Romanian Journal for Plant Protection, Vol. XII, 2019
ISSN 2248 – 129X; ISSN-L 2248 – 129X

IDENTIFICATION OF CROWN GALL ASSOCIATED BACTERIA IN CHERRY TREES (*Prunus avium* L.)

Lavinia Diana Nicoleta Barbu [1,2], *Sorina* Dinu,[1] *Oana-Alina Boiu-Sicuia* [*1,2]

[1] Research-Development Institute for Plant Protection Bucharest, Romania
[2] University of Agronomic Sciences and Veterinary Medicine in Bucharest, Faculty of Biotechnology, Romania

Correspondence address:
Research-Development Institute for Plant Protection,
8 Ion Ionescu de la Brad Blvd., 013813, Bucharest, Romania,
phone: 004-021-2693231;
e-mail: sicuia_oana@yahoo.com

Abstract: Crown gall of cherry trees (*Prunus avium* L.) is mainly caused by the pathogenic bacteria *Rhizobium radiobacter* carrying the tumor-inducing (*Ti*) plasmids. The causal agent has a controversial taxonomy suffering different nomenclature changes based on the biochemical and genetic discoveries over the time. At first, this pathogenic bacterium was known as *Agrobacterium* biovar 1 or *Agrobacterium tumefaciens*, and currently as *Rhizobium radiobacter* carrying the *Ti* plasmid. The aim of this study was to identify the causal agent responsible for the crown gall symptoms of young cherry trees. Analyzed plants were provided from young orchards from Călărași County, between 2015 and 2019. The pathogen was foreseen based on its characteristic disease symptoms. However, pathogen identification was sustained by several microbiologic and biochemical tests and confirmed through Biolog GEN III technique. The pathogenicity test has revealed the isolated strains' virulence. Some other bacterial species were also associated with tumors.

Keywords: *crown gall, cherry trees*

INTRODUCTION

Cherry is an important fruit species in Romania, our country being in the top 5 in Europe regarding productivity, with 9.2 t/ha in 2017 (www.fao.org). Among the plant diseases affecting the cherry trees, crown gall has great economic impact in both nurseries and fruit orchards (Ali et al., 2010). The disease is caused by pathogenic bacteria carrying the tumor - inducing (Ti) plasmids. Bacteria carrying this plasmid have a controversial taxonomy suffering different nomenclature changes based on the biochemical and genetic discoveries over the time. At first, this pathogenic bacterium was known as *Agrobacterium* biovar 1 or *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942, and currently as *Rhizobium radiobacter* carrying the Ti plasmid (Young et al., 2001, 2003).

The crown gall formation is correlated with the transfer of specific DNA material (called T-DNA or transfer-DNA), from the pathogenic bacteria carrying the Ti plasmid in the genome of the infected plant cells. Therefore, it can be said that bacteria carrying the Ti plasmids mediate a natural process of genetic plant-transformation. According to Hwang et al (2017) the major steps of this process are: (i) the recognition of wounded plants, which release phenolic compounds that act as chemo-attractants for *A. tumefaciens*; (ii) bacteria attachment through cellulose fibers to the surface of plant cells (Escobar & Dandekar, 2003); (iii) *A. tumefaciens* up-regulates its virulence genes as a result of plant signal recognition, allowing the transduction and transport of T-DNA from the bacterial cells to plant cells; (iv) effector proteins trick the plant cells to insert the T-DNA in their genome; (v) nuclear integrated T-DNA express the agrobacteria-originated oncogenes into the plant cells; (vi) crown galls start to develop in plants, due to phytohormone synthesis encoded by the oncogenes of integrated T-DNA. Among the oncogenes detected in the T-DNA, only *IaaH*

(indole-3-acetamide hydrolase), *IaaM* (tryptophan monooxygenase) and *Ipt* (isopentenyl transferase) were found to be essential for crown gall development. These genes are involved in auxin (*IaaH*, *IaaM*) and cytokinin (*Ipt*) biosynthesis, which induces cell proliferation and differentiation (Zhang et al., 2015).

