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Anatomical changes caused by *Ralstonia solanacearum* Smith and *Pectobacterium carotovorum* (Jones) in *Solanum tuberosum* L. stems

Cambios anatómicos provocados por *Ralstonia solanacearum* Smith y *Pectobacterium carotovorum* (Jones) en tallos de *Solanum tuberosum* L.

Alterações anatômicas causadas por *Ralstonia solanacearum* Smith e *Pectobacterium carotovorum* (Jones) em caules de *Solanum tuberosum* L.

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Abstract

Potato (*Solanum tuberosum* L.) is a crop notably affected by various pathogens, including bacterial, and it is important to study the histological changes that they produce to understand the symptoms associated with each disease. The objective of this research was to determine the anatomical changes that occur in potato stems due to the infection caused by *Ralstonia solanacearum* and *Pectobacterium carotovorum*. An assay was carried out with 45-day-old plants of the Kennebec variety, which were infiltrated in the basal part of the main stem with a suspension of 10^8 CFU.mL^{-1} of *R. solanacearum* and *P. carotovorum,* including a control treatment in which the plants were treated with sterile distilled water. Twelve days after inoculation, segments were taken from the second internode of the main stem and they were fixed in FAA (formaldehyde-acetic acid-ethanol 70 %) until processing to obtain cross sections of the stem. In the plants inoculated with *R. solancaearum*, invasion of the xylem vessels by the bacterium and formation of tyloses in some of them was observed. In the case of *P. carotovorum*, necrosis in the form of discontinuous bands in the epidermal cells, cortex, vascular cylinder, and pith was observed, as well as obstruction in xylem vessels by the bacteria and tylosis in some of them. The anatomical changes in the stem, induced by the two bacteria studied, are directly linked to the symptomatology of the disease that each of them causes in the potato crop.

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Resumen

La papa (*Solanum tuberosum* L.) es un cultivo notablemente afectado por diversos patógenos, incluyendo los bacterianos, y es importante el estudio de las alteraciones histológicas que éstos producen para comprender los síntomas asociados a cada enfermedad. En esta investigación se determinaron los cambios anatómicos provocados por *Ralstonia solanacearum* y *Pectobacterium carotovorum* en tallos de *Solanum tuberosum*. Se realizó un ensayo con plantas de la variedad Kennebec de 45 días de edad, las cuales fueron infiltradas en la base del tallo principal con una suspensión de 10^8 UFC.mL^{-1} de *R. solanacearum* y *P. carotovorum*, incluyendo un tratamiento testigo en el que las plantas fueron tratadas con agua destilada estéril. Doce días después de la inoculación, se tomaron segmentos del segundo entrenudo del tallo principal y se fijaron en FAA (formaldehido-ácido acético-etanol 70 %) hasta su procesamiento para la obtención de secciones transversales del tallo. En las plantas inoculadas con *R. solancaearum*, se observó invasión en los vasos xilemáticos por la bacteria y formación de tílides en algunos de ellos. En cuanto a *P. carotovorum*, se evidenció necrosis en forma de bandas discontinuas en las células de la epidermis, corteza, cilindro vascular y médula, así como obstrucción en vasos del xilema y tilosis en algunos de ellos. Los cambios anatómicos en el tallo, inducidos por las dos bacterias estudiadas, están directamente vinculados con la sintomatología de la enfermedad que cada una de ellas provoca en el cultivo de papa.

Palabras clave: histología caulinar, infección bacteriana, papa

Resumo

A batata (*Solanum tuberosum* L.) é uma cultura notavelmente afetada por vários patógenos, incluindo bacterias, sendo importante estudar as alterações histológicas que eles produzem para entender os sintomas associados a cada doença. O objetivo desta pesquisa foi determinar as alterações anatômicas que ocorrem em caules de batata devido à infecção causada por *Ralstonia solanacearum* e *Pectobacterium carotovorum*. Foi realizado um ensaio com plantas da variedade Kennebec com 45 dias de idade, que foram infiltradas na base do caule principal com uma suspensão de 10^8 UFC.mL^{-1} de *R. solanacearum* ou *P. carotovorum*, e um tratamento foi incluído. controle em que as plantas foram tratadas com água destilada estéril. Doze dias após a inoculação, foram retirados segmentos do segundo entrenó do caule principal e fixados em FAA (formaldeído-ácido acético-etanol 70 %) até o processamento, para obtenção de cortes transversais do caule. Nas plantas inoculadas com *R. solancaearum*, observou-se a invasão dos vasos do xilema pela bactéria e a formação de tilos em algumas delas. Em relação ao *P. carotovorum*, foi encontrada necrose na forma de bandas descontínuas nas células da epiderme, córtex, cilindro vascular e medula, além de obstrução nos vasos do xilema pela bactéria e tilose em algumas delas. Los cambios anatómicos en el tallo, inducidos por las dos bacterias estudiadas, están directamente vinculados con la sintomatología de la enfermedad que cada una de ellas provoca en el cultivo de papa.

