, vv2, vv3, vv4) for their identical or similar colony morphology to a phytopatogenic reference strain of Agrobacterium tumefaciens. The isolates vv1, vv2 and vv3 were positive for esculinase and urease, but negative for 3-ketolactose. In the tumour inducing tests, the isolates vv1, vv3, vv4 and the reference At12 were found to induce hyperplasia on carrot slices. The isolates vv1, vv2 and vv3 were identified as Rhizobium vitis bv 3 (formerly known as Agrobacterium biovar 3 or Agrobacterium vitis, comb. nov. Allorhizobium vitis). The fourth isolate (vv4) was identified as Pantoea agglomerans. In vitro assay revealed that cooper sulphate inhibits bacterial growth at less than 5% concentration, and completely suppresses their growth at 5% or higher concentration.

Key words*: A. vitis, Pantoea agglomerans, grapevine crown gall.*

INTRODUCTION

Romania is currently ranked as the 4th in Europe, considering the area planted with grapevine (www.fao.org). The Food and Agriculture Organization of the United Nations - FAOSTAT has mentioned that, in 2016, Romania was having 175 057 ha of grapevine. This placed Romania as the tenth state worldwide in growing grapevine, after Spain (920 108 ha), France (757 234 ha), China (843 407 ha), Italy (668 087 ha), Turkey (435 227 ha), United States of America (409 947 ha), Argentine (223 944 ha), Israel (207 329 ha), and Chile (203 127 ha).

Crown gall is a serious grapevine disease worldwide causing vine decline and mortality in vineyards with important economic losses. In cold climate regions, grapevine is more susceptible to infection, expressing a particularly severe attack (Vizitiu & Dejeu, 2011). The causal agent is the phytopathogenic bacteria *Agrobacterium vitis*, syn. *Agrobacterium* biovar 3, named *Rhizobium vitis* by Young et al. (2001), and currently proposed to be renamed *Allorhizobium vitis* (Mousavi et al., 2015). However, in some cases, there was also found *Agrobacterium tumefaciens* (Szegedi et al., 2005; Bazzi et al., 2008), currently named *Rhizobium radiobacter* (Young et al., 2001, 2003). These pathogens are causing hyperplasia and hypertrophy in the infected plant tissue, generating tumours or galls (Burr et al., 1998, 1999; Kawaguchi et al., 2017). The infection process can occur naturally through the wounds caused by frost. Thus, colder and humid climate increase plant vulnerability to infection, especially in less winter-hardy cultivars. Severe winter weather, as well as the current climatic changes with extreme temperature fluctuations, late spring frosts or early winter frosts, damage the grapevine trunks increasing infection risks. Tumorigenic infection and pathogen spread is higher during grafting, pruning and other mechanical activities in the vineyard. Therefore, careful monitoring of plant phytosanitary status is important in order to minimize the pathogenic infection and disease

spread. Preventive measurements and treatments are of great importance since the infections are established before the symptoms occur.

Taking into consideration the impact of crown gall infection in young grapevine, we analysed the presence of the pathogen in some small tumours, collected from young plants of Miniş-Măderat vineyard in order to characterize the causal agent of the tumour induction.

MATERIALS AND METHODS

Grapevine canes and cordons with small or abundant tumours (Figure 1) were collected from young plants of Miniş-Măderat vineyard in order to characterize the causal agent of the crown gall. The samples were taken from young plants of *Vitis vinifera* L., Italian Riesling and Fetească neagră cultivars, grafted on SO4 rootstocks. The samples were collected in 2016, when the vineyard was seven to eight year-old.

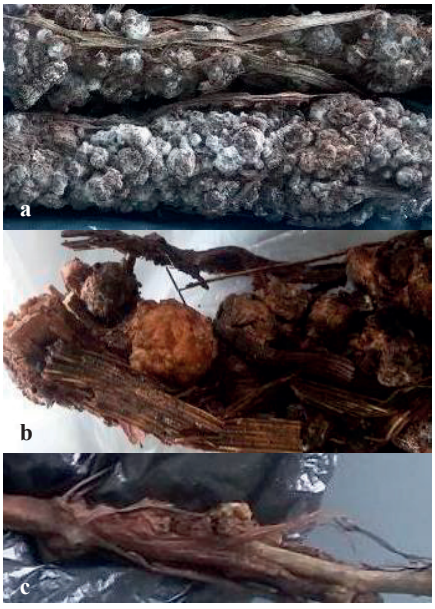

Figure 1. Tumours on canes and cordons from Italian Riesling (a, c) and Fetească neagră (b) cultivars

In order to detect the causal agent of the tumours, the samples were subjected to the analysis. Bacterial isolation was carried on MG-Te medium (Ophel & Kerr, 1990). This is an Agrobacterium semi-selective medium based on mannitol and glutamic acid, supplemented with potassium tellurite. To purify the bacterial cultures we used the streak plate method on MG-Te and Yeast extract Mannitol Agar (YMA) supplemented with Congo red. These two semi-selective media were also used to grow the reference strains *Agrobacterium tumefaciens* At12 and *Agrobacterium rhizogenes* Ar8196, which allowed a comparative analysis between the bacterial growths. The newly isolated strains were selected based on their morphological characteristics and growth similarities with the reference strains.

