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# ISOLATION, IDENTIFICATION AND ANTIBIOTIC SUSCEPTIBILITY OF *CURTOBACTERIUM FLACCUMFACIENS* STRAIN PM_YT FROM SEA DAFFODIL (*PANCRATIUM MARITIMUM* L.) SHOOT CULTURES

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**ABSTRACT**

After eight years of cultivation of Sea daffodil (*Pancratium maritimum* L.) shoot cultures at *in vitro* conditions, bacterial contamination on the plant tissues and medium surface appeared. The contamination was due to endophytic bacterium later isolated as a bacterial strain PM_YT. The colonial characteristics and cell morphology of the isolate were determined after cultivation on YEB-agar medium and coloring of microscopic preparations. The strain identification was implemented by biochemical methods (oxidase and catalase standard strip tests) and nucleotide sequencing of the 16S rRNA gene. The results from the morphological and cultural identification showed that the isolate was Gram-positive bacterium presented from small, short rods, forming small (size of 1-3 mm), round-shaped, yellow- to slightly orange-colored colonies. The results from biochemical identification showed that the strain was oxidase-negative and catalase-positive. The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed 99% pairwise similarity of the strain PM_YT to the bacterial species *Curtobacterium flaccumfaciens*. Antibiotic susceptibility was implemented by Bauer-Kirby disc diffusion method and the results demonstrated that the isolate was most sensitive to the antibiotics belonging to inhibitors of protein synthesis group, especially to tetracycline, doxycycline, amikacin, rifampin, lincomycin, chloramphenicol, erythromycin, streptomycin and ciprofloxacin.

**Keywords:** *Curtobacterium flaccumfaciens*, *Pancratium maritimum*, antibiotic susceptibility, nucleotide sequencing

## INTRODUCTION

The bacterial species *Curtobacterium flaccumfaciens* encompasses a group of closely related plant pathogens of agricultural and economic importance subdivided into several pathovars that exhibit differences in their host range - *C. flaccumfaciens* pv. *flaccumfaciens* (*Phaseolus vulgaris*), *C. flaccumfaciens* pv. *betae* (*Betae vulgaris*), *C. flaccumfaciens* pv. *oortii* (*Tulipa gesneriana*) and *C. flaccumfaciens* pv. *poinsettiae* (*Euphorbia pulcherrima*) with a further pathovar *C. flaccumfaciens* pv. *basellae* (*Basella alba*) **(Guimaraes *et al.*, 2003)**.
Bacteria belonging to *Curtobacterium flaccumfaciens* sp. are Gram-positive, short rods that may be straight to slightly curved or wedge shaped. In older cultures coccoid cells are usually presented. They form colonies of 2-4 mm in diameter, with smooth margins, slightly convex and wet-shiny appearance, pigmented in shades of yellow and orange. Some variants produce a blue to purple water-soluble pigment. *Curtobacterium flaccumfaciens* strains are motile and do not form endospores **(Hsieh *et al.*, 2004)**. They are aerobic, oxidase-negative, catalase-positive and possess lateral flagella **(Chen and Yin, 2007)**.
*C. flaccumfaciens* pv. *flaccumfaciens* was the first member described in the USA by **Hedges (1921)**, causing "bacterial wilt disease" of *Phaseolus* spp. This disease occurs in several European countries, as well as in Australia, Canada, Mexico and Colombia. In some countries as Brazil, bacterial wilt has been verified in several regions resulting in loses in bean production. Typical symptoms of the disease in bean plants are mainly wilting, vascular darkening and death of the aerial parts of the plant. Under field conditions during mild temperature seasons the infected bean plants may develop without bacterial disease symptoms **(Maringoni *et al.*, 2006)**. Natural infections also occur on *Vigna* spp., *Glycine max* (L.) and *Pisum sativum* (L.). Disease symptoms are described as gradual wilting of the leaves of the seedlings, appearance of broad irregular yellow areas starting from the leaf margin and extending inwards, followed by necrosis of the parenchymal tissue. Bacterial wilt appears to be more severe on stressed plants and can be lethal on young plants. In adult plants the symptoms are less pronounced, with slower development of the disease, and the bacterium is also able to colonize the seeds via vascular tissue **(Tegli *et al.*, 2002)**.
*Curtobacterium flaccumfaciens* pv. *poinsettiae* was first described in the USA by **Starr and Pirone (1942)**, which was associated with a disease causing leaf spots, stem rots, cankers, defoliation and brown discoloration of the vascular tissues on poinsettia also known as Christmas flower (*Euphorbia pulcherrima*). In the next decades, this bacterium has been reported from several European countries – United Kingdom, Germany, the Netherlands and Slovenia, as well as in South America (Venezuela) **(Trujillo *et al.*, 1989).**
*C. flaccumfaciens* pv. *basellae* is a phytopathogen that causes bacterial leaf spot disease of malabar spinach (*Basella rubra* [*B. alba*]) and described in 1994 in Jiangsu Province, China **(Fang *et al.*, 2000)**. This pathovar as well as *C. flaccumfaciens* pv. *oortii* have not yet been studied enough.
*C. flaccumfaciens* pv. *betae* (*beticola*) is the causative agent of silvering disease in red beet (*Beta vulgaris* var. *rubra*) and also may be pathogenic to feed-stuff beet (*Beta vulgaris* var. *lutea*) and to sugar beet (*Beta vulgaris* var. *saccharifera*) causing the disease called "bacterial leaf spot". *C. flaccumfaciens* pv. *betae* was discovered for a first time in 1995 in Inner Mongolia autonomous region, China. This strain damages the sugar beet, invaded from wounds of the leaves. Initially, the leaf spots are yellow and small, and in a few weeks become brown with a yellow halo. After one month the spots merge into brown patches followed by dehydration and death. The roots of affected sugar beets are observed to be much smaller than the roots of healthy plants. The disease symptoms occur on the leaves of sugar beet from June to October **(Chen and Yin, 2007)**.
Presently, *Curtobacterium flaccumfaciens* has been identified in neither *Pancratium maritimum* L. plants nor their *in vitro* cultures. Therefore, the aim of the current study was to identify by cultural, biochemical and molecular-genetic methods the bacterial strain *Curtobacterium flaccumfaciens* PM_YT isolated from Sea daffodil (*Pancratium maritimum* L.) shoot cultures and to evaluate its antibiotic susceptibility in order to apply the suitable antibiotics in the growth medium and to eliminate the infection in these *in vitro* plant systems.

