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PARTIAL RESULTS REGARDING THE DETECTION AND IDENTIFICATION OF PATHOGENS ON DENDROFLORIC PLANTS IN DIFFERENT GREEN SPACES OF CRAIOVA MUNICIPALITY

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Abstract

The purpose of the study in this paper was to detect and identify pathogens in some dendrofloric plants in different green spaces of Craiova. The detection and identification of the attack of different phytopathogens consisted both in periodic visual observations in the experimental area on certain organs of the host plants that showed symptoms of disease, and in performing laboratory tests. Regarding the detection of certain bacterial diseases, in the monitored period, following bacteria were identified: Agrobacterium tumefaciens, Erwinia carotovora, Pseudomonas marginalis, Xanthomonas hyacinthi, Xanthomonas campestris pv. campestris, Xanthomonas hortorum pv. pelargoni. For proper identification, not only macroscopic observations, but also laboratory tests are required to certify the initial identification in the field and, where possible, a pathogenicity test on susceptible plants was performed, obtaining the reproduction of the disease in the test plants.

Key words**:** *observation, test, isolation, detection, identification.*

INTRODUCTION

From ancient times the man has lived in close contact with nature showing interest in it and especially in dendrofloric plants, the wonders of the world that enrich our lives with the scent, color, shape and health it offers us by producing oxygen, an indispensable gas of life. The most important role in bacteriology was played by Erwin Smith (1854-1927) who laid the foundations of the research method in phytobacteriology, being recognized as the father of this branch of science (Alexandri et al., 1967; Hatman et al., 1989). In Romania, Săvulescu, Rădulescu, Olga Săvulescu, Severin, Preda, Popescu, Pop, Lazăr, Alexandri, Ghimpu and Iliescu laid the foundations of phytobacteriology and had important contributions in this field. As a result of the interaction between bacteria and the host, smaller or larger changes appear in the host plant, so that various symptoms may occur (Rădulescu & Negru, 1967). More than 200 species of phytopathogenic bacteria are known worldwide (Mitrea R., 2006).

The ,,Fire blight" caused by the pathogen *Erwinia amylovora* was first reported in the United States in 1780 as one of the oldest phytopathological diseases described, and only a century later it was shown that the disease was caused by a bacteria capable of killing destroys considerable crops in just a few weeks (Burte, 1992). It was conducted the first study to investigate *Erwinias'* relationships using several strains of *E. chrysanthemi* and all *E. carotovora* subspecies (Avrova et al., 2002). In a study conducted in 2018 on the assortment of flowering plants cultivated in different green spaces of Craiova Municipality the attack of twenty-seven pathogens was reported on the nineteen host plants (Sălcudean (Ioniță) et al., 2019).

According to (Severin & Iliescu, 2006), the main symptoms caused by bacteria in plants are:

- spots and burns of different shapes and sizes, with or without halo - may occur on all aerial organs of the plant; are produced by bacteria of the genera *Erwinia, Pseudomonas and Xanthomonas*;
- wilting - are due to the invasion of the xylem by bacteria, which subsequently multiplies and then migrates throughout the plant; wilting usually kills part or all of the plant; are produced by some bacteria of the genera *Clavibacter, Curtobacterium, Pseudomonas, Ralstonia* and *Xanthomonas*;

- soft rot - may occur in vegetation or storage, giving off a characteristic odor; are due to bacteria of the genera *Erwinia* and *Burkholderia*;
- hypertrophies - are represented by galls and tumors that can develop on roots, leaves and stems; bacteria of the genera *Agrobacterium* and *Rodococccus* are responsible for such manifestations;
- ulcers - usually appear on the stem and branches of the plant, being represented by necrotic areas, longitudinal cracks of the scab, growths or cavities; bacteria of the genera *Clavibacter, Pseudomonas* and *Xanthomonas* are responsible for the existence of ulcers.

In general, for bacteriosis, the main means of movement and dispersal is considered the propagating material, and trade is an important factor in the spread of pathogens.

Another important aspect is related to the use of their own propagating material, obtained from infected crops and which led to the spread and growth of areas where bacterial diseases have been reported in ornamental plants.

