Biology and histopathology of *Ustilago filiformis* (=*U. longissima)*, a causal agent of leaf stripe smut of *Glyceria multiflora*

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Received: March 16, 2015
Accepted: November 12, 2015

Abstract: The aims of this study were to clarify the reproductive biology of the *Ustilago filiformis* Schrank, as causal agent of the stripe smut of *Glyceria multiflora*, determine the infection process of the pathogen and analyze the histological changes associated host-*Glyceria* any fungus attack. Moreover, the life cycle of the fungus was elucidated for the first time. Both teliospores and basidiospores were found to be equally efficient in producing the infection in *Glyceria* plants after the plants had been inoculated. These results constitute an important contribution for the understanding of the epidemiology of the disease.

Key words: basidiospores, infection cycle, stripe smut, teliospores

Introduction

Smut fungi are Basidiomycota fungi belonging to the order Ustilaginales. They received this name due to the very conspicuous symptoms of black teliospore masses resembling smut, which they often produce on the host plants. There have been over 1,100 smut species recognized in over 93 genera. Smuts infect more than 4,000 plant species belonging to approximately 75 different plant families. The biggest group of this fungi infects monocots belonging to the Graminea, which include cereal crops. The smuts encompass, among others, the genera *Ustilago* and *Tilletia*. The latter cause, the so-called, bunt diseases of various cereal crops. Between the species genus *Ustilago*, the name of *Ustilago longissima* (based on *Uredo longissima* Sowerby 1799) should be replaced by *Ustilago filiformis* (based on *Lycoperdon filiformis* Schrank, in Hoppe 1793), accepted by the 13th International Botanical Congress in Sydney, Australia, 1981 (Vánky 1985; MycoBanK 2015).

Ustilago filiformis is the causal agent of the stripe smut in many species of the genus *Glyceria*, such as *G. fluitans, G. grandis, G. multiflora, G. nemoralis, G. obtusa, G. plicata,* and *G. striata* (Mordue 1991). Stripe smut produces chlorosis and necrosis on the leaves. The infected plants are slightly stunted, and pale or yellowish-green. They produce new tillers partially atrophied of a pale greenish yellow color, and have a weak aspect. The plants usually fail to reach the flowering stage due to fungus damage. The geographical distribution of the disease is generalised to temperate areas of the world such as: Asia – Northern India, the USSR (Siberia); Europe – Austria, Bulgaria, Czech, Denmark, Finland, France, Germany, Hungary, Italy, Poland, Romania, Spain, Sweden, Switzerland, the United Kingdom, the former Yugoslavia; and North and South America.

In Argentina, the disease was detected in the cities of Jeppener and La Plata which are in the Buenos Aires Province. The pathogen is transmitted through teliospores spreading by wind and water (Hirschhorn 1986; Mordue 1991; Astiz Gassó *et al*. 2006).

Glyceria multiflora Steudel is a native species. It is aquatic, glabrous, perennial, rhizomatous with long tillers, and it has wide ligule, narrow panicle lax, 20–60 cm long, and reddish–dark fruit cariopsis (Burkart 1969). In Argentina, it inhabits marginal areas of low grasslands of Mesopotamia and "Cuenca del Salado", which are in the Buenos Aires Province. In these areas, it is of high value as cattle forage (Vidal and Piergentili 1973). The high productivity and good palatability of this species make it an interesting alternative in marginal farming areas of the country (Rossi *et al*. 2006, 2009; Alonso *et al*. 2009).

Other smut species are recognised in terms of their life cycle and histopathology e.g. *Sporisorium reilianum* f. sp. *zeae* on *Zea mays* and *Sorghum bicolor* (L.) Moench (Wilson and Frederiksen 1970; Matyac 1985; Martinez *et al*. 2002).

The first aim of this work was to explain the reproductive biology of the fungus *U. filiformis*. The second aim was to determine the pathogen's process of infection.

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The third aim was to analyse the histological changes of the host-*Glyceria* associated with the fungus attack.

Materials and Methods

The methodology used in this research was adapted and modified based on previous techniques used for the study of smut fungi as mentioned by Fernández *et al*. (1978), Matyac (1985), Craig and Frederiksen (1992), Maurya *et al*. (2010) and Kosiada (2011).

