# A NEW BACTERIAL DISEASE OF CARNATION IN PORTUGAL CAUSED BY *BURKHOLDERIA ANDROPOGONIS*

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## ABSTRACT

The occurrence of a leaf spot disease of carnation caused by *Burkholderia andropogonis* is recorded for the first time in Portugal. Symptoms consisted of 'eyespot' lesions on all aerial plant parts, often bordered by water-soaked halos on the leaves. As the disease progressed lesions became dark brown and affected areas dried out. Phenotypic studies and Polymerase Chain Reaction using specific primers Pf/Pr targeted to 16S rDNA of *B. andropogonis* were used to identify the pathogen. Pathogenicity tests on china pink plants, re-isolation of the pathogen from inoculated plants and further PCR testing confirmed the identification of the bacterium. Infected plants came from an open air nursery and the whole production was destroyed to avoid dissemination of the pathogen.

**Key-words**: Leaf spot of carnation**;** *Dianthus caryophyllus*; first record.

## RESUMO

A ocorrência da mancha bacteriana do craveiro causada por *Burkholderia andropogonis* é pela primeira vez assinalada em Portugal. Os sintomas observados consistiam em manchas em forma de olho-de-perdiz em todos os órgãos aéreos das plantas afectadas, frequentemente circundadas por halos hidrópicos nas folhas. À medida que a doença progredia, as lesões adquiriam uma coloração castanha escura, acabando os órgãos afectados por secar. A identificação do agente causal da doença baseou-se no estudo dos seus caracteres fenotípicos e na Reacção em Cadeia da Polimerase (PCR), utilizando os iniciadores específicos Pf/Pr dirigidos à região 16S rDNA de *B. andropogonis*. A identificação foi confirmada por ensaios de patogenicidade em cravinas, reisolamento do agente causal da doença a partir das plantas inoculadas e novos ensaios PCR. As plantas infectadas provinham de um viveiro ao ar livre e toda a produção foi destruída a fim de evitar a disseminação do patogéneo.

**Palavras-chave**: Mancha bacteriana do craveiro**;** *Dianthus caryophyllus*; primeira detecção.

## INTRODUCTION

In June 2007, severely diseased potted carnations (*Dianthus caryophyllus* L.) of different cultivars, including cvs. 'Fragansia' and 'Resues', were found in an open air nursery in the Setúbal region. The propagating material used to produce those plants had different origins, but no symptoms were detected upon their arrival at the nursery.

The disease affected almost all carnation plants where 'eyespot' lesions on stems, leaves and even flowers were observed. On the leaves, those spots were frequently bordered by water-soaked halos. As the disease progressed, older lesions became dark brown and affected areas dried out (Figures 1, 2 and 3).

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These symptoms were similar to those caused by *Burkholderia andropogonis* (Smith) Gillis *et al.* on carnations and several other host plants, including different ornamental plants (Moffett *et al.*, 1986; Sivaplalan & Hamdan, 1997; Bagsic-Opulencia *et al.*, 2001; Cother *et al.*, 2004; Takahashi *et al.*, 2004; Li & De Boer, 2005).

All potted plants were kept outdoors, close to each other and overhead-watered by sprinklers. No weeds were found in the premises as the ground over which the pots were laid down was covered with black polyethylene. No other plant species with symptoms of a bacterial disease were observed in the vicinity of the carnations plot or in other plants neighbouring the nursery.

**Figures 1, 2 and 3 –** Plants of *Dianthus caryophyllus* showing 'eyespot' lesions on all aerial plant parts frequently bordered by water-soaked halos on the leaves. Older lesions became dark brown and affected areas dried out.

## MATERIAL AND METHODS

### Plant material

Affected leaves and stems from carnations cvs. 'Fragansia' and 'Resues' from a nursery located in the Setúbal region were used to isolate the pathogen. Pathogenicity tests were performed on china pink (*Dianthus chinensis* L.) plants.

### Isolation of the pathogen

After a previous rinse in sterile distilled water (SDW), small pieces from the margins of the stems and leaves lesions of affected carnations were dissected in SDW and left at room temperature for 30 min. Loopfuls of the resulting suspensions and their $10^{-1}$ dilutions were streaked onto King's medium B (KB) (King *et al.,* 1954) to isolate the pathogen. Plates were incubated at 27ºC for 4-5 days. Single colonies were then selected and restreaked on KB medium for further purification.

### Identification of the pathogen

Identification of the pathogen was based on its phenotypic characters, complemented by PCR amplification, using the species-specific primer pair Pf/Pr (Bagsic *et al*., 1995).

To evaluate the phenotypic characters, the selected isolates were grown on KB medium (King *et al.,* 1954) for 48 hours at 27ºC and tested for oxidative/fermentative metabolism of glucose following Hugh and Leifson (1953). Gram stain, fluorescence on KB medium, oxidase, catalase, urease and phosphatase activity, pectate degradation on CVP medium, arginine dihydrolase and indole production were evaluated according to Lelliott

& Stead (1987). Further testing, performed according to Bradbury (1986) and Chun & Jones (2001), included nitrate reduction, starch hydrolysis and acid production from adonitol, arabinose, cellobiose, fructose, galactose, glucose, glycerol, lactose, maltose, manitol, manose, melibiose, sucrose, L-ramnose, D-sorbitol, D-tartrate and trehalose. All tests were run in duplicate for each isolate. Negative controls using SDW were included in every test.