The crown gall pathogens can be isolated from different habitats: soil, rhizosphere and crown galls of various hosts (Collins, 2001). In diseased plants, many others are still identifying the causal agent of crown gall by biochemical tests, after a previous isolation of the pathogen through classical microbiologic techniques (Finer et al., 2016).

In our study we analyzed several young cherry trees with small and medium size tumors on their roots, collar, grafting point, or at the base of their stock. The aim of our study was to detect de causal agent. The classic techniques were mainly used for this purpose, although we validated the results using a microbial identification system based on the pathogen physiological profile.

MATERIALS AND METHODS

Biologic material. Young cherry trees were imported as planting material in two different orchards, of Călăraşi County. Some of these trees were found to have crown gall symptoms. Tree samples (Figure 1) were brought for laboratory analysis in order to identify the causal agent.

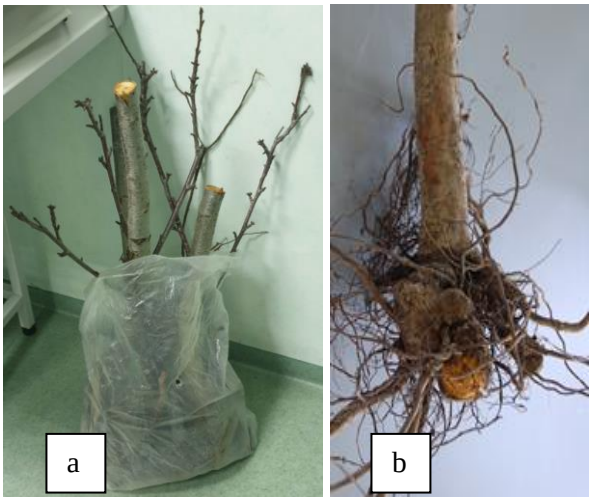

Figure 1. Cherry tree samples (a) brought for laboratory analysis and their crown gall symptoms (b)

Pathogen isolation. Tumor samples were collected from each cherry tree brought for analysis. The tissue was washed and disinfected with 4% sodium hypochlorite, then rinsed with sterile distilled water (SDW). Tissue samples were subsequently crushed in SDW and infused for 30 minutes. The obtained suspension was plated on solid media for agrobacteria isolation*:* MG supplemented with potassium tellurite, and YEM with Congo red. Samples were incubated at 28 ± 0.5°C for 72 hours. Isolated colonies having typical morphology for agrobacteria were purified on YEM.

Microbilogical and biochemical tests. Several laboratory tests presumptive for agrobacteria identification were used. Among these, we used the Gram stain reaction and motility (Constantinescu et al., 2010), oxidase test, urease and esculinase production, and 3-

ketolactose test (Islam et al., 2010). For each test we used one to three days old bacterial biomass from pure cultures.

The motility test was made on semi-solid medium with 0.3% agar. The plates were inoculated in the center with a small amount of bacteria biomass. The swimming motility was evaluated after 24h of static incubation at 28°C.

In the oxidative test, we used fresh Kovacs reagent, and the samples were evaluated as positive if a violet-indigo color reaction appeared in the first 10 to 30 seconds of bacterial exposure to the reagent.

The urease test was carried on urea broth medium, based on 3g/L L-tryptophan; 20 g/L urea; 1g/L KH_2PO_4; 1g/L K_2HPO_4; 5g/L NaCl; 10ml/L ethanol 95% and 25 mg/L phenol red. Positive reaction was attributed to those cultures that changed their color from yellow to fuchsia. The esculinase test was carried on broth medium, based on 10 g/L peptone; 1 g/L esculine and 20 g/L ferric ammonium citrate. Positive reactions were considered for those cultures that changed their color into dark brown after 24-48h of incubation at 28°C (Shams et al., 2012).

For the 3-keto-lactose test, bacteria must be grown on lactose medium (1% lactose, 0.1% yeast extract, 1.8% agar) for three days, at 28°C. After incubation, the plates were flooded with Benedict reagent. Positive reaction appears after 1h in biovar 1 agrobacteria are surrounded by an yellow ring caused by CuO_2 precipitation (Cubero & López, 2004).