The isolated bacteria were subjected to some biochemical tests for agrobacteria (Campillo et al., 2012), like urease, β-glucosidase (esculinase), and 3-ketolactose. For the urease test fresh bacterial biomass was suspended in specific medium containing 2% urea, 0.3% L-tryptophan, 0.1% monopotassium phosphate, 0.1% dipotassium phosphate, 0.5% sodium chloride, 0.95% of pure ethanol, and 0.0025% phenol red in distilled water. The esculinase test was performed on broth medium containing 1% peptone, 0.1% esculine, and 2% ferric ammonium citrate in distilled water. These two specific solutions were filter sterilized, through 0.22µm membrane. For 3-ketolactose test, bacteria were grown on Lactose Agar medium for two days and cultures were flooded with Benedict's reagent. The yellow colour around microbial growth reveal lactose conversion to 3-ketolactose. This positive reaction is specific to *A. tumefaciens* (Shams et al., 2012).

The purified strains were biochemical characterized and identified based on their physiological profile using the Biolog GEN III microbial identification system and the standard IFA protocol, according to the manufacturer guidelines.

The pathogenicity test of the isolated strains was carried out *in vitro* on carrot slices inoculated with fresh bacterial suspension (10^7cfu/ml), using o similar protocol as described by Milijašević et al. (2007). Carrots were disinfected with Dakin's solution (0.5% sodium hypochlorite) and aseptically cut in slices with a sterile scalpel. The carrot disks were placed in sterile humid chambers of moistened filter paper in Petri dishes. For this

test, a negative control of sterile distilled water was used. As positive control, the reference strain *A. tumefaciens* At12 was also used. Inoculated carrot slices were incubated in moistened chambers for three weeks at room temperature. The pathogenicity test was repeated two times, each performed in four replicates.

Bacterial sensitivity to copper based treatments was also studied. In this study a modified version of disc diffusion method was used. For these test, 100 µl of fresh bacterial suspension was plated on Yeast Mannitol Agar (YMA). Several paper disc impregnated with 10 µl of fungicide solution, in different test concentrations, were placed on top of the agar plate. The fungicide solution was based on different concentrations of copper sulphate, neutralized with slaked lime, $Ca(OH)_2$. The Bordeaux mixture was used as reference. Thus, ten different concentrations of copper sulphate solution were tested: 0.75%, 1%, 1.5%, 2%, 3%, 4%, 5%, 6%, 6.5%, and 7% $CuSO_4$. Plates were incubated at 28ºC for 5 days, before analysing the growth inhibition zone.

RESULTS AND DISCUSSIONS

Four bacterial isolates were purified from the grapevine crown galls: vv1, vv2, vv3 and vv4 (Table 1). During isolation, bacterial growth obtained on MG-Te was compared with the reference strains *A. tumefaciens* At12 and *A. rhizogenes* Ar8196. Bacterial colonies having identical or similar colony morphology with the reference strains were harvested and bacterial cultures were purified on MG-Te and YMA with Congo red.

Table 1. Bacterial strains isolated from grapevine tumours

Bacterial isolates	Vineyard	ID code	Harvest area (ha)	Cultivar	Rootstock	Grapevine age	Disease symptoms
vv1	Miniș-Măderat vineyard	BRA-M	1.2	Italian Riesling	SO4 (*V. riparia* × *V. berlandieri*)	7 years	Crown galls on cane
vv2		ELI-M	3.5	Fetească neagră		8 years	Tumours on cane and cordons
vv3		BRA-M	1.2	Italian Riesling		7 years	Tumours on cane
vv4			1.2				Corky galls and cracking of bark on grapevine cordon

On MG-Te media, the bacterial isolates vv1, vv2 and vv3 exhibited black coloured, smooth, circular, convex, isolated colonies, identical with the reference agrobacteria (Figure 2a). Bacterial isolate vv4 presented similar morphology; however it had a much abundant growth, of dark grey colour and semi-translucent appearance (Figure 2b).