Palavras chave: histologia de caulina, infecção bacteriana, papa.

Introduction

Potato (*Solanum tuberosum* L) is the fourth most important crop in the world after corn (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.), with an estimated world production of 359.07 million metric tons by 2020 (FAOSTAT, 2022).

In Venezuela, this crop is located in the Andean region, as well as in the Central and Eastern zone of the country. In the Central area, ‘‘High Valleys of Carabobo’’ stand out where the sowing is carried out between November to January, when night temperatures are below 20°C, favoring the good development of the crop (Olivares and Hernández, 2019).

In field visits and through sampling analysis of sick plants, the presence of bacterial infections in potato has been detected, the most recurrent being soft rot by *P. carotovorum* and wilt by *R. solanacearum*. These two bacterial pathogens constitute a threat to the crop because as they spread, they affect plantations, drastically reducing yields (Fiers *et al*., 2012; Yuliar *et al*., 2015; Charkowski *et al*., 2020).

The host range of *R. solanacearum* is quite wide and includes 53 different botanical families, being distributed at various altitudinal levels (Karim *et al*., 2018; Lowe-Power *et al*., 2018a). The bacterium penetrates through the roots, either through wounds or natural openings, causing plant wilting due to its rapid colonization and multiplication in vascular tissues (Yuliar *et al*., 2015; Lowe-Power *et al*., 2018b). According to Buddenhagen and Kelman (1964) and Hayward (1991), bacterial colonization of the stem results in xylem browning, leaf epinasty, and generalized lethal wilting

In sections of tomato stems (*Solanum lycopersicum* L.) observed under the light microscope, the presence of bacterial masses of *R. solanacearum* densely stained in the xylem and in microscopic preparations studied under the transmission microscope was observed; in addition, the bacterium was detected in the primary xylem of a resistant material, but not in the secondary xylem; while in sensitive material, it was visualized in both primary and secondary xylem (Grimault *et al*., 1994; Nakaho *et al*., 2000).

In an anatomical study on tomato stems infected with *R. solanacearum,* Hernández *et al.* (2005) detected bacterial invasion in the xylem and part of the phloem, mainly in the conducting cells with the largest diameter, while observations made under the electron microscope showed that the invasion also occurred in the parenchyma cells associated with the conducting tissue.

In this same crop, it has been shown that the invasion of this bacterium in a single lateral vascular bundle of the petiole causes leaf decay (epinasty), while the invasion of all vascular bundles causes wilting (Alvarez *et al*., 2010; Karim *et al*., 2018), likewise, it has been indicated that in tomato stems whose vascular bundles are invaded by *R. solanacearum*, there is a tendency to form adventitious roots that develop externally to the invaded vascular bundle.

The wilting caused by this bacterium may be due to the gradual mechanical obstruction of the xylem vessels, or to metabolites produced by the pathogen; among them, putrescine has been mentioned, which acts as well as physical factors in the production of symptoms (Khokhani *et al*., 2017; Lowe-Power *et al*., 2018b; Xue *et al*., 2020).

In the case of *P. carotovorum,* a polyphagous pathogen with a wide host range (Czajkowski *et al*., 2009; Pérombelon, 2002; Charkowski, 2018), its mode of action is associated with a set of depolymerizing activities such as the production of pectinases, cellulases, proteases, phospholipases and xylanases that can cause degradation of cell wall components in cells of different plant tissues (Pérombelon, 2002; Charkowski, 2015; Agyemang *et al*., 2020). Rot symptoms vary according to the initial bacterial concentration in seed potato tubers, cultivar susceptibility, and environmental conditions, particularly temperature and soil moisture content (Pérombelon, 2002; Charkowski, 2015; Charkowski *et al*., 2020).

Based on the above remarks and considering the scarce documented information on histological alterations in *S. tuberosum* stems affected by *R. solanacearum* and *P. carotovorum* in varieties commonly used by farmers in the country, the present research was proposed with the objective of determining the anatomical changes in the stem of potato plants infected with *R. solanacearum* and *P. carotovorum* bacteria.

Materials and methods

Plant material. To carry out the trial, 45-day-old potato plants of the Kennebec variety, were grown in pots of 1.5 kg capacity, containing a substrate composed of a mixture of sand and sterile soil (3:1), which were kept until inoculation in a shade house with protection against insects, located at the Institute of Agricultural Botany of the Faculty of Agronomy, in Maracay, Aragua state, Venezuela.

