

Histopathological and morphological alterations caused by *Plasmodiophora brassicae* in *Brassica oleracea* L.

Alteraciones histopatológicas y morfológicas causadas por *Plasmodiophora brassicae* en *Brassica oleracea* L.

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ABSTRACT

Plasmodiophora brassicae is a plant pathogen of the Brassicaceae family, which presents a remarkable ability to survive in soil and high capacity of infection, significantly reducing crop yields. The present histopathological study conducted with the aim of contributing to knowledge of the infection cycle of the pathogen, showed the presence of multinucleated plasmodia at cortex and periderm cells level in infected cabbage roots, as well as thickening and disruption of cell wall. As a result of this disarray was observed in diseased tissues, mainly in the cortex, compared with healthy tissues in uninoculated plants. The inoculation cabbage seedlings with dormant spores of *P. brassicae* at concentrations of $1 \cdot 10^7$ and $1 \cdot 10^8$ spores mL⁻¹ by immersion and spray, induced a higher growth and less growth longitudinal lateral roots compared with uninoculated plants as well as presence of young plasmodia at 28 days after inoculation.

Key words: *Plasmodiophora brassicae*, *Brassica oleracea* L., histopathology, gall, plasmodia, inoculation, hypertrophy.

RESUMEN

Plasmodiophora brassicae es un patógeno de plantas de la familia Brassicaceae que presenta notoria habilidad de sobrevivencia en suelo y alta capacidad de infección, lo cual reduce considerablemente el rendimiento de los cultivos. El presente estudio se realizó para contribuir al conocimiento del proceso infeccioso de este patógeno en el repollo, una especie de interés económico para Colombia, pues la información disponible es incipiente y desactualizada. A fin de determinar el daño a nivel celular se practicaron cortes histológicos en plantas con infecciones naturales; adicionalmente, se hicieron pruebas de patogenicidad con el fin de hacer un seguimiento de los síntomas iniciales de la enfermedad bajo condiciones controladas. Los resultados indicaron la presencia de plasmodios multinucleados en las células del cortex y la peridermis, así como engrosamiento y rotura de la pared celular en raíces de repollo infectadas, además de pérdida en la diferenciación de los tejidos, principalmente del cortex, comparado con tejidos sanos de plantas no infectadas. La inoculación de plántulas de repollo con esporas latentes de *P. brassicae* en concentraciones de $1 \cdot 10^7$ y $1 \cdot 10^8$ esporas mL⁻¹, mediante inmersión y adición de inóculo en el sustrato, indujo mayor crecimiento lateral y menor crecimiento longitudinal de las raíces comparado con plantas no inoculadas, además de la presencia de plasmodios jóvenes a los 28 días de la inoculación.

Palabras clave: *Plasmodiophora brassicae*, *Brassica oleracea* L., histopatología, agallas, plasmodios, inoculación, hipertrofia.

Introducción

The club root disease in crucifers is caused by *Plasmodiophora brassicae* Woronin. In Colombia it has been reported on the main crucifer growing areas in the departments of Cundinamarca, Antioquia and Caldas, in crops of broccoli, cabbage, cauliflower, Brussels sprouts, radishes and Chinese cabbage among others, reducing yields of 20

- 50 %, becoming a major constraint to the production of these vegetables (Jaramillo and Diaz, 2006).

P. brassicae is an inhabitant of the soil, pathogenic on plants of the Brassicaceae family (Karling, 1968; Voorrips, 1995), which cannot grow in any state saprophytic life cycle, and this is divided in two stages, a primary phase occurs in root hairs and the secondary phase in the cells of root cortex,

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associated with cellular hyperplasia and hypertrophy, consequently inducing abnormal root growth and formation of club root or hernia in susceptible hosts (Cao *et al.*, 2007; Friberg *et al.*, 2008; Karling, 1968; Voorrips, 1995). The disease cycle begins with the germination of dormant spores (Friberg *et al.*, 2005), which can survive in soil for more than 15 years in the absence of a host plant (Friberg *et al.*, 2008). It is assumed that the germination of dormant spores is stimulated due to the presence of host plants who release specific substances from the roots, the same way, increases in moisture and temperature promote the germination of dormant spores which affect host root hairs during primary infection phase (Ingram and Tommerup, 1972; Manzanares *et al.*, 2001).