PCR amplification was performed according to Bagsic *et al*. (1995). A total volume of 50 µL per reaction contained 1 x buffer (supplied by the manufacturer [Invitrogen, Carlsbad CA 92008] with the *Taq* DNA Polymerase), 1.5 mM $MgCl_2$, 200 µM dNTP (Invitrogen, Carlsbad CA 92008), 0.25 µM of each primer (Pf: 5'-AAG TCG AAC GGT AAC AGG GA-3' and Pr: 5'-AAA GGA TAT TAG CCC TCG CC-3'), 0.25U *Taq* DNA Polymerase and 5 µL of lysates from suspensions of the isolates under study, previously heated at 100º C for 4 min for bacterial cell lysis. For each lysate two replicates were tested. Two negative controls of sterile double distilled water were also included in the test. Amplification was carried out on a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Waltham, USA) under the following conditions: a first denaturation step at 95º C for 5 min, followed by 25 cycles of denaturation at 94º C for 15 sec, annealing at 62º C for 15 sec and extension at 96º C for 5min. Final extension was at 72º C for 45 sec. Primer pair Pf/Pr specifically targets *B. andropogonis* 16S rDNA, producing a 410-bp amplicon with genomic DNA templates from the pathogen.

## Confirmation tests

Pathogencity of the selected isolates obtained from diseased carnations was evaluated by pricking leaves of china pink plants, three plants per isolate, with toothed forceps, the tips of which were wrapped in cotton wool and dipped in $10^6$ cells.$mL^{-1}$ suspensions of those isolates. SDW was used as a negative control.

Plants were incubated at 22º C with a photoperiod of 16hrs light. During the first 48hrs they were kept in closed polyethylene bags to assure high humidity levels. Symptoms were observed over a 4-week period.

Re-isolation of the pathogen was performed on KB medium (King *et al.,* 1954) from typical lesions on inoculated leaves and stems of china pink plants.

PCR amplification of genomic DNA of the isolates obtained from the inoculated plants, using the species-specific primer pair Pf/Pr (Bagsic *et al*., 1995), completed the confirmation tests.

# RESULTS

A bacterium was consistently isolated on KB medium (King *et al.,* 1954) from the diseased carnation plants. Two isolates were selected for identification: isolate 1 obtained from the stems and isolate 2 from the leaves.

The phenotypic characters, including cultural, morphologic and physiologic characteristics of both isolates, are summarised on Table 1.

PCR amplification using the species-specific primer pair Pf/Pr (Bagsic *et al*., 1995) produced the expected 410-bp amplicon with genomic DNA templates from isolates 1 and 2 obtained from carnation plants (Fig. 4, lanes 3, 4, 5, and 11).

China pink leaves inoculated with isolates 1 and 2 developed the typical 'eyespot' lesions, frequently bordered by water-soaked halos on the leaves (Figs 5, 6 and 7). These lesions were similar to those observed on diseased carnations and described in the literature as caused by *B. andropogonis* on several hosts, including carnations (Moffett *et al*., 1986; Sivaplalan & Hamdan, 1997; Cother *et al*., 2004; Takahashi *et al*., 2004; Li & De Bauer, 2005).

Although the inoculations were only performed on china pink leaves, 'eyespot' lesions started to show up on stems and flowers from the inoculated plants after an incubation period of about 10-15 days.

**Table 1 –** Phenotypic characters of isolates1 and 2 obtained, respectively, from stems and leaves of infected carnation plants.

| TEST | ISOLATE 1 | ISOLATE 2 | ACID FROM | ISOLATE 1 | ISOLATE 2 | ACID FROM | ISOLATE 1 | ISOLATE 2 |
|---|---|---|---|---|---|---|---|---|
| Gram | - | - | Adonitol | + | + | Manitol | + | + |
| H & L | Ox | Ox | Arabinose | + | + | Manose | + | + |
| Fluorescence on KB | - | - | Cellobiose | - | - | Melibiose | - | - |
| Levan | - | - | Fructose | + | + | L-Ramnose | + | + |
| Pectate hydrolysis | - | - | Galactose | + | + | D-Sorbitol | + | + |
| Oxidase | - | - | Glucose | + | + | Sucrose | - | + |
| Catalase | + | + | Glycerol | + | + | D-Tartrate | - | - |
| Argine dihydrolase | - | - | Lactose | + | + | Trehalose | + | + |
| Fosfatase | + | + | Maltose | - | - | | | |
| Urease | + | + | | | | | | |
| Indol | - | - | | | | | | |
| Nitrate reduction | - | - | | | | | | |
| Starch hydrolysis | - | - | | | | | | |