Inoculation. Bacterial cultures of *R. solanacearum* and *P. carotovorum* of 48 h of growth on YCA medium (yeast extract calcium carbonate agar) were used. From each bacterial strain, a suspension adjusted to a concentration of 10^8 $CFU.mL^{-1}$ was made, using tube number 3 of the McFarland scale (Gayathiri *et al*., 2018).

The inoculation of plants was made by infiltrating them with the bacterial suspension in the basal part of the stem using a hypodermic needle. Three treatments were used: (i) inoculation with *R. solanacearum*; (ii) inoculation with *P. carotovorum* and (iii) control (inoculation with distilled water) and a total of 6 plants/treatments were arranged. After inoculation, the plants were placed in a humid chamber for 48 hours and then transferred back to the shade house where they were distributed according to a completely randomized design and watered daily with tap water. During the trial, the average climatic conditions inside the shade house were: minimum temperature 20 ºC, maximum temperature 32 ºC, and relative humidity 66 %.

Anatomical study. At 12 days after inoculation, approximately 1.5 cm long segments were taken from the second internode (base-to-apex direction) of the main stem of four plants per treatment. Stem segments were gently washed with distilled water and fixed in FAA (formaldehyde-acetic acid-ethanol 70 %) until processing. With the fixed material, freehand cross sections approximately 20 µm thick were made using a razor blade; the sections obtained were stained with 0.1 % aqueous toluidine blue, mounted in a water-glycerin solution (v:v), and sealed with transparent enamel, to obtain semi-permanent slides that were examined under a NIKON E-200 optical microscope to make the corresponding descriptions, and additionally, digital images were taken of the different sections using an Evolution LC Color camera incorporated to the microscope.

Results and discussion

Anatomical description of the stem of *S. tuberosum* var. Kennebec

The control plants did not show any symptomatology and when analyzing the cross sections under the microscope, a uniseriate epidermis, cortex made up of 6-7 layers of angular collenchyma and 4-5 layers of parenchyma with cells of variable size were observed; the pith was totally parenchymatous and the vascular system consisted of open bicollateral bundles, as is characteristic in Solanaceae (Metcalfe and Chalk, 1950), further distinguishing a poorly developed secondary structure with a greater proportion of secondary vascular tissue in the fascicular zone, and in the case of the xylem, the conduction elements presented empty lumen, without any obstruction (Figures 1A, 1B), as was expected in healthy plants. These histological characteristics coincided with those reported by Shtein *et al*. (2020), who studied the anatomical structure in mature internodes of *Solanum tuberosum* cv. Nicola.