MATERIALS AND METHODS

The researches was carried during the period 2018-2021, the studied material being represented by dendrofloric plants from different green spaces of Craiova Municipality. Field phytosanitary checks show that several pathogens can attack a variety of dendrofloric species, and the manifestations can be similar, even if they are attacked by different pathogens. The biological material was represented by plants and parts of plants with symptoms belonging *Prunus nigra*, *Hyacinthus orientalis, Brassica oleracea var. acephala* and *Pelargonium* sp.

In addition to macroscopic observations, the detection and identification of some of the phytopathogenic agents was performed in the biology laboratory of the high school "Dimitrie Filișanu" - Filiași, where they were planted in pots and grown in laboratory conditions in order to be able to make several observations until the appearance of obvious symptoms of the disease, part of the samples taken from the field. The detection and identification of the attack of different phytopathogens consisted both in periodic visual observations in the experimental area on certain organs of the host plants that showed symptoms of disease, and in performing laboratory tests. The different test methods used were developed according to Janse (2005) and Schaad et al. (2001), as follows:

- **macroscopic observation -** the material suspected of being infected with bacteria was subjected to visual examination; thus, if visible changes were detected - rotten areas or areas with spots and necrotic lesions, tumor like growths, additional investigations were performed to highlight various bacterial diseases;
- **isolation method on culture medium -** isolation was made from plant material suspected of being infected with phytopathogenic bacteria (examples: leaves, stems, bulbs etc.); the first step was washing, followed by disinfection (with 70% ethanol) of the infected material; then small portions of the affected areas were taken; sampling was performed at the boundary between the healthy and diseased material, and the pieces of material obtained were placed in extraction bags and crushed with a hammer (Figure 1); sterile water was added until the material was covered and then stirred at room temperature for 30 minutes for the soft plant material (leaves, bulbs) and 1-2 hours for the wood material (tumors); the isolation of the obtained suspensions was made in sterile conditions, in a vertical laminar air flow cabinet by streaking media with o loop; semi-selective culture media (King´B medium) were used for the detection of *Pseudomonas* and non-selective media (NA - Nutrient Agar, YPGA - Yeast Peptone Glugose Agar) for the detection of other bacteria; incubation of inoculated plates was performed at 27°C for at least 5 days; suspicious colonies were subjected to additional tests, such as rapid serological tests, biochemical tests, hypersensitivity tests and/or pathogenicity tests;
- **rapid serological tests -** Fast Lateral Flow test was used, which is very easy to perform and is based on an antigen-antibody reaction; it allowed rapid detection of antigens using specific antibodies. This test was used only to

detect *Xanthomonas hortorum* pv. *pelargonii*, using a kit from Loewe-Germany (Figure 2); a suspicious colony was resuspended in a buffer provided by the manufacturer; the mixture was stirred for a few seconds and then 3-4 drops of it were placed in the well of the test box; the result was read and interpreted after 5-15 minutes at room temperature;

Figure 1. Test preparation for analysis (original)

Figure 2. Fast Lateral Flow Test (original)

- **biochemical tests -** young colonies were needed to perform biochemical tests. They were either resuspended in sterile water to obtain slightly turbid suspensions, or were used as such; the following biochemical tests were performed:

- *test on liquid Beef medium* (meat broth) - was performed by pipetting 500 µL suspicious bacterial suspension in a tube with liquid Beef medium; after inoculation, the tubes were incubated for 3-5 days at an optimum growth temperature of 27°C;
- *oxidative band method* - a suspicious young colony was chosen and placed on an oxidative band; interpretation of the result was performed after 1 minute;
- *hydrogen sulfide* (H_2S) *production* - suspicious colonies were transferred using a sterile loop and inoculated by pricking in a tripton medium; the inoculated tubes were incubated for 3-5 days at 27°C; a strip of paper soaked in lead acetate was then inserted into the tube, securing it with a stopper so that the tripton medium would not be touched; the tubes were further incubated and followed for 1-2 days until a reaction occurred;
- *starch hydrolysis* - a suspicious colony was streaks on a Petri dish with solid soluble starch medium; the plates were incubated at 27°C for 3-5 days, until bacterial growths appeared; Lugol iodine solution was pipetted onto the Petri dish and waited a few minutes for the results to be interpreted;
- *mobility* - young, suspicious colonies were taken with a loop; they were inoculated by stabbing in a tube with motility-specific medium (medium with peptone and sodium chloride); interpretation of the results was performed after 3-5 days of incubation at 27°C;