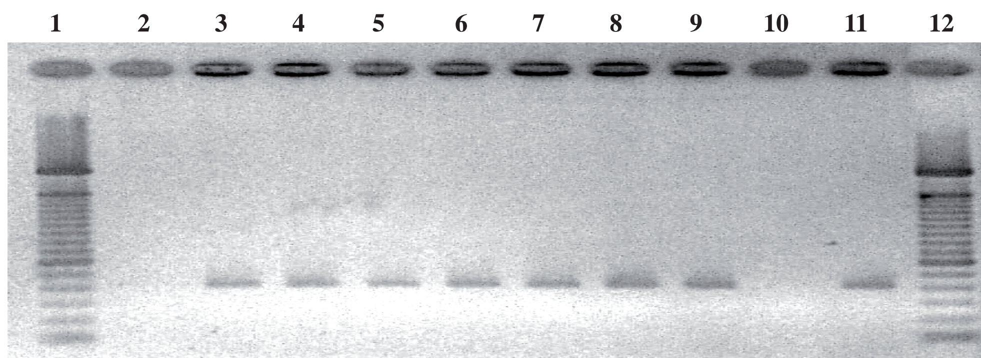


**Figure 4 –** PCR test on isolates from *Dianthus caryophyllus* (isolates 1 and 2) and *D. chinensis* plants (isolates 3 and 4). Lanes 1 and 12: 100bp Ladder; Lanes 2 and 10: negative controls; Lanes 3 and 4: Amplified products from isolate 1; Lanes 5, and 11: Amplified products from isolate 2; Lanes 6 and 7: Amplified products from isolate 3; Lanes 8 and 9: Amplified products from isolate 4.

As the disease progressed, older lesions became dark brown and affected areas dried out.

A bacterium was consistently re-isolated on KB medium (King *et al.,* 1954) from the typical lesions of china pink plants. Two isolates were selected, one from the leaves (isolate 3) and the other from the stems (isolate 4). The morphology on KB medium of the corresponding colonies was identical to the morphology of the colonies used in the pathogenicity tests, previously obtained from the diseased carnations (isolates 1 and 2).

PCR amplification using primer pair Pf/Pr produced the expected 410-bp amplicon with genomic DNA templates from isolates 3 and 4 obtained from the inoculated *D. chinensis* plants (Fig. 4, lanes 6, 7, 8 and 9).

**Figures 5, 6 and 7 –** Pathogenicity tests on Dianthus chinensis. 'Eyespot' lesions on leaves, stems and flowers of plants inoculated with isolates of *Burkholderia andropogonis* from infected carnations. Fig. 5: First symptoms after inoculation. Fig. 6: Detail of leaves with lesions bordered by water-soaked halos. Fig 7: Infected flowers, some of them already dried out.

## CONCLUSIONS

*Burkholderia andropogonis* is responsible for one of the most important bacterial disease affecting carnations (Diatloff & Rochecoust, 1991; Li *et al.*, 1993; Cother *et al.*, 2004).

According to Moffett *et al.* (1986) and Bagsic-Opulencia *et al.* (2001), isolates of *B. andropogonis* are highly uniform in cultural, morphologic and physiologic characteristics, in contrast with a high level of genetic diversity (Bagsic-Opulencia *et al.*, 2001). As the phenotypic characters of the Portuguese isolates were identical to those mentioned by Bradbury (1986), Lelliott & Stead (1987), Sivapalan Hamdan (1997) and Chun & Jones (2001) for *B. andropogonis*, the isolates from carnations were identified as *B. andropogonis*. PCR amplification using the species-specific primer pair Pf/Pr (Bagsic *et al.*, 1995) further confirmed the identification.

Koch's postulates were fulfilled by re-isolating bacteria from typical lesions on inoculated china pink plants that were identical to inoculated strains in colony morphology. Furthermore, the expected 410-bp amplicon was produced with genomic DNA templates from china pink isolates after PCR amplification using the species-specific primers Pf/Pr.

Although only the leaves of china pink plants were inoculated in the pathogenicity tests, typical symptoms could also be detected on stems and flowers after an incubation period of about 10-15 days, thus suggesting the possible systemic character of the infection.

The inoculum source could not be established as the propagating material from where the diseased carnations derived had different origins, and no symptoms had been noticed upon arrival at the nursery. However, it is possible that bacterial leaf spot was already present in some of that material at a very low incidence and that the disease proliferated within and between plants under the favorable conditions of the nursery. In fact, overhead irrigation and the proximity of all carnation pots, together with favorable weather conditions for disease development could be pointed out as the most probable factors responsible for the rapid dissemination of the pathogen that affected almost all carnations in that nursery.

In what concerns control measures, only some prophylactic measures could be suggested. Taking into account the pathogen is disseminated by infected propagating material, handling of infected plant material and contaminated water splash and wind driven rain, the use of disease-free plant propagating material, together with good sanitation measures, avoiding wet foliage and removing of all infected plant hosts from the growing areas will certainly contribute to minimize the risk of infection of healthy plants.

Despite the high economic losses, the nursery destroyed all carnation plants to prevent the possible establishment and dissemination of the pathogen, considering its wide host range.

This is the first record of *B. andropogonis* in Portugal and, to our knowledge, *Dianthus chinensis* L. is now reported for the first time as a possible new host for this pathogen, notwithstanding the typical symptoms were observed after artificial inoculation.

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