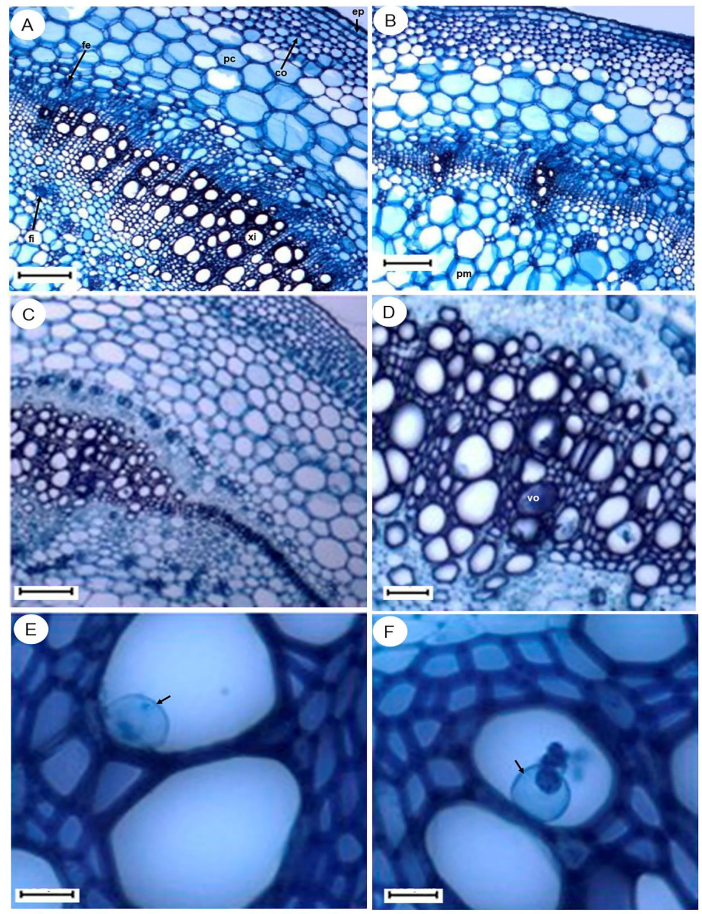

Figure 1. Details of the cross-section of the stem in control plants of *S. tuberosum* and inoculated with *R. solanacearum*. A-B: control; C-D: inoculated plant; E-F: xylem tissue of the inoculated plant, with arrows indicating tyloses in formation. ep: epidermis; co: collenchyma; pc: cortical parenchyma; fe: external phloem; fi: internal phloem; xi: xylem; pm: medullary parenchyma; vo: obstructed xylem vessel. Scale bars: 300 µm (A, B, C); 100 µm (D); 30 µm (E, F).

Symptomatology and stem anatomical changes in plants of *Solanum tuberosum* inoculated with *R. solanacearum*

The plants inoculated with *R. solanacearum,* started to show symptoms 6 days after inoculation, with decay and subsequent wilting of lower leaves, which then spread to the other leaves and newer branches, while 12 days after inoculation, total wilting was already observed in some plants. These symptoms coincide with those reported by several authors in plants infected by *R. solanacearum* (Karim *et al*., 2018; Charkowsky *et al*., 2020).

Likewise, Hernández *et al*. (2005) observed in tomato plants a total collapse of them at 12 days after inoculation with *R. solanacearum*.

In relation to the stem anatomy, in plants inoculated with *R. solanacearum* only changes were observed in the xylem conduction elements, specifically in the xylem vessels, where obstruction of their lumen was detected (Figures 1C - 1F). This alteration in the xylem structure coincides with what was observed by other researchers, who point out that *R. solanacearum* affects water conduction and consequently causes wilting, due to the blockage in the xylem conducting cells (Álvarez *et al*., 2010; Yadeta and Thomma, 2013; Planas-Marqués *et al*., 2020). It has been indicated that the multiplication of the bacterium and the production of large amounts of extracellular polysaccharides, would be the cause of the blockage of xylem conducting cells and the typical wilting caused by this bacterium in infected plants of different species, including *S. tuberosum* (Van der Wolf and De Boer, 2007; Genin and Denny, 2012, Khokhani *et al*., 2017).

In plants inoculated with *R. solanacearum*, the presence of balloon-shaped tyloses was also evident in some of the vessels, mainly in the secondary xylem (Figures 1E, 1F), structures to avoid the advance of the bacterium towards the upper portions of the stem, however, the evident wilting observed in potato plants of the Kennebec variety inoculated with this bacterium showed that the formation of tyloses in the xylem vessels was not effective in stopping the invasion of the bacterium and therefore the progress of the disease.

The formation of this type of structure is reported as a response of the plant to the attack by vascular pathogens, to avoid their invasion through the conducting tissue (Kashyap *et al*., 2021). Similarly, to what was observed in this research, Ferreira *et al*. (2017) detected the presence of vessels plugged by balloon-shaped tyloses in potato clone 09509.6 inoculated with *R. solanacearum* bacteria, suggesting that infected xylem vessels may induce the formation of these.

Symptomatology and stem anatomical changes in plants of *S. tuberosum* inoculated with *P. carotovorum*

Three days after the inoculation of the plants with *P. carotovorum,* softening and maceration of the tissue around the point of inoculation was observed, which spread to cover the entire circumference of the stem; being also notorious the presence of certain areas with necrosis, and additionally wilting occurred in some plants. This symptomatology has been reported in potatoes as well as in other species such as paprika (*Capsicum annuum*), eggplant (*Solanum melongena*), tobacco (*Nicotiana tabacum*) (Pérombelon, 2002; Charkowski, 2015; Charkowski, *et al*., 2020), and it has been indicated that, under favorable conditions for the development of this bacterium, the affected organs rot in two to three days, and the plants can wither (Charkowsky, 2018).

The stem anatomy was severely affected in plants inoculated with *P. carotovorum* (Figures 2B - 2F), in relation to control plants (Figures 1A, 1B, 2A). One of the most evident changes was the lysis and disintegration of the cells of the epidermis, as well as those of the cortex, vascular cylinder, and pith tissues, observing severe necrosis in the form of discontinuous bands in those areas of the stem cross-section, being also notorious the obstruction in the xylem vessels by the attack of this bacterium (Figures 2B - 2D). According to Charskowsky (2018), high concentrations of *P. carotovorum* in the xylem can cause necrosis in the vascular tissue.