Isolation of *Ustilago filiformis* and *in vitro* assays

Glyceria samples of healthy plants and plants infected with *U. filiformis*, were collected in 2009 at Jeppener (35°1639.5″S; 58°1153.7″ O/–35.277639, –58.19825), Buenos Aires Province, Argentina. Subsequently, symptomatic plants were taken to the laboratory for morphological and biometrical observations and to identify the smut. In addition, isolates of the fungus on agar media were performed to study the type of teliospore germination and the colony development of the fungus. Fungal spores (0.5 g) were treated for 3 min in a 2% (w/v) hypochlorite sodium solution (55 g Cl/l), and afterwards washed in sterile distilled water. After that, teliospores were seeded on two agar media: (1) 2% Water Agar (AA) for observing the germination type, and (2) 2% Potato Dextrose Agar (PDA) for the study of colony characteristics and fungus morphology. Cultures in PDA were kept in the refrigerator at 5±2°C to be used in the artificial inoculations carried out on the host, *Glyceria* (Craig and Frederiksen 1992).

Inoculum preparation

For this experiment, basidiospores axenic cultures were obtained using the methodology described above. In Erlenmeyer flasks with a 200-ml Potato Broth medium 2% (PB), the fungal cultures were seeded. They were subsequently placed in a shaker 200 rpp, and placed in an oven (BINDER KBF LQC) at 25±2°C in the dark. Every 24 h, a sample was taken to quantify the basidiospores concentration. The quantification was done with the aid of a Neubauer haemocytometer. When the basidiospore concentration reached $10^{6–8}$ basidiospores · ml^{-1}, the samples were placed in the refrigerator (5°C) to be used for future inoculations (Wilson and Frederiksen 1970; Martinez *et al*. 2002).

Glyceria plants

Two containers (2 m wide × 10 m long) coated with thick black plastic 200 µ were conditioned to transplant the *Glyceria* plants in environmental conditions similar to those where this species develops. Twelve healthy *Glyceria* plants were obtained from the wetlands of Jeppener. In spring (September–October), as soon as the emergence of tillers began, the samples were kept for laboratory assays. Four other containers, twelve plants in total, were selected for being inoculated under field conditions.

Laboratory inoculation assay

Small bits of *Glyceria* leaves (2 cm) were inoculated with a suspension of (1) basidiospores ($10^{6–8}$ spores · ml^{-1}) and (2) teliospores ($10^{6–8}$ spores · ml^{-1}) in sterile distilled water obtained from the culture media (PB). The control plants received the PB media alone without the fungus. Inoculated leaf samples were placed in Petri dishes with sterile filter paper moistened with sterile distilled water to maintain a relative humidity of 80–90%. Then, the dishes were kept in an oven (BINDER KBF LQC) at 25±2°C photoperiod 12 : 12 (L : D) h. After 24 h, samples were mounted in cotton blue lactophenol for observation and histopathological examination under light microscope.

Field inoculation assay

The inoculum was prepared in Erlermeyer flasks with 200 cm^3 of PC media, and sprayed at a concentration of $10^{6–8}$ basidiospores · ml^{-1} on the *Glyceria* plants at the seedling stage (shoot apical meristem with axillary buds). The inoculation was repeated every time plants produced new buds during the vegetative stage (approximately every 20 days). Untreated plants (the controls) received PB media alone. Inoculation was performed during spring 2009 (November-December). Two containers of six plants each one (replications) of *G. multiflora* were selected for field inoculation assays. Another two containers were selected as the control. For both the inoculated plants and the controls, the observations and sampling started 24 h after the inoculations were done. Macro- and microscopic observations were performed to check if any new tillers were infected.

Histopathological study

Samples of *G. multiflora* that had been inoculated under field conditions were collected for the histopathological study and compared with the untreated controls. Samples were fixed in a mix of formalin-acetic acid-alcohol (FAA) and later treated with 10% hydrofluoric acid for 24 h to soften the tissue and remove the silica from the epidermis. Inclusion was performed in paraffin. Transverse and longitudinal sections (10–12 µ thick) were obtained using a microtome type Minot (Senior Rotary Microtome Arcane Brand Model RTM-30). Samples were double stained with safranin-fast green. The technique described by Bracegirdle and Miles (1975) and D'Ambrogio Argüeso (1986) was used for the histopathological determination.