In the secondary phase infection extends into the root cortex, where plasmodium, develops a multinucleated structure that is fractionated (Karling, 1968; Suwabe *et al.*, 2003). Each piece is surrounded by a membrane and develops within zoosporangia, however, some, produces dormant spores zoosporangia (Vidhyasekaran, 2004), and consequently causing cellular hyperplasia and hypertrophy, reflected it in the galls on the roots of the plant. Subsequently, dormant spores are released into the soil when galls formed by host tissue are disintegrated (Ingram and Tommerup, 1972). The dormant spores are highly resistant structures, formed approximately 25% chitin, 2.5% other carbohydrates, 34% protein and 18% lipids (Moxham and Buczacki, 1983), which allows survives and remain viable in soil for long periods of time (Karling, 1968; Voorrips, 1995).

The aims of this study were observe and compare different histopathological changes produced by *P. brassicae* in cabbage, *Brassica oleracea* var. *capitata* and a wild host (turnip, *Brassica rapa*) and determinate initial morphological changes in cabbage plants inoculated with the pathogen.

Materials and methods

Plant material

Mature plants of turnip (*B. rapa*) and cabbage (*B. oleracea* var. *capitata*), healthy and with the presence of club root were collected in the greenhouses of the Faculty of Agronomy of the Universidad Nacional de Colombia, Bogotá, in order for studies histology changes. For patogenicity tests were used green cabbage seeds *B. oleracea* L. cv. Früher Dithmarscher.

Processing of tissues for histological observations

The root samples of turnip (*B. rapa*) and cabbage (*B. oleracea* var. *Capitata*), with natural infections were rinsed with water to remove soil residues. Subsequent, were cutted

of 1 cm and fixed in 20 mL of FAA (10% formalin - acetic acid 5%, 85% - alcohol 70%) for 24 h. Then, slices were dehydrated through successive steps in alcohol at 70, 80, 90, 96 and 96%, each with 24 hours. Tissues were cleared in xylene for 24 hours and then melted paraffin, imbibes from 56 to 58 C for 24 h. Finally, were prepared paraffin blocks, cutting with rotating type Minot microtome model 820 Spencer (American Optical, Delhi) and staining with safranin and fast green. The observations and imaging were performed in light microscopy (Olympus 31X).

Preparation *P. brassicae* of suspension

Roots of cabbage plants with the presence of mature galls induced by *P. brassicae* dormant spores were removed. Spore suspension was prepared mashing galls in deionized sterile water (1:10 w/v). The homogenate was filtered as described by Narisawa and Hashiba (1998). The filtrate was centrifuged at 300 rpm for 20 minutes according to Suzuki *et al.* (1992). The button was removed and supernatant was centrifuged twice, obtaining a spore suspension, from this, was verified the presence of dormant spores. The final concentration of suspension was adjusted to two levels: $1 \cdot 10^7$ spores mL^{-1} and $1 \cdot 10^8$ spores mL^{-1} , by counting on hemacytometer.

Inoculation of cabbage seedlings

Sterile peat was Pro Mix was used as substrate for growing seeds of cabbage seeds *B. oleracea* L. cv. Früher Dithmarscher. For the inoculation of cabbage seedlings were used two methods, first consisted in immersion of plant in the spore suspension and then were sown in the nursery, others were planted directly, and then added the suspension of dormant spores to the substrate. Controls of seedlings were immersed in distilled water and then planted, resulting in the following treatments: T1) direct addition of 2 mL of inoculum in the soil at a concentration of $1 \cdot 10^7$ spores mL^{-1} ; T2) roots immersed in 2 mL of inoculum at a concentration of $1 \cdot 10^7$ spores mL^{-1} ; T3) direct addition of 2 mL of inoculum in the soil at a concentration of $1 \cdot 10^8$ spores mL^{-1} ; T4) roots immersed in 2 mL of inoculum at a concentration $1 \cdot 10^8$ spores mL^{-1} ; T5) uninoculated control. The seedlings were maintained for 30 days a temperature 18° C and ambient brightness. Frequent irrigation was provided to field capacity and fertigated to 15 and 21 days after inoculation (dpi) with 20 mL of Hogland solution.