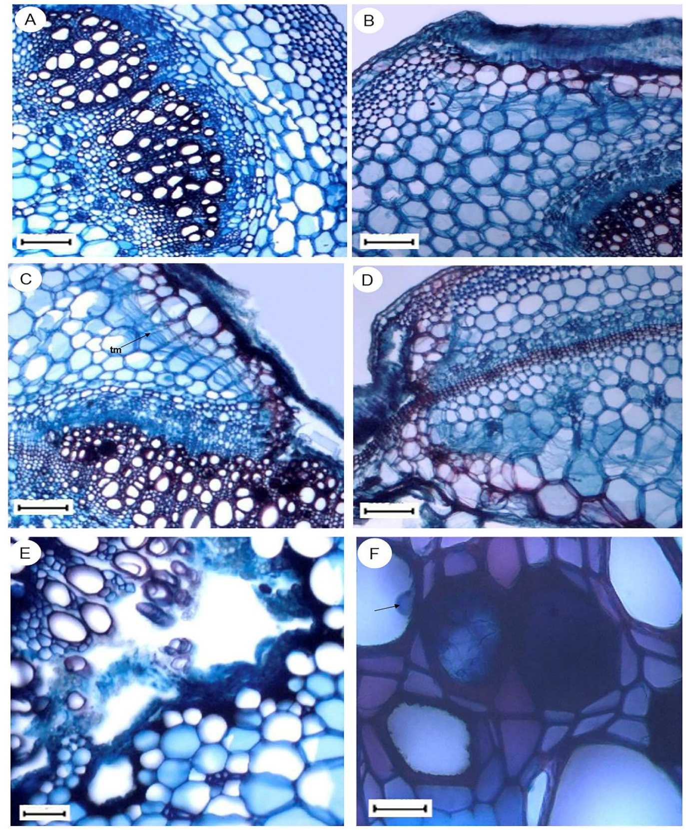

Figure 2. Details of the cross-section of the stem in control plants of *S. tuberosum* and inoculated with *P. carotovorum*. A: control plant; B-D: inoculated plant; note tissue lysis in the epidermis, cortex, vascular cylinder, and pith; E-F: conducting tissue in the inoculated plant, note maceration and necrosis in the xylem tissue (E) and obstruction of vessels and tyloses in formation indicated by arrow (F). tm: meristematic tissue. Scale bars: 300 µm (A, B, C); 100 µm (D); 30 µm (E, F).

These degenerative changes in the anatomical structure of the stem correspond to the symptoms of soft rot that characterize the damage by *P. carotovorum* (Pérombelon, 2002; Charskowsky, 2015; Charskowsky, 2018), a bacterium that produces a series of enzymes such as cellulases, pectinases, proteases, phospholipases, among others, which cause degradation of the cell wall components that form different tissues (Barras *et al.,* 1994).

The appearance of layers of meristematic tissue was also observed in some regions of the bark, possibly originating from the dedifferentiation of cortical parenchyma cells, which seems to be a defense mechanism of the plant to ensure the development of new tissues.

It should be noted that in potato plants affected by "blackleg" produced by *P. atrosepticum,* Artschwager (1920) found an extensive lignification of vascular tissues and the development of sclereids in the stem bark and pith, which according to Pérombelon (2002) tends to increase the plant resistance to infection since lignification offers greater resistance to cell wall degradation by peptic enzymes; however, this response was not observed in the present study.

Similarly, to what was observed in plants inoculated with *R. solanacearum*, in those inoculated with *P. carotovorum* the formation of globose tyloses in the conducting cells of the xylem was also noticeable (Figures 2E, 2F). The systemic invasion of bacteria of the *Pectobacterium* and *Dickeya* genus through the xylem has been documented by several authors (Kastelein *et al*., 2020; Czajkowski *et al*., 2010; Pérombelon, 2002).

Regarding the presence of tyloses in the xylem of plants affected by *P. carotovorum*, there are no previous reports in the available literature; however, it is assumed that it is possibly a defense reaction of the plants of the potato variety studied to the attack of the bacterium, as occurs with *R. solanacearum* and other vascular pathogens (Yadeta and Tomma, 2013). It should be noted that the fact that tylosis is due to a reaction to mechanical damage is ruled out since these were not observed in the plants of the control treatment.

Conclusions

R. solanacearum and *P. carotovorum* caused histological changes in the stem of *S. tuberosum* of the Kennebec variety. In the case of *R. solanacearum*, obstruction was predominantly observed in the secondary xylem due to the formation of tyloses, which explains the wilting of the plants, while in the case of *P. carotovorum*, the most noticeable anatomical change was maceration of the stem tissues, in addition to the obstruction in the conducting cells of the xylem due to tylosis.

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