- **hypersensitivity test** - this test was performed on tobacco plants, *Nicotiana tabacum* in the stage of 3-4 true leaves; the suspicious bacterial colonies were placed in 2 mL of sterile water, obtained a slight turbidity bacterial suspensions; they were inoculated, using a hypodermic needle syringe, into the intracellular space of the leaves of the tobacco test plants, which were then incubated at room temperature and monitored for 1-2 days;

- **pathogenicity test** - the pathogenicity test may be performed on carrot or potato slices, on detached leaves or on test plants susceptible to target bacteria; bacterial suspensions were made from suspicious colonies and inoculated on the material used to establish pathogenicity:

- *vegetal tissue test* - was performed on carrot slices for the detection of *Agrobacterium tumefaciens* and on detached leaves from *Pelargonium* sp. for the detection of *Xanthomonas hortorum pv. pelargonii*; the surface of the plant material was disinfected with 70% ethanol for a few seconds, after which the

excess ethanol was removed with a napkin; in the case of using carrots, they were sliced to obtain 0.5 cm thick rounds, which were placed in Petri dishes with 1% agar medium; then were made bacterial suspensions from the suspicious colonies; the plant tissue of the test plant was injured by scratching in the case of carrot slices and by stinging in the case of geranium leaves; between 100-1000 µL of bacterial suspension was pipetted over the injured wounds; the plates thus inoculated were placed in a box with a lid to maintain a moist environment and then incubated at 27°C for several days, until symptoms appeared.

- *vegetal tissue maceration test* - was performed to establish the pectolytic activity of bacteria: *Xanthomonas campestris pv. campestris* on *Brasica oleracea var. acephala* (decorative cabbage), *Erwinia carotovora*, *Pseudomonas marginalis* and *Xanthomonas hyacinthi* on *Hyacinthus orientalis*; this test was performed on potato slices; thus, after the surface of the potato tubers was disinfected with 70% ethanol and the excess alcohol was removed with a napkin, the vegetable tissue was sliced and placed in a Petri dish with 1% agar; bacterial suspensions were made in sterile water from suspicious colonies; then about 1000 µL of bacterial suspension was pipetted over the tissue used; to maintain a moist environment, inoculated Petri dishes were placed in a container with a lid and incubated for 48 hours at 27°C;
- *plant test* - was performed for the artificial reproduction of the disease on sensitive test plants. The test was performed on plants of *Pelargonium sp.* for *Xanthomonas hortorum pv. pelargonii* and plants of *Solanum lycopersicum* 'Money Maker' for *Agrobacterium tumefaciens* and *Xanthomonas hortorum* pv. *pelargonii*; plants were used in the stage of 1-2 true leaves; in the case of confirmation of the bacterium *Agrobacterium tumefaciens*, bacterial suspensions obtained directly from the extract; in the case of reproduction of the disease caused by *Xanthomonas hortorum* pv. *pelargoni* were used bacterial suspensions made by resuspending suspicious young colonies in sterile water; the suspensions were inoculated into the stem and the plants were incubated at 26-28°C until the onset of disease symptoms.

RESULTS AND DISCUSSIONS

Regarding the detection of certain bacterial diseases, following macroscopic observations, in the monitored period, various symptoms produced by bacteria were identified (Table 1).