Results and Discussion

Identification of the species and colony morphology

Macro- and microscopic observations of infected *Glyceria* plants confirmed that the sori observed in the inoculated diseased plants agree with the ones described for the species *U. filiformis*. This species produces sori in leaves resulting in a dark stripe of brown to black color, 12 mm wide to several cm in long, at first covered by epidermis which later rup-

tures to expose the powdery dark olivaceous-brown spore mass (Figs. 1A and B) (Hirschhorn 1986; Mordue 1991).

Teliospores are simple, round to sub-globose, 4–7 × × 3–5 μm in size; smooth and light yellow to dark brown episporio under light microscope (Fig. 2A). Scanning electron microscope showed a spore ornamentation with a finely granulated episporio (Fig. 2B).

After 3 days in AA, the teliospores produced basidiospores of 10–100 μ long with only a few septa. Basidiospores (sporidia) may be eitherlateral or terminal. Their multiplication is by shooting and the shoots are abundant in quantity (Figs. 2C and D).

Laboratory tests

In PDA, the colony is a yeast-like type, light brown in color, with roughness on the surface. Basidiospores formed rough, yeast-like, light brown to ocher colonies covering the Petri dishes up to 9 cm, after 10 days (Fig. 3A). The methodology applied for the inoculation of both basidiospores (Fig. 3B) and teliospores were successful for the production of symptoms on *Glyceria* leaves under *in vitro* conditions and the elucidation of the infective process of *U. filiformis* on *Glyceria* plants (Figs. 3C and D).

After the basidiospores' inoculation, new yeast-like basidiospores were formed by budding within 6–8 h after inoculation. Mating was observed between compatible basidiospores (–n + n) and, consequently, the infective hypha was produced (Fig. 4A). On the other hand, the germination of the teliospores occurred within 3–6 days after the inoculations and produced fast-spreading, lateral proliferation of the basidiospores. This process continued until an infective hypha was formed, as happened for basidiospores (Fig. 4B).

The methodology used in the *in vitro* inoculations on *Glyceria* leaf pieces, as in the use of basidiospores inoculated as teliospore, were efficient. In figures 3B, 3C, 4D: (1) the procedural steps that allowed for visualisation were recorded. For basidiospores, rapid multiplication and production of a new basidiospora-type budded yeast on the leaf blade within 6–8 h, was observed. Meanwhile, between compatible mating basidiospores (−n + n), subsequently, an infective hyphae originated was observed (Figs. 4A and B); (2) teliospore germination occurred in 3–6 days; it was inoculated quickly and the basidiospore side-proliferation process continued until the formation of an infective hypha started, as was noted with the basidiospores (Figs. 4C and D).

This methodology allowed us to elucidate the infection process of *U. filiformis* on *Glyceria* and the infection process interaction with the host tissues.

Field tests of pathogenicity

Using basidiospores as inoculum of *Glyceria* plants under field conditions the first lesions induced by the pathogen attack were observed within 10–15 days after inoculations (Figs. 5A and B). Symptoms corresponding to the first stage of infection by *U. filiformis* were sub-epidermal lesions parallel to the veins. Chlorosis, and at a later stage (Fig. 5C), necrosis of the epidermal tissue, were observed (Fig. 5D). However, the latter is not commonly found in biotrophic pathogens like this smut. Dark streaks and/or blisters were observed on the leaves. When they burst, they release an olivaceous-brown spore mass composed of teliospores. Sterile spikelets in the panicles were also observed (Figs. 5E and F). No symptoms or signs of the disease were observed on the non-treated controls. The symptoms registered in this study are similar to those previously found in other species of the smuts. The striations elongated in the flag smut in *Urocystis agropyri* (=*Urocystis tritici*) and the sterile panicles in *Sporisorum reiliana* (=*Sphacelotheca reiliana*) (Wilson and Frederiksen 1970; Bhatnagar *et al*. 1978; Mordue and Walker 1981; Beniwal 1992; Vánky and Shivas 2008).

Histopathological examination of infected tissues

In the control plants, it was observed that the leaf anatomy corresponded to normal plants. The glyceria sheet has a cross sectional structure corresponding to the anatomy of festucoide type having two sheaths around vascular bundles. The mesophyll is formed by a underdeveloped chlorenchyma with remarkable intercellular spaces between secondary vascular bundles. In these spaces, the presence of stellate cells is observed corresponding to the parenchymal tissue with an air reserve called aerenchyma. The aerenchyma allows oxygenation and allows that these floating plants can live in flooded areas. The parenchymal sheath of the vascular bundles is poorly developed and has cells with thin walls (Figs. 6A and B).