## MATERIAL AND METHODS

### Plant *in vitro* cultures

The Sea daffodil (*Pancratium maritimum* L.) shoots formation was initiated by transferring of the sterile explants (pieces of young fruits) on Murashige and Skoog medium, supplied with 3% sucrose (Duchefa, The Netherlands), 5.5 g/L "Plant agar" (Duchefa, The Netherlands) and 1.15 mg/L NAA and 2.0 mg/L BAP (Duchefa, The Netherlands) **(Georgiev *et al.*, 2010)**. The cultivation was carried out at 26°C, under photoperiod of 16 h on light and 8 h on darkness. Obtained shoots were cultivated for more than 8 years with the subcultivation period of 28 days at the same conditions.

### Studied microorganism

*Curtobacterium flaccumfaciens* strain PM_YT, isolated from Sea daffodil (*P. maritimum* L.) shoot cultures.

### Culture media

***YEB-agar medium.*** This agar medium was prepared by the following prescription (g/L): beef extract – 5.0; yeast extract – 1.0; peptone – 5.0; sucrose – 5.0; $MgCl_2$ – 0.5. The final pH was adjusted to 7.2 and 15.0 g agar/L was added. The medium was sterilized by autoclaving at 121ºC for 20 min. Then YEB-agar medium was transferred in quantities of 16 mL in sterile Petri dishes (d = 9 cm) and allowed to harden.

### Morphological and cultural methods

#### *Isolation and cultivation of the strain*

The isolate from the shoot cultures was streak plated on YEB-agar medium. The Petri dishes were incubated at 30°C for 48 hours.

#### *Cellular and colonial morphology*

The description of the cellular morphology of the isolated strain was done by microscopic observation of a colored smear on a slide. The Gram affiliation of the studied strain was determined by the classical Gram staining. The colonial characteristics were determined by microscopic observation of single colonies developed on YEB-agar.

#### *Cellular motility*

The cellular motility was determined by deep inoculation of the strain using a bacteriological needle in tubes containing YEB-agar medium.

### Biochemical methods

The oxidase and catalase activity of the strain were determined by standard strip tests (Merck, Germany). For positive controls were used *Pseudomonas aeruginosa* ATCC 9027 (for oxidase test) and *Staphylococcus aureus* ATCC 25923 (for catalase test).