In all of these tests we used a positive control of *A. tumefaciens* At12 strain, provided by the Faculty on Biotechnology, from the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

Pathogenicity test. The pathogenicity test was carried on carrot discs (Aysan & Sahin, 2003). Disinfected carrots were sliced (0.5 to 0.7 cm thickness) and placed on sterile humid chambers. Each disc was covered with a cotton plug carrying 200µl of fresh bacterial inoculum (10^8 cfu/ml). Plates were incubated at room temperature 3 to 4 weeks. Positive and negative controls were also prepared. The positive control consisted of At12 inoculum, and the negative control was made of sterile distilled water. The pathogenicity was appreciated based on the tumor size, indexed as: 0 = no tumors, 1 = single, very small tumor, 2 = small tumors, incomplete ring, 3 = medium size tumors, in almost complete ring, 4 = confluent large tumors, in a ring shape (Islam et al., 2010).

Biolog phenotypic microarray for bacteria identification. The identification test was carried out in order to confirm biovar 1 agrobacteria. The test was made in Biolog GEN III microplates, inoculated with 100µl bacterial suspension prepared in IFA fluid at 95-97% turbidity. Plates were incubated less than 48h at 33°C, then spectrophotometric analyzed with the semi-automat Biolog Microstation Plate Reader and MicroLog3 software, connected to the Biolog microorganisms database.

RESULTS AND DISCUSSIONS

From all 9 trees brought with crown gall symptoms, we were able to obtain agrobacteria characteristic growth on semi-selective MG-Te and Congo red YEM media. From the Mannitol Glutamate (MG) medium supplemented with potassium tellurite we selected only isolated colonies that reduced tellurite and developed black, glistening, convex and circular colonies. From Yeast Extract Mannitol (YEM) Agar supplemented with Congo red, we selected only translucent-reddish colonies with convex, glistening and circular shape that did not change the pigmentation of the medium.

A. tumefaciens At12 strain was used as positive reference. This bacterial strain was also grown on MG-Te medium to reveal the bacterial colony morphology and the positive reaction

of tellurite reduction (Figure 2a). On Congo red YEM medium the agrobacteria developed reddish colonies with convex, glistening aspect (Figure 2b).

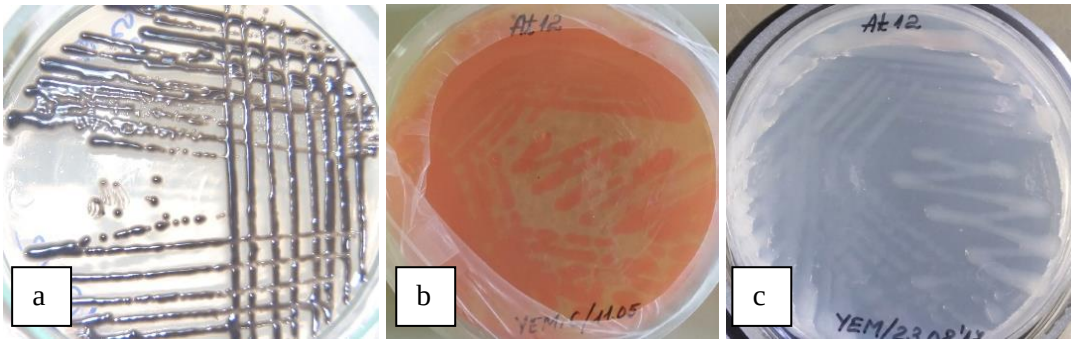

Figure 2. Colony morphology of *Agrobacterium tumefaciens* At12 reference strain on MG-Te (a), Congo red-YEM (b) and YEM (c) media

Selected isolates with agrobacterium colony morphology were purified on YEM or MG agar and macroscopic characterized as smooth, whitish or semi-translucent colonies, glistening, convex and circular shaped (Figure 3).