Morphological observations of infection with *P. brassicae* in cabbage

On plants of cabbage *B. oleracea* L. cv. Früher Dithmarscher, were carry out three destructive sampling roots at 15 dpi (1st sampling), 21 dpi (2nd sampling) and 28 dpi (3rd sampling). Tissues were washed with water to remove

residual peat and morphological observations were made by direct observation under a stereoscope and for pathogen structures assess tissues were immersed in 10% KOH for 8 h, later in 12% KOH for 2 h-bath. Then, those were stained with trypan blue solution for 2 h, and washed with water. Finally the roots were spread on glass slides and observed by light microscopy optics.

Results and discussion

Histopathology of cabbage root galls *P. brassicae*

In the plates with tissue sections of roots affected by *P. brassicae*, plasmodia were observed in different stages of development in cabbage (Fig. 1A) and turnip roots (Fig. 1B).

The observed structures are part of the life cycle of primary and secondary disease (Suwabe *et al.*, 2003). It can be seen elongation and/or increased cell size associated with hypertrophy and rupture processes of the cells containing the pathogen (Fig. 2A), which is consistent with what was mentioned by Donald *et al.* (2008) and Kobelt *et al.* (2000), this being a mechanism of spread of the pathogen within the host tissue.

Symptoms of cortical invasion by *P. brassicae* included disruption of the cell wall, presence of vesicles or inclusion bodies inside the cell wall, cell wall thickness in combination with plasmodesmata and elongation and/or disorganization host nuclei. Ameboidal shape of pathogen has been reported in root cortical cells and, it is often found in association with disruption of cell walls, and is presumed that it is the structure of the parasite that penetrates and invades the cortical cells (Donald *et al.*, 2008; Kobelt *et al.*, 2000). The observations carried out can be seen in the cell wall thickening (Figure 2B).

At the level of epidermis, periderm and cortex become visible a loss of tissue differentiation (Figs. 3 A, B).

Kobelt *et al.* (2000) described compatible interaction: Cvi ecotype of *Arabidopsis* and *P. brassicae* founding that spore-forming protozoan parasite are located in large cells of the cortex near the periderm, while young secondary plasmodia are found mainly in small cells next to central cylinder, which corroborates the histological differences found in this study.

Observations of artificial infection of *P. brassicae* in cabbage seedlings

At 15 dpi, plants inoculated at a concentration of $1 \cdot 10^8$ spores mL^{-1} had a lower longitudinal growth of the root system (4 - 6.7 cm), compared with plants inoculated at a concentration of $1 \cdot 10^7$ spores mL^{-1} (3 - 8.5 cm) and control (5.5 - 8.5 cm). Regardless of inoculation method,

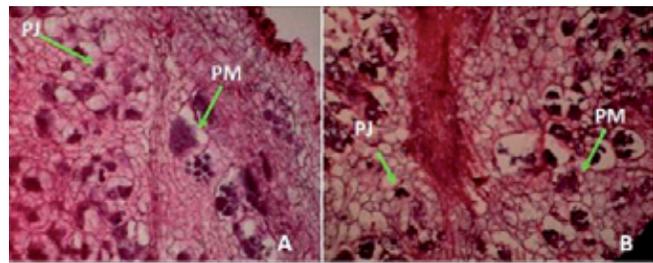


FIGURE 1. Detail of ruptured cells containing plasmodia: PJ, young plasmodia; PM, mature plasmodia 40X. (A) roots of cabbage, (B) turnip roots.

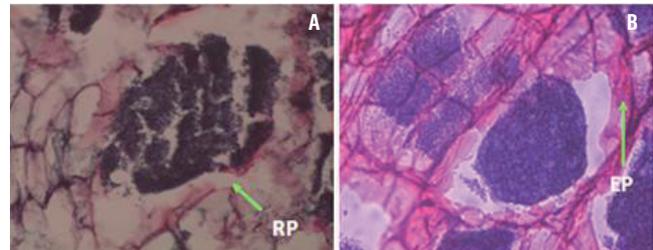


FIGURE 2. Damage to the cell wall level: (A) RP, breakage of cell wall in *B. rapa* (40X). (B) EP, cell wall thickening (40X).