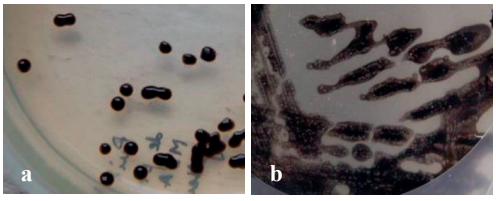

Figure 2. Bacterial growth on MG-Te semi-selective media: a) typical agrobacteria colonies of the vv1 isolate; b) vv4 bacterial growth

On YMA with Congo red, *A. tumefaciens* At12 reference developed bright red colonies. *A. rhizogenes* Ar8196 produced smooth, circular, convex colonies, whitish-pink in colour, with a central reddish spot after prolonged incubation on YMA with Congo red. Bacterial strains vv1, vv2 and vv3 developed pink colonies, with red pigmentation in the centre. Bacterial strain vv4 developed pink, punctiform colonies (Figure 3).

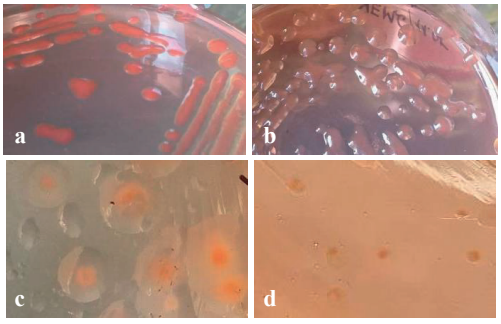

Figure 3. Bacterial growth morphology on YMA with Congo red: a) At12; b) Ar8196; c) vv1 strain; d) vv4 strain

Bacterial maintenance was made on YMA growth medium, on which luxuriant growth was obtained. Bacterial strains vv1, vv2, vv3 presented translucent, gummy, glistening, elevated colonies with entire margins, and vv4 strain had a fluid, translucent growth (Figure 4).

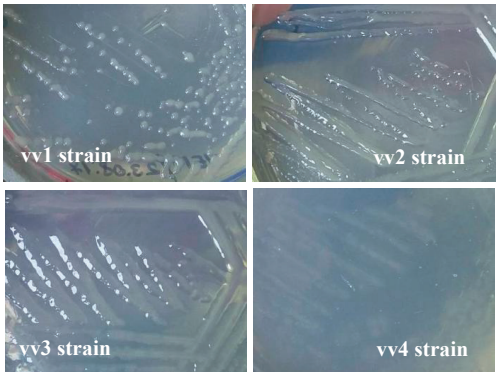

Figure 4. Bacterial strains on YMA growth media

The urease, esculinase and 3-ketolactose tests were performed for all isolates, and compared to the reference strain At12. Urease production was detected in At12 reference, vv1 and vv3 isolates. Regarding vv2, urease reaction was weak-positive after overnight incubation. The reaction was clear negative in vv4 (Figure 5). All bacterial strains, including the reference, were esculinase positive. Regarding these two test, a positive reaction should be obtained for agrobacteria detection (Campillo et al., 2012). Production of 3-ketolactose was detected only by the reference strain At12, the isolates tested being negative. According to several authors, 3-ketolactose is produced only by *A. tumefaciens*, *A. vitis* showing a negative response (Ophel & Kerr, 1990; Argun et al., 2002). Corroborating the results obtained for urease, esculinase and 3-ketolactose it is suggested that vv1, vv2 and vv3 could be affiliated to *A. vitis*.

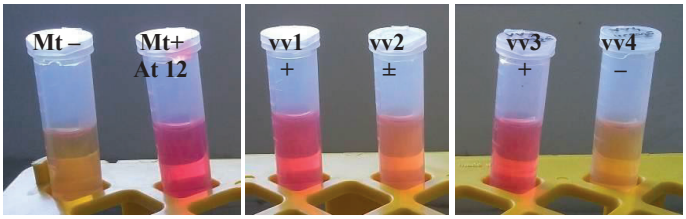

Figure 5. Urease production by different bacterial strains isolated from grapevine tumours

Species identification was made using the Biolog Gen III phenotypic microarray, based on 96 tests revealed by a redox reaction. Among these tests, 71 are biochemical tests and 23 are chemical tolerance tests. This technique is able to detect bacteria, at species and biovar level. The vv1, vv2 and vv3 isolates were assigned to *Rhizobium vitis* bv.3, also known as *Agrobacterium vitis* or *Agrobacterium* biovar 3, and currently reclassified as *Allorhizobium vitis* (Mousavi et al., 2015), which is the main pathogen involved in grapevine crown gall. This identification confirmed the results revealed by the previous tests. Unlike the first three isolates, vv4 strain was assigned to *Pantoea agglomerans*.