### Molecular-genetic methods

#### *Isolation of total DNA*

The isolation of DNA was performed using E.Z.N.A. DNA isolation kit according to the manufacturer's instructions.

#### *16S rDNA amplification*

All PCR reactions were performed using the PCR kit – PCR VWR in a volume of 25 µl in a Progene cycler (Techne, UK) according to the instructions of the manufacturer. In each PCR reaction 50 ng total DNA of the tested strain and 10 pmol praimers were used. DNA of the studied strain was amplified using universal primers for the 16S rDNA - 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') **(Lane, 1991)**. The amplification program included: denaturation - 95°C for 3 min.; 40 cycles - 93°C for 30 s, 55°C for 60 s, 72°C for 2 min.; final elongation - 72°C for 5 min.

#### *Purification of the product of the PCR-reaction – 16S rDNA – from TAE agarose Gel*

The purification of the 16S rDNA was conducted using DNA-purification kit (GFX MicrospinTM) according to the manufacturer's instructions.

#### *Sequencing of the 16S rRNA gene*

The partial sequencing of the 16S rRNA gene with two universal primers (27F and 1492R) was performed by the method of **Sanger *et al.* (1977)** at "Macrogen Europe Laboratory", The Netherlands. Using the CLC Sequence Viewer software, the entire sequence of the 16S rRNA gene was obtained, and the resulting whole sequence was compared with the on-line database sequences via the BLASTn algorithm. Thus, the studied strain was identified to the species level with the corresponding confidence level.

### Antibiotic susceptibility test

Antibiotic susceptibility test was performed by Bauer-Kirby *in vitro* disc diffusion method **(Bauer *et al.*, 1966; Drew *et al.*, 1972)** with impregnated paper discs of 21 antibiotics ("Bul Bio - NCIPD" Ltd., Bulgaria). The strain suspension was spread plated on YEB-agar medium. Then four discs of different antibiotics per Petri dish were put on the surface of the agar medium. The Petri dishes were incubated at 30°C for 48 hours. Zones of inhibition were measured and recorded at 24-th and 48-th h of incubation. One unit (U) of antimicrobial activity was defined as equal to 1 $mm^2$ of the zone of inhibition beyond the disc diameter (d = 6 mm) **(Xie *et al.*, 2009)**.

## RESULTS AND DISCUSSION

Sea daffodil (*Pancratium maritimum* L.) is a characteristic for sandy coastal habitats of the Mediterranean and Black sea regions where it is endangered and protected. Recent studies demonstrated that some alkaloids synthesized by *P. maritimum* L. are acetylcholinesterase inhibitors **(Georgiev *et al.*, 2010; Georgiev *et al.*, 2011)**. As such, they are of pharmaceutical importance and hence they are in the focus of research to investigate the potential of *in vitro* cultures as a sustainable system for producing these secondary metabolites under controlled conditions. Plant *in vitro* systems are a promising technology for both fundamental investigations of plant cells and for the production of bioactive metabolites **(Pavlov, 2014; Berkov *et al.*, 2014).**

In the case with rare and threatened plants, such as *P. maritimum* L., they are prospective ecologically friendly experimental matrix for searching new bioactive metabolites. However, one of the biggest problems, concerning plant *in vitro* cultures initiation from dune plants are microbial contaminations. The same problem appeared frequently also during their subcultivation. Growing deeply in the low content of organic matter and nutritional substances sand, *P. maritimum* L. is in close relations with many symbiotic microorganisms, which possess great difficulties with sterilization and increase the risk of explants contamination **(Georgiev *et al.*, 2010)**. To avoid this risk, we used young fruits for initiation of *in vitro* systems by *P. maritimum* L. which led to obtaining of shoot cultures free of contaminations. However, after 8 years of subcultivation, bacterial contamination on the plant tissues appeared, which rapidly spread on the medium surface. This fact put at risk the survival of *P. maritimum* L. *in vitro* shoots. As far as we spent a lot of time for shoot line selection, we decided to isolate and identify the bacterium responsible for the contamination and to solve this problem using the most suitable for the purpose antibiotics.

The infected shoot cultures exhibited different symptoms varying from dehydration, wilting and necrosis of the plant tissues to appearance of slightly orange-colored bacterial colonies on the plant surface, followed by death of the aerial parts (Figure 1).