Table 1. Bacteriosis identified in the experimental area

Host plant	Harmful organism	Disease - Symptoms
Prunus nigra	*Agrobacterium tumefaciens*	**Bacterial cancer** - tumors like cauliflower inflorescences, at the base of the stem (Figure 3)
Hyacinthus orientalis	*Erwinia carotovora*	**The soft rot of the bulbs** - small plants, bulbs soft, macerated, mucilaginous and blackened, with an unpleasant odor, characteristic of rot (Figure 4)
	Pseudomonas marginalis	**Marginal burning of leaves** - stagnation of plant growth and necrotic lesions at the top of the leaves (Figure 5)
	Xanthomonas hyacinthi	**The yellow rot of hyacinth bulbs** - areas of macerated tissue and necrotic areas inside the bulbs; yellowed and browned areas towards the top of the leaves or leaf rot (Figure 6)
Brassica oleracea var. acephala	*Xanthomonas campestris pv. Campestris*	**The black nervation of the cruciferous leaves** - yellowing and internervurian necrosis; vascular ring necrosis (Figure 7)
Pelargonium sp.	*Xanthomonas hortorum pv. Pelargonii*	**Staining of leaves and rot of geranium stalks** - leaves with internervurian necrotic areas, with yellowed edge of parenchima; whole dried leaves (Figure 8)

To confirm the disease caused by ***Agrobacterium tumefaciens***, pathogenicity tests were performed on *Daucus carota* slices and on plants a test of *Solanum lycopersicum* cv. 'Money Maker'. On the carrot slices, after about 3 weeks, whitish tumors appeared near the vascular ring (Figure 9. a).

Also, on the stems of the test plants, after 4-6 weeks after inoculation, tumors of different sizes, light green-whitish color were observed (Figure 9. b).

Erwinia carotovora was detected by the hydrolysis of starch, a test in which clear

areas appeared around the bacterial growths, the rest of the medium being stained due to the activity of Lugol's iodine. Also, following the establishment of the pectolytic activity of the suspicious bacteria, it was found that they showed such an activity that led to changes in the substrate (Figure 10.a). The color and consistency of the potato slices changed, leading to the maceration of the vegetal tissue (Figure 10.b)

Figure 3. Tumors of *Agrobacterium tumefaciens* on *Prunus nigra* (original)

Figure 4. Symptoms of *Erwinia carotovora* on *Hyacinthus orientalis* (original)

Figure 5. Symptoms of *Pseudomonas marginalis* on *Hyacinthus orientalis* (original)

Figure 6. Symptoms of *Xanthomonas hyacinthi* on *Hyacinthus orientalis* (original)

Figure 7. *Xanthomonas campestris* pv. *campestris* on *Brasica oleracea* var. *acephala* (original)

Figure 8. Symptoms of *Xanthomonas hortorum* pv. pelargonii on *Pelargonium* sp. (original)

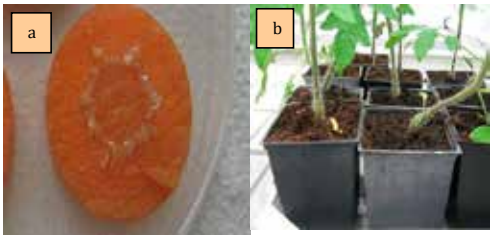

Figure 9. *Agrobacterium tumefaciens* - laboratory tests a - pathogenicity test on carrots; b - pathogenicity test on tomato plants (original)

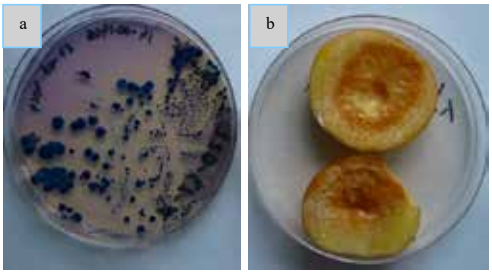

Figure 10. *Erwinia carotovora* - laboratory tests a - hydrolysis of starch; b - vegetal tissue maceration test (original)

Pseudomonas marginalis was isolated on King´B medium, obtaining fluorescent, translucent colonies (Figure 11.a). The first test performed after obtaining suspicious bacterial colonies was the oxidative band test. After the test was completed, a purple coloration was observed (Figure 11.b). The

purple color indicates a positive reaction. Another test performed was the vegetal tissue maceration test which was also positive. The vegetable tissue of the potato slices used was macerated and browned (Figure 11.c). Being a bacterium of the genus *Pseudomonas*, the hypersensitivity test of tobacco leaves was also performed. At the injection point of the bacterial suspension appeared a yellow area that extended into the parenchyma of the leaf, followed by necrosis and drying (Figure 11.d).