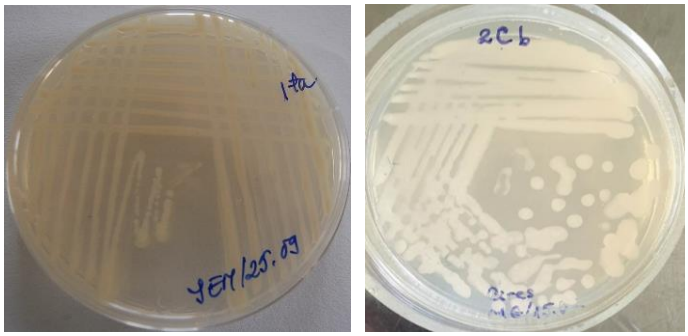

Figure 3. Colony morphology of some selected isolates: 1ta on YEM (left) and 2Cb on MG (right) media

All 23 selected isolates were Gram negative bacteria. Out of these, 20 isolates (~87%) were oxidase positive, but only 16 isolates (69.6%) having swimming motility were kept, as they were similar to At12 reference strain (Figure 4).

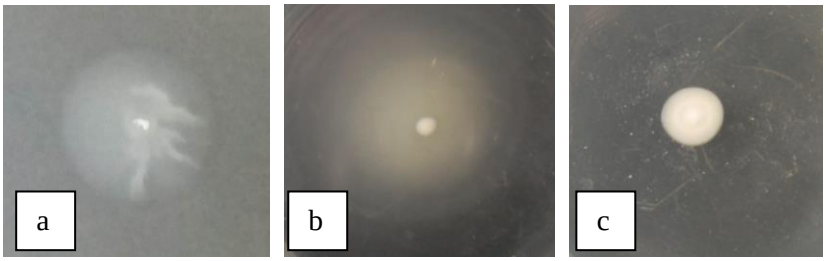

Figure 4. Swimming motility of At 12 strain (a) and 2Cb isolate (b) on soft agar medium compared to a non-motile bacteria (c)

The biochemical tests for urease, esculinase and 3-keto-lactose were performed only with the 16 selected bacteria. Among these, 11 isolates were urease positive (Figure 5 a), 13 were esculinase positive (Figure 5 b) and 8 produced 3-keto-lactose (Figure 6).

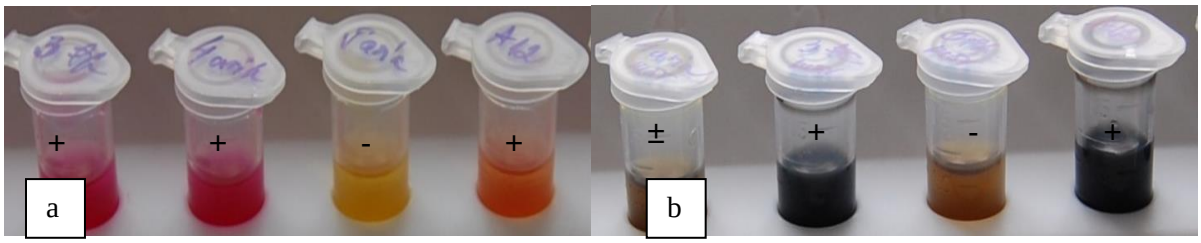

Figure 5. Biochemical test revealing the bacterial strains with positive and negative reactions for urease (a) and esculinase (b) production, compared to the positive At12 reference.

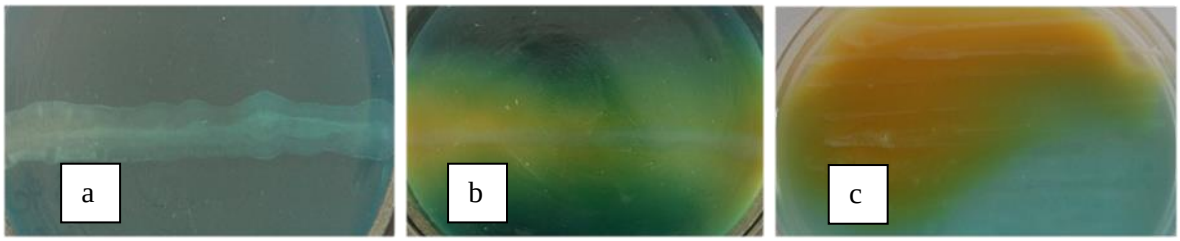

Figure 6. Screening for 3-ketolactose production:
(a) negative reaction, (b) positive reaction, (c) positive reaction in At12 reference strain

According to the pathogenicity test, the reference strain At12 was highly virulent, inducing ring tumors on the carrot slices, with an index 4 (Figure 7). Comparing the newly isolated strains with this reference, all 11 strains tested were pathogenic. Five of these isolates revealed high virulence (index 4), while all the rest revealed moderate virulence (index 2 and 3).