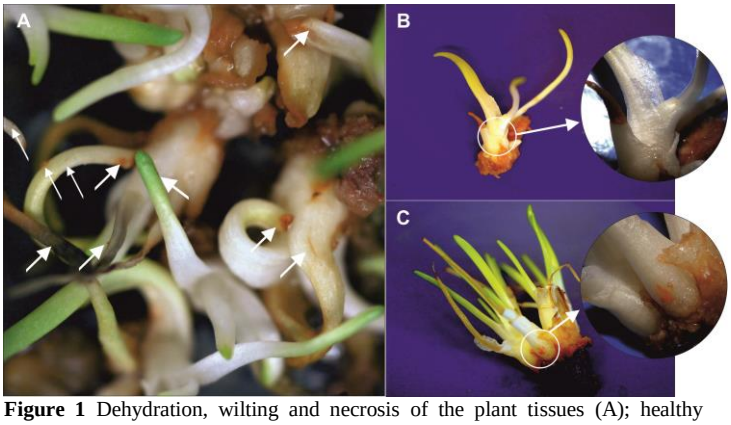
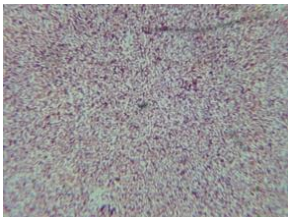



**Figure 1** Dehydration, wilting and necrosis of the plant tissues (A); healthy (normal) plant (B); appearance of orange-colored bacterial colonies on the plant surface (C).

The first step of isolation and identification of the etiological agent was streaking of the material on YEB-agar medium. After incubation at 30°C for 48 h, colored smears were prepared and observed under a microscope in order to determine the Gram affiliation and cellular characteristics. The cell morphology of the studied strain PM_YT is presented in Table 1.

The second step was to determine the colonial characteristics of the isolate by microscopic observation of single colonies. As seen in Table 1, the colonial characteristics of the isolated strain PM_YT were typical for *Curtobacterium flaccumfaciens* **(Holt *et al.*, 1997)**.

**Table 1** Colonial characteristics and cell morphology of *C. flaccumfaciens* strain PM_YT

| Colonial characteristics | | Cell morphology | |
|---|---|---|---|
| **Description of the colonies** | **Visualisation** | **Description of the cells** | **Visualisation** |
| Round shape with smooth edges, convex surface, yellow to slightly orange-colored, sticky consistency and size of 1 – 3 mm |  | Gram-positive, motile, small, short rods, arranged singly, in pairs or forming V-shaped configurations |  |

The next two steps were connected with the biochemical and molecular-genetic identification of the isolated strain. The results obtained from biochemical tests showed that the strain PM_YT was oxidase-negative and catalase-positive – properties typical for *C. flaccumfaciens* strains. The implemented test for cellular motility demonstrated that the cells of the strain PM_YT were motile - also a typical feature for *C. flaccumfaciens* strains **(Holt *et al.*, 1997)**.

The comparative 16S rRNA gene sequence-based phylogenetic analysis revealed that the isolate PM_YT belonged to the bacterial species *Curtobacterium flaccumfaciens* with 99% pairwise similarity of the sequence of 16S rDNA of the studied isolate and the partial sequence of 16S rDNA of the reference strain *Curtobacterium flaccumfaciens* LMG 3645 (Table 2 and Figure 2).

**Table 2** 16S rRNA gene partial sequence of the reference strain *C. flaccumfaciens* LMG 3645. Range 1: 141 to 1317

**Alignment statistics for match #1**

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 2053 bits (2276) | 0.0 | 1163/1178 (99%) | 1/1178 (0%) | Plus/Plus |

The results obtained from antibiotic susceptibility test showed that the isolate *C. flaccumfaciens* PM_YT was resistant to nalidixic acid and β-lactam antibiotics or penicillin-derivates (penicillin, ampicillin, oxacillin and amoxicillin). The susceptibility to other antibiotics of the β-lactam group (piperacillin) and the broad-spectrum cephalosporin antibiotic cefamandole was determined as moderate. The results demonstrated that the strain *C. flaccumfaciens* PM_YT was highly susceptible to the acylampicillin antibiotic azlocillin, antibiotics of aminoglycoside group (gentamicin, kanamycin, tobramycin, amikacin and streptomycin), glycopeptide antibiotic vancomycin, tetracyclines (tetracycline and doxycycline), ansamycines (rifampin), lincosamide antibiotics (lincomycin), the broad-spectrum antibiotic chloramphenicol, macrolide antibiotic erythromycin and fluoroquinolones (ciprofloxacin) (Table 3).