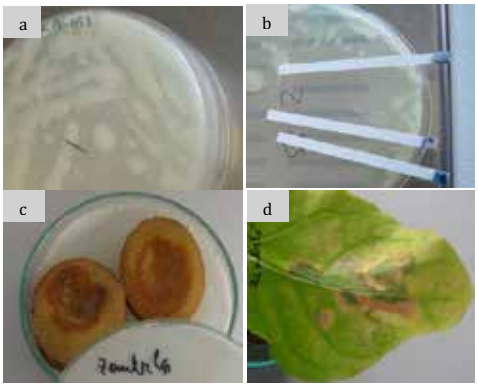

Figure 11. *Pseudomonas marginalis* - laboratory tests
a - isolation on King´B medium; b - oxidase test;
c - vegetal tissues maceration (on potato slices);
d - hypersensitivity test on tobacco leaves (original)

Xanthomonas hyacinthi was identified by the following tests: isolation, biochemical, hypersensitivity and vegetal tissue maceration. On the culture medium the colonies were glossy and light yellow. Four biochemical tests were performed and all corresponded to the biochemical profile of this bacterium.

Thus, the bacteria did not produce hydrogen sulfide (H_2S) (Figure 12.a), hazing the Beef medium (Figure 12.b), were mobile (Figure 12.c) and weakly hydrolyzed the starch (Figure 12.d).

Therefore, the liquid Beef medium was hazing due to the growth of bacteria and the paper soaked in lead acetate did not turn black, so no H_2S was produced.

The test of vegetal tissues maceration leds to the total maceration of the potato slices used, which indicated a very high pectolytic activity (Figure 12.e). The hypersensitivity test on tobacco leaves revealed the appearance of necrotic areas in the area of the parenchyma injected with bacterial suspension (Figure 12.f).

Xanthomonas campestris pv. campestris was identified by isolation on non-selective YPGA (Yest Peptone Glucose Agar) culture medium. After a few days of incubation, small, circular, smooth, yellow, shiny colonies appeared (Figure 13.a, b). The colonies also showed pectolytic activity leading to maceration and blackening of the plant tissue used in the test (Figure 13.c, d).

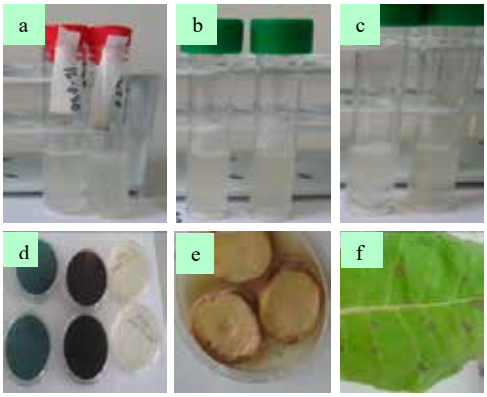

Figure 12. *Xanhomonas hyacinthi* - laboratory tests
a - H_2S production; b - test on liquid beef medium (broth); c - mobility test; d - starch hydrolysis;
e - vegetal tissue maceration test;
f - hypersensitivity test (original)

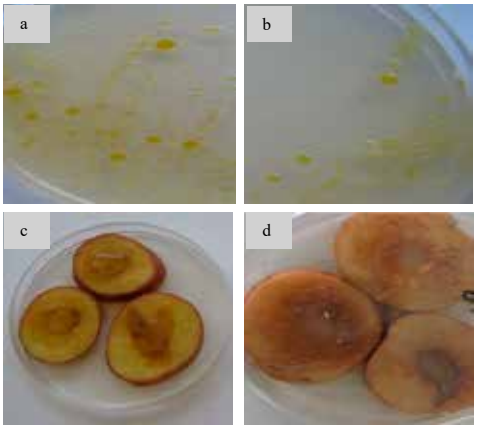

Figure 13. *Xanthomonas campestris pv. campestris* - laboratory tests
a, b - isolation on non-selective culture medium;
c, d - vegetal tissue maceration test (on potato slices) (original)