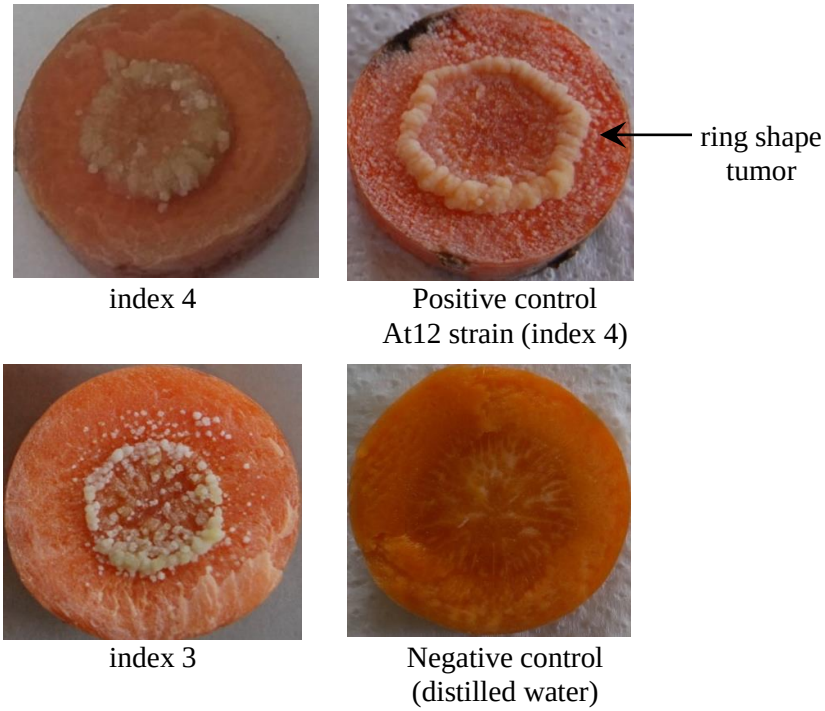

Figure 7. Pathogenicity test on carrot slices

According to these laboratory tests, all 11 strains tested comprised the pathogenic (tumor inducing) characteristics of agrobacteria. To validate these suppositions, two isolates, one from each orchard, were subjected to phenotypic identification with the Biolog Microbial Identification System. Both strains (2Cb and 3tb) were assigned to *Rhizobium radiobacter*

(Figure 8), also known as *Agrobacterium tumefaciens* biovar 1, the main pathogen involved in crown gall.

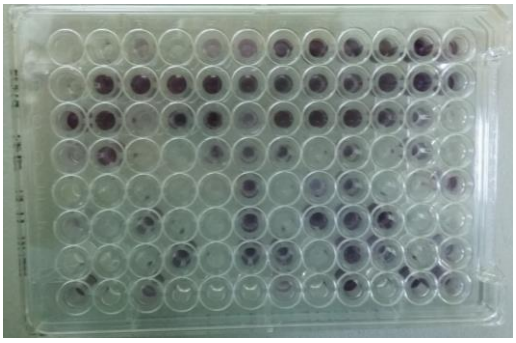

Figure 8. The physiological profile of 1Da strain identified as *Rhizobium radiobacter*

For 2Cb strain, the probability of the identification process was estimated as 99.6%, with a similarity of 87.8% between the phenotype of our strain and those from the database. In the case of 3tb strain, the probability of the identification process was estimated as 95.3%, with a similarity of 78.7%. The phenotypic identification of these strains validate results revealed by the previous microbiological and biochemical tests, and confirm that the crown gall symptoms observed on the cherry trees are caused by agrobacteria infections.