Studying the metabolic profile of vv1, vv2 and vv3 strains compared to *Rhizobium vitis* from the system database, it was shown that these newly isolated strains can metabolize dextrine faster than the references. Compared to vv1, vv3 and the database references, vv2 strain metabolised slower the fucose. Another difference among strains is that vv1 used L-arginine slower than vv2, vv3 and the database references. The vv1 and vv2 strains used L-histidine substrate slower compared to the references; vv1 and vv3 revealed a less intense redox reaction on p-hydroxyphenylacetic acid. All three strains (vv1, vv2 and vv3) presented rifamycin SV, lincomycin and potassium tellurite resistance, and vv1 was tolerant to fusidic acid.

Regarding the identified *Pantoea agglomerans* vv4 strain, it presented more rapid redox reactions on fucose, L-aspartic acid and citric acid compared to references from the system database. Although we isolated vv4 strain from grapevine tumours, *P. agglomerans* is however mentioned as a saprophytic specie with a cosmopolitan distribution, being an epiphyte colonizer of the vegetal material or an

endophyte symbiont (Nadarasah & Stavrinides, 2014; Walterson & Stavrinides, 2015). Although *P. agglomerans* is known to be a good competitor against *Erwinia amylovora* plant pathogen (Wright et al., 2001, Pusey et al., 2011), some strains are associated to plant, insects and human infections (Dutkiewicz et al., 2016), such as seed-born decay of maize (Silva-Rojas et al., 2016), or onion bulb rot (Edens et al., 2006).

Virulence or tumour induction capacity was evaluated for each newly isolated strain, in comparison with the reference bacterial strain At12. Therefore, artificially infected carrot slices were analysed after 3 weeks of incubation in humid chamber, at room temperature. Tumorigenic agrobacteria are able to induce tumour-like callus form the meristematic tissue. The carrot disks were checked for tumours in the pericycle area. Bacterial virulence was visually evaluated, as follows: grade 0 - for isolates that are not capable to induce tumours, grade 1 - for induction of solitary tumours in the pericycle area, grade 2 - for induction of several differentiated tumours, grade 3 - for confluent tumours in arc circle shape, grade 4 - for abundant tumours on the pericycle ring. The At12 positive control induced tumours classified as grade 3 and 4. In the negative control, were carrot slices were treated with sterile distilled water, no tumour was generated (Figure 6).

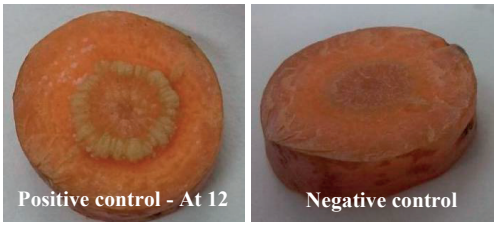

Figure 6. Young galls around the central vascular system induced by virulent agrobacteria

The vv1 and vv3 strains generated tumours of 3 and 4 grade, suggesting an increased virulence. The vv2 strain presented a lower virulence, as it generated small isolated tumours, of grade 1, in not more than two slices of each replicate (Figure 7).

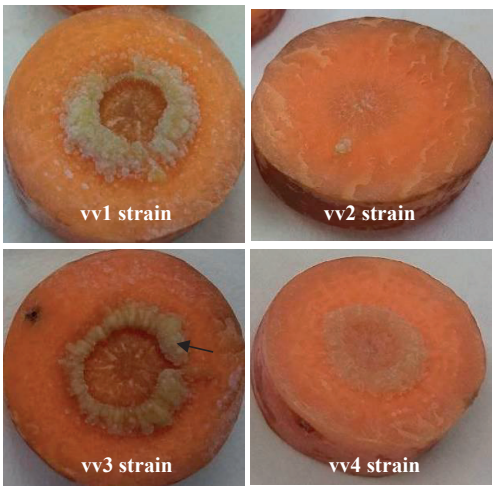

Figure 7. Hyperplasia and hypertrophies of the meristematic tissue from carrot pericycle, symptoms were induced by *R. vitis* vv1, vv2 and vv3 strains, and by *P. agglomerans* vv4

Although vv4 strain is not an agrobacteria, it produced foal, laced hypertrophies on the carrot slices (Figure 7). Since the result was not to be expected from a *P. agglomerans* strain, we hypothesized that meristematic tissue was stimulated to grow due to a high amount of auxine phytohormones, probably produced by vv4 strain. In *P. agglomerans* bacteria, formerly known as *Enterobacter agglomerans*, auxins production is a commonly found feature (Ludwig-Muller, 2014). These plant hormones regulate cell division and increases cells size (Perrot-Rechenmann, 2010).