Xanthomonas hortorum **pv.** ***pelargonii*** was identified by the following tests: isolation on

culture media, hypersensitivity, vegetal tissue test and plant pathogenicity. Isolation on culture medium was performed on non-selective media (YPGA - Yeast Peptone Glucose Agar and NA - Nutrient Agar).
After 3 days of incubation, yellow, smooth and translucent colonies appeared. The color of the colonies was more intense on the YPGA medium than on the NA (Figure 14.a, b). Bacterial suspensions were made from the suspected colonies, which were tested serologically, obtaining a positive reaction to the Fast lateral flow test (Figure 14.c).

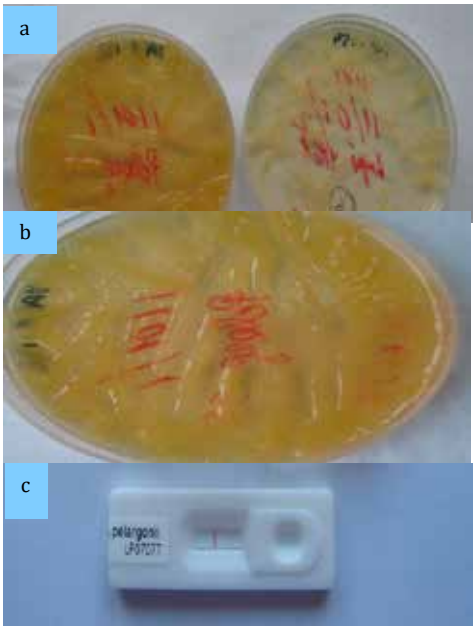

Figure 14. *Xanthomonas hortorum* pv. *pelargonii* - laboratory tests
a, b - isolation test on non-selective culture media;
c - Fast lateral flow test (original)

Also, the suspensions that were inoculated by injection into *Nicotiana tobacco* and *Pelargonium* sp. leaves led after 24-48 h to the collapse of the inoculated parenchyma (Figure 15.a and 15.b).
During the pathogenicity test, after inoculation of the seedlings of *Solanum lycopersicum*, it led to necrotic lesions on the leaves and to the upward rolling of the leaf (Figure 15.c).

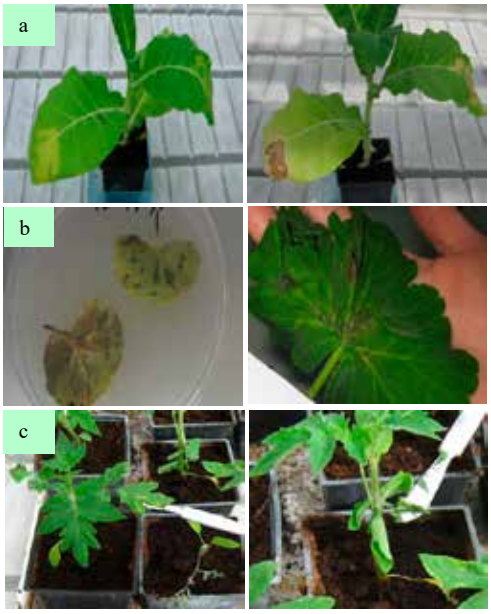

Figure 15. *Xanthomonas hortorum pv. pelargonii* - laboratory tests
a - hypersensitivity test; b - pathogenicity test on leaves detached from *Pelargonium* sp.; c - test on *Solanum lycopersicum* (original)

CONCLUSIONS

The starting point in the detection of diseases caused by bacteria were the macroscopic observations made in the field that showed the existence of symptoms, but without being able to make an exact diagnosis.
For proper identification, not only macroscopic observations but also laboratory tests are required to certify the initial identification in the field.
Regardless of the host plant or the bacterium suspected of producing the present symptoms, the first laboratory test was to isolate the bacteria from the suspicious material on various non-selective or semi-selective culture media.
After isolation, bacterial colonies were used for identification by additional tests (serological, biochemical and hypersensitivity tests) and, where possible, was performed a confirmation test (pathogenicity test on susceptible plants) in order to reproduces the disease in test plants under laboratory conditions.

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