CONCLUSIONS

Laboratory diagnostics of plant pathogens can influence the phytosanitary measurements in the orchard. Therefore, early identification of pathogenic infections reduces disease spread. Although molecular techniques are quicker and accurate, classical identification methods could also be used for pathogen detection. However, for precise diagnostic, if a pathogenic infection is detected in a plant, the disease must be confirmed through a second analysis method, in order to validate the result.

In our study, after classical and phenotypic analyses we were able to confirm that the young cherry trees sampled from two orchards of Călărași County were infected with pathogenic *Rhizobium radiobacter* (syn. *Agrobacterium tumefaciens*) the causal agent of crown gall disease.

REFERENCES

ALI, H., KHALIL, A., HUSSAIN, A., IMRA, N. (2010). Incidence and severity of crown gall disease of cherry, apple and apricot plants caused by *Agrobacterium tumefaciens* in Nagar Valley of Gilgit-Baltistan, Pakistan. *Pakistan Journal of Nutrition*, 9, Doi: 10.3923/pjn.2010.577.581.

AYSAN, Y., SAHIN, F. (2003). An outbreak of crown gall disease on rose caused by *Agrobacterium tumefaciens* in Turkey. *Plant Pathology*, 52: 780-780.

CONSTANTINESCU, F., SICUIA, O.A., ZAMFIROPOL, R., DINU, S., OANCEA, F. (2010). Beneficial characteristics of some bacterial strains isolated from rhizosphere. Romanian Journal of Plant Protection, 3: 6-11.

CUBERO, J., LÓPEZ, M.M. (2004). *Agrobacterium* persistence in plant tissues after transformation, In: Peña L. (ed.). *Trans-genic Plants*, 351-364. Humana Press, Totowa, NJ, USA.

ESCOBAR, M.A., DANDEKAR, A.M. (2003). *Agrobacterium tumefaciens* as an agent of disease. *Trends in Plant Science*, 8, 380-386. Doi: 10.1016/S1360-1385(03)00162-6.

FINER, K.R., FOX, L., FINER, J.J. (2016). Isolation and characterization of *Agrobacterium* strains from soil: A laboratory capstone experience. *Journal of Microbiology & Biology Education*, 17, 3, 444-450. Doi:10.1128/jmbe.v17i3.1124.

HWANG, H.H., YU, M., LAI, E.M. (2017). *Agrobacterium*-mediated plant transformation: Biology and applications. *The Arabidopsis Book*, 15, Doi: 10.1199/tab.018.

ISLAM, S., AKTER, M., RAHMAN, A., RAHMAN, M., AKHTAR, M., ALAM, F. (2010). Isolation of *Agrobacterium tumefaciens* strains from crown gall sample of dicot plants in Bangladesh. *Current Research in Bacteriology*, 3, 27-36. Doi: 10.3923/crb.2010.27.36

SHAMS, M., CAMPILLO, T., LAVIRE, C., MULLER, D., NESME, X., VIAL, L. (2012). Rapid and efficient methods to isolate, type strains and determine species of *Agrobacterium spp.* in pure culture and complex environments. *In Biochemical Testing*, 3-16. Edited by Jimenez-Lopez J.C., *InTech*. Croatia. Doi: 10.5772/36068.

YOUNG, J.M. (2001). Classification, naming, and plant pathogenic bacteria – what is to be done? *In Plant Pathogenic Bacteria*, 30-37. Proceedings of the 10th International Conference on Plant Pathogenic Bacteria, 23-27 July 2000, Charlottetown, Prince Edward Island, Canada. Edited by S. H. De Boer. London: Kluwer Academic Publisher

YOUNG, J.M., KUYKENDALL, L.D., MARTÍNEZ-ROMERO, E., KERR, A., SAWADA, H. (2003). Classification and nomenclature of *Agrobacterium* and *Rhizobium*. *International Journal of Systematic and Evolutionary Microbiology*, 53, 5, 1689-1695.

ZHANG, Y., LEE, C.W., WEHNER, N., IMDAHL, F., SVETLANA, V., WEISTE, C., DRÖGE-LASER, W., DEEKEN, R. (2015). Regulation of oncogene expression in T-DNA-transformed host plant cells. *PLoS Pathogens*, 11, 1, e1004620. Doi: 10.1371/journal.ppat.1004620