Although copper bactericides could kill pathogenic agrobacteria by contact, they do not penetrate systemically in the vine (Burr, 2004). Studies performed by Vizitiu (2016) revealed that combination of copper sulphite with garlic tincture decreased gall apparitions caused by agrobacteria in grapevine and stopped the proliferation of pathogenic *A. tumefaciens*. Previous studies of Vizitiu & Rădulescu (2013) showed that the application of copper sulphate in combination with garlic tincture can reduce *A. tumefaciens* density in soil. Moreover, increased copper concentrations enhance some plant enzymes activity, like Phenylalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), and caffeic acid peroxidase (CA-POD), triggering lignin synthesis (Liu et al., 2018). Therefore copper

applications can help plants in the healing process of wounds caused by frost, or mechanical injuries. Thus reducing the incidence of bacterial infections.

Considering the tumorigenic potential of the isolated strains, bacterial sensitivity to copper based products, including Bordeaux mixture, was studied. Therefore, ten different concentrations of copper sulphate solution (0.75% to 7% $CuSO_4$) were used. All tested copper sulphate solutions were neutralized with calcium hydroxide, in equal concentration. Results suggested that in concentrations lower than 5% bacterial growth was inhibited. The inhibition zone being proportional with the tested concentration. At 0.75% CuSO4, reduced bacterial inhibition zones, of 0.1 to 0.3cm, were obtained (Figure 8b).

Treatment solutions based on copper sulphate in 5% or higher concentrations generated clear inhibition zones, were bacterial growth was completely blocked (Figure 8c), However, at less than 5% concentration bacterial inhibition zones were detected, but bacterial cells remained viable, and started to develop a weak/pale growth. Higher concentrated copper solutions (≥ 5%) induced clear zones, with no bacterial growth in the first 2-3 cm, surrounded by a pale growth, due to copper diffusion through agar.

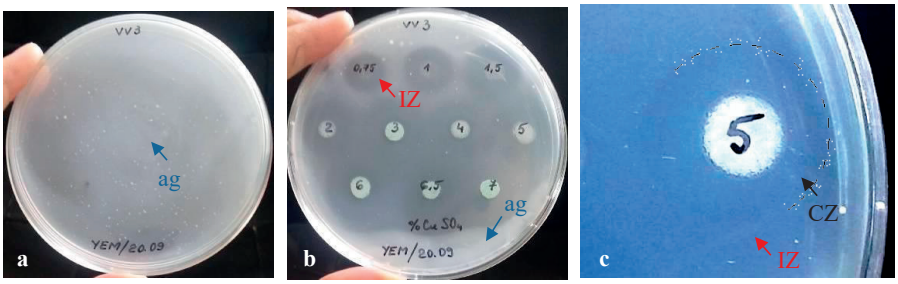

Figure 8. The inhibitory activity of copper based treatments against bacterial growth:
a) Bacterial growth in untreated culture; b) Inhibited bacterial growth, due to the copper sulphate solutions, tested in different concentrations; c) Clear zone with blocked bacterial growth neat the copper treated disks revealed at ≥ 5% $CuSO_4$ concentrations
Legend: ag = abundant bacterial growth; CZ = clear zone with blocked bacterial growth;
IZ = inhibition zone, were bacterial growth was diminished but not completely blocked;
- - - - = marking line between CZ and IZ.

Similar results were obtained among all bacterial strains (vv1, vv2, vv3 and vv4), including the reference strains At12 and Ar8196. This confirming that copper based products have a wide antibacterial spectrum.

These results support farmers' effort to reduce agrobacteria infections during winter and spring, when high concentrations copper treatments are limiting the spread of bacterial infection, favouring plants to heal their natural or mechanical wounds without contacting an infection.

CONCLUSIONS

A. vitis was found to be the causal agent of the grapevine crown gall of Italian Riesling and Fetească neagră cultivars, from Miniş-Măderat vineyard. Although *P. agglomerans* is generally considered not to be a threat to plants health, our tests suggested that *P. agglomerans* vv4 strains could induce proliferation in meristematic tissues most probably due to an increased phytohormone production. The isolated bacteria were identified through classical microbiological assays and Biolog Gen III microarray. Regarding farmer's need to treat and prevent pathogenic bacterial infection we recommend a spring treatment of 5% Copper sulphate solution before budding. These would reduce plant phyto-toxicity, but will eradicate the pathogen, preventing the spread of crown gall disease.

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