The isolation, identification and determination of antibiotic susceptibility of *C. flaccumfaciens* is of great significance in agriculture due to its substantial role as one of the most important and spread plant pathogens. The occurrence of infections with *C. flaccumfaciens* has been recorded in diverse geographical areas in Europe **(González *et al.*, 2005)**, Asia **(Chen and Yin, 2007; Osdaghi *et al.*, 2015)**, North America **(Venette *et al.*, 1995; Hsieh *et al.*, 2002)**, South America **(Trujillo *et al.*, 1989; Maringoni *et al.*, 2006)**, Africa **(Allen, 1995)** and Australia **(Wood and Easdown, 1990)**, where this pathogen affects the bean seeds, red and sugar beet, spinach, poinsettia and tulips crops and causes diseases with great economic loses. This leads to the necessity for the development of fast, effective and low-cost methods for the routine detection and identification of one of the major phythopathogens such as *C. flaccumfaciens*.

The successful eradication of *C. flaccumfaciens* infections is associated with application of effective diagnostic procedures. The diagnostic procedure for *C. flaccumfaciens* includes extraction from plant material or seeds, presumptive diagnosis with rapid tests and simultaneous isolation of bacterial colonies, identification of *C. flaccumfaciens* putative isolates and where relevant - determination of their pathogenicity. The screening (rapid) tests include PCR and isolation on agar media (detection of colonies with typical or suspected morphology). Identification tests on pure culture perform two tests with different biological principle: IF/PCR/Genetic fingerprints (BOX-PCR) and biochemical characteristics (which only allow identification to species level and further tests are required for confirmation).

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Query  121   TACTGGATAACATCGATGGCCGCATGGTCTGGTGGTGGAAAGATTTTTTGGTTGGGGATG  180
             |||||||||   |||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  141   TACTGGATATGATCACTGGCCGCATGGTCTGGTGGTGGAAAGATTTTTTGGTTGGGGATG  200

Query  181   GACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201   GACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGC  260

Query  241   CGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   CGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG  320

Query  301   GCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321   GCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGGG  380

Query  361   ATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381   ATGACGGCCTTCGGGTTGTAAACCTCTTTTAGTAGGGAAGAAGCGAAAGTGACGGTACCT  440

Query  421   GCAGAAAAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCG  480
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Query  481   TTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAT  540
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Sbjct  501   TTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAT  560

Query  541   CCCGAGGCTCAACCTCGGGCCTTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAG  600
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Sbjct  561   CCCGAGGCTCAACCTCGGGC-TTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAG  619

Query  601   ATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAA  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  620   ATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAA  679

Query  661   GGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATT  720
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Sbjct  680   GGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATT  739

Query  721   AGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGATGTAGGGACCTTTCCACGG  780
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Query  781   TTTCTGTGTCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAA  840
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Query  901   AACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCC  960
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Sbjct  920   AACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCC  979

Query  961   CTTGTGGTCGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG  1020
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Query  1021  TTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCGGGTTATGCCGGGGACT  1080
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Query  1141  CCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCGATACCGTA  1200
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Query  1201  AGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTAGGAATGAGGTCAGCAACTCGACATC  1260
             |||||||||||||||||||||||||||||||||||| ||| ||||||| |||||||||| ||
Sbjct  1220  AGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCTC  1279

Query  1261  ATGAAGTCGGAGTCGTTAGTAATTACAGATGAACCACG  1298
             ||||||||||||||| |||||||  ||||| | | |||
Sbjct  1280  ATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACG  1317
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**Figure 2** Comparison between the nucleotide sequence of 16S rDNA of the isolate *C. flaccumfaciens* PM_YT and the partial sequence of 16S rDNA of the reference strain *C. flaccumfaciens* LMG 3645