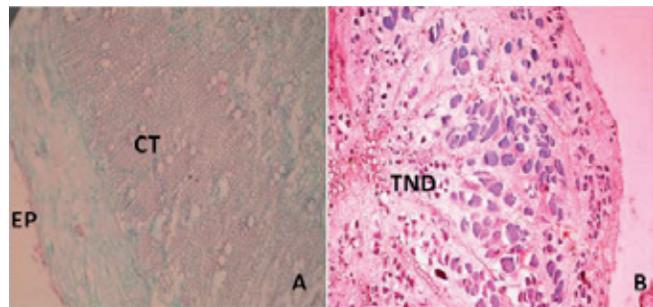


FIGURE 3. Contrast healthy and diseased tissues: (A) Differentiated tissues healthy root of *B. rapa*: EP, epidermis; CT: cortex (10X). (B) Undifferentiated tissue (TND) diseased root of *B. oleracea* (4X).

there was increased lateral growth (1 - 1.5 cm) as shown in Figure 4, which could be related to an imbalance of growth regulators in host-pathogen interaction, such as auxins and cytokinins, (Ludwig-Müller and Schuller, 2008). Epicotyl length was higher in control plants compared to inoculated plants, suggesting that *P. brassicae* affects the growth and development of infected plants.

Samples taken 21 dpi revealed that plants inoculated at a concentration of $1 \cdot 10^8$ spores mL^{-1} by direct addition and immersion (T3 and T4) showed abundant lateral growth compared with control plants (T5), with values of 0.4 cm, 0.5 cm and 0.3 cm, respectively. As seen in the stereoscope was evident in the axis tissue loss and darkening of the main root in the inoculated treatments, mainly in T4 (Figure 5). At this time young plasmodia were found in treatments of immersion.



FIGURE 4. Plants infected with *P. brassicae*, 15 dpi. T1: direct addition of inoculum to $1 \cdot 10^7$ spores mL^{-1} , T2: immersion $1 \cdot 10^7$ spores mL^{-1} , T3: direct addition of inoculum to $1 \cdot 10^8$ spores mL^{-1} , T4: immersion $1 \cdot 10^8$ spores mL^{-1} , T5: control.

The results at 28 dpi were consistent with those obtained for the first and second sampling, for showing lower growth and higher longitudinal lateral growth for treatments T3 and T4. According to Devos *et al.* (2005) and Siemens *et al.* (2006), infection with *P. brassicae* results in the formation of adventitious roots, resulting in swelling, due to the involvement of auxins, cytokinins and xiloglucan enzyme transglycosylase/hydrolase (XTH).

According to Voorrips and Kanne (1997) symptoms in infected plants may have variations from small swellings with ovoid or globular in the roots to the formation of large galls on the entire root system, which is consistent with that observed in this work. The growth rate of infected plants is delayed and chlorosis occurs because the presence of large galls usually causes a reduction in nutrient uptake and water. As a result, infected plants grow abnormally and production is reduced (Karling, 1968; Voorrips, 1995).

Discolored roots showed the presence of young plasmodia the beginning of infection associated with loss of main root axis and increased lateral root growth, being more noticeable at a concentration of $1 \cdot 10^8$ spores mL^{-1} by immersion. In advanced stages was observed a higher number of

multinucleated plasmodia with different sizes and shapes. It is noteworthy that other treatments except the controls were also positive for the presence of pathogen structures.

Methods developed in this study allow us to describe damage induced by *P. brassicae* at histologic level and its relationship to physiological alterations in susceptible hosts. Based on the results of pathogenicity tests, morphological changes registered are an indicator of the presence of the pathogen in early phenological stages, therefore, this information is important for those who produce plant material, it allows recognition of initial signs of the disease.

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FIGURE 5. Cabbage roots of plants infected with *P. brassicae* (28 dpi): View of the top of the taproot; from left to right. T1: addition of inoculums $1 \cdot 10^7$ spores mL^{-1} ; T2: immersion $1 \cdot 10^7$ spores mL^{-1} ; T3: addition of inoculums $1 \cdot 10^8$ spores mL^{-1} ; T4: immersion $1 \cdot 10^8$ spores mL^{-1} ; T5: control.

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