**Table 3** Antibiotic susceptibility of *C. flaccumfaciens* strain PM_YT

| Antibiotic | | Concentration/ disc | Mode of action | Inhibition zones, mm[a] | Activity, U[b] |
|---|---|---|---|---|---|
| **Penicillin** | P | 10 E | Inhibitors of the cell wall synthesis | - | - |
| **Azlocillin** | Az | 75 µg | | 23 | 387 |
| **Piperacillin** | Pi | 100 µg | | 20 | 285.7 |
| **Ampicillin** | A | 10 µg | | - | - |
| **Oxacillin** | O | 1 µg | | - | - |
| **Amoxicillin** | Ax | 25 µg | | - | - |
| **Vancomycin** | V | 30 µg | | 32 | 775.6 |
| **Cefamandole** | Cm | 30 µg | | 18 | 226.1 |
| **Tetracycline** | T | 30 µg | Inhibitors of the protein synthesis | 35 | 933.4 |
| **Doxycycline** | D | 30 µg | | 35 | 933.4 |
| **Gentamicin** | G | 10 µg | | 27 | 544 |
| **Kanamycin** | K | 30 µg | | 32 | 775.6 |
| **Tobramycin** | Tb | 10 µg | | 23 | 387 |
| **Amikacin** | Am | 30 µg | | 34 | 879.2 |
| **Rifampin** | R | 5 µg | | 42 | 1356.5 |
| **Lincomycin** | L | 15 µg | | 35 | 933.4 |
| **Chloramphenicol** | C | 30 µg | | 42 | 1356.5 |
| **Erythromycin** | E | 15 µg | | 42 | 1356.5 |
| **Streptomycin** | St | 30 µg | | 35 | 933.4 |
| **Nalidixic acid** | Nx | 30 µg | Inhibitors of the DNA synthesis or cell division | - | - |
| **Ciprofloxacin** | Cp | 5 µg | | 33 | 826.6 |

**Legend:** $d_{zone}$ < 8 mm – resistance; $d_{zone}$ = 8 ÷ 16 mm – intermediate susceptibility; $d_{zone}$ > 16 mm – high susceptibility; $d_{disc}$ = 6 mm;
[a] measured at 48$^{-th}$ h; [b] U – units.

The molecular methods are among the most suitable for fast detection and identification of this pathogen. Two PCR tests for the detection of *C. flaccumfaciens* pv. *flaccumfaciens* in bean seeds were described by **Guimaraes *et al.* (2001)** and **Tegli *et al.* (2002)**. The authors developed specific primers for *C. flaccumfaciens* pv. *flaccumfaciens* that can be used for detection of this bacterium in naturally-infected bean seeds by the PCR technique. When required, additional confirmation and verification of the pathogenicity of identified *C. flaccumfaciens* isolates have to be performed on bean plantlets or been seeds **(*Curtobacterium flaccumfaciens* pv. *flaccumfaciens* diagnostic protocol, OEPP/EPPO, 2011)**.
Besides from plants, some authors reported isolation of *Curtobacterium* spp. from specimens of human origin. *Curtobacterium* strains from five patients with symptoms of respiratory distress, chronic bronchitis, wound infection, conjunctivitis and lymphadenopathy have been isolated for a first time and identified by 16S rRNA gene sequence analysis from **Funke *et al.* (2005)**. The authors reported that the phylogenetic analysis of the complete 16S rRNA genes of these clinical isolates with homologies to *Curtobacterium* sp. ranging from 99.2 to 100%, confirmed the identification of the strains as *Curtobacterium* spp. The results from the antimicrobial susceptibility test of these isolates demonstrated that all strains were susceptible to macrolide antibiotics and rifampin, showing very low MICs values. **Francis *et al.* (2011)** reported for isolation and identification of a strain *C. flaccumfaciens* from synovial fluid and knee tissue of a 7-year-old boy with septic arthritis. The isolated strain was susceptible to the antibiotics penicillin, cefotaxime and vancomycin.

## CONCLUSION

This is the first report describing the isolation and identification of *Curtobacterium flaccumfaciens* from the plant Sea daffodil (*Pancratium maritimum* L.) and its shoot cultures used for laboratory purposes. This is of great scientific interest due to the wide spread of *C. flaccumfaciens* and its important role in the field of agriculture, causing significant crop and economic loses in different countries worldwide. The determination of antibiotic susceptibility of the strain *C. flaccumfaciens* PM_YT is also of great significance in the agricultural and laboratory practice in order to avoid or eliminate the microbial contamination with this phytopathogen of the plants and plant *in vitro* systems.

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