In infected plants there were differences in the anatomy of sheets. Observations on infected material showed that the fungus penetrates the sheet through the stomata cell walls or middle lamella and the cell walls by the action of lytic enzymes (Figs. 6C and D). Then the fungal mycelium colonise clorenchyma and this mycelium laterally invades tissue, even tissue below the vascular bundles (Figs. 7A and B). Hyphae occurs primarily on the abaxial face of the phyllode. Once the mycelium fragments are induced, the sporogenesis occurs in the intercellular spaces between vascular bundles where the aerenchyma is located. The aerenchyma is observed as being expanded by tissue hypertrophy. Aerenchyma was fully occupied by dark brown teliospores of the fungus (Figs. 7C and D). Macroscopic observation of the infected area showed symptoms or brown-streaked dark blisters between the veins of the leaves (Fig. 5). There was no fungal presence after the observation of the cuts made in the stem or vegetative meristem and panicles. Therefore, in these studies, artificially infected plants with *U. filiformis* were used to allow us to state that the route of infection is of a local type. This localized infection was also found in other species of smuts, like *Ustilago maydis* (Hirschhorn 1986; Bannuett and Herskowitz 1996), *Thecaphora solani* (Vilaró 2005), and *Thecaphora frezii* (Astiz Gassó *et al*. 2010 a, b).

This study is the first result and contribution to the histopathology of *U. filiformis* in *Glyceria* sp. (Astiz Gassó *et al*. 2012).

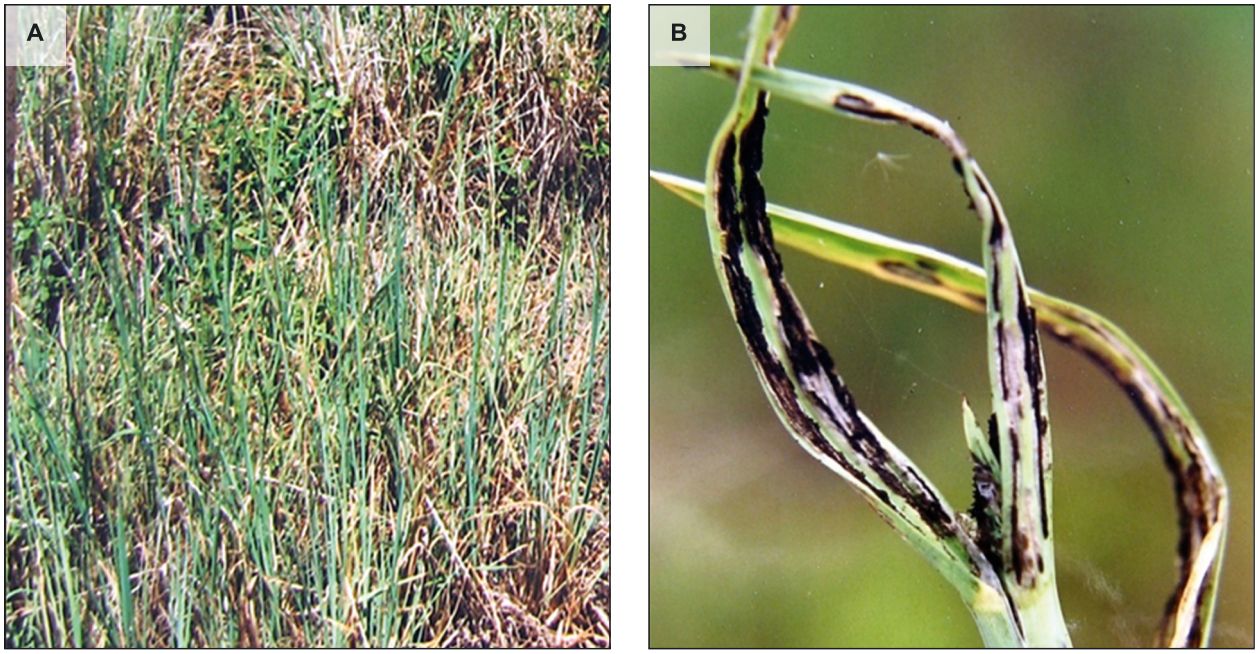

Fig. 1. A – Plants of *Glyceria* naturally infected with *Ustilago filiformis;* B – Macroscopic observation of symptoms – chlorosis and necrosis – on leaves. The black teliospores of the fungus are a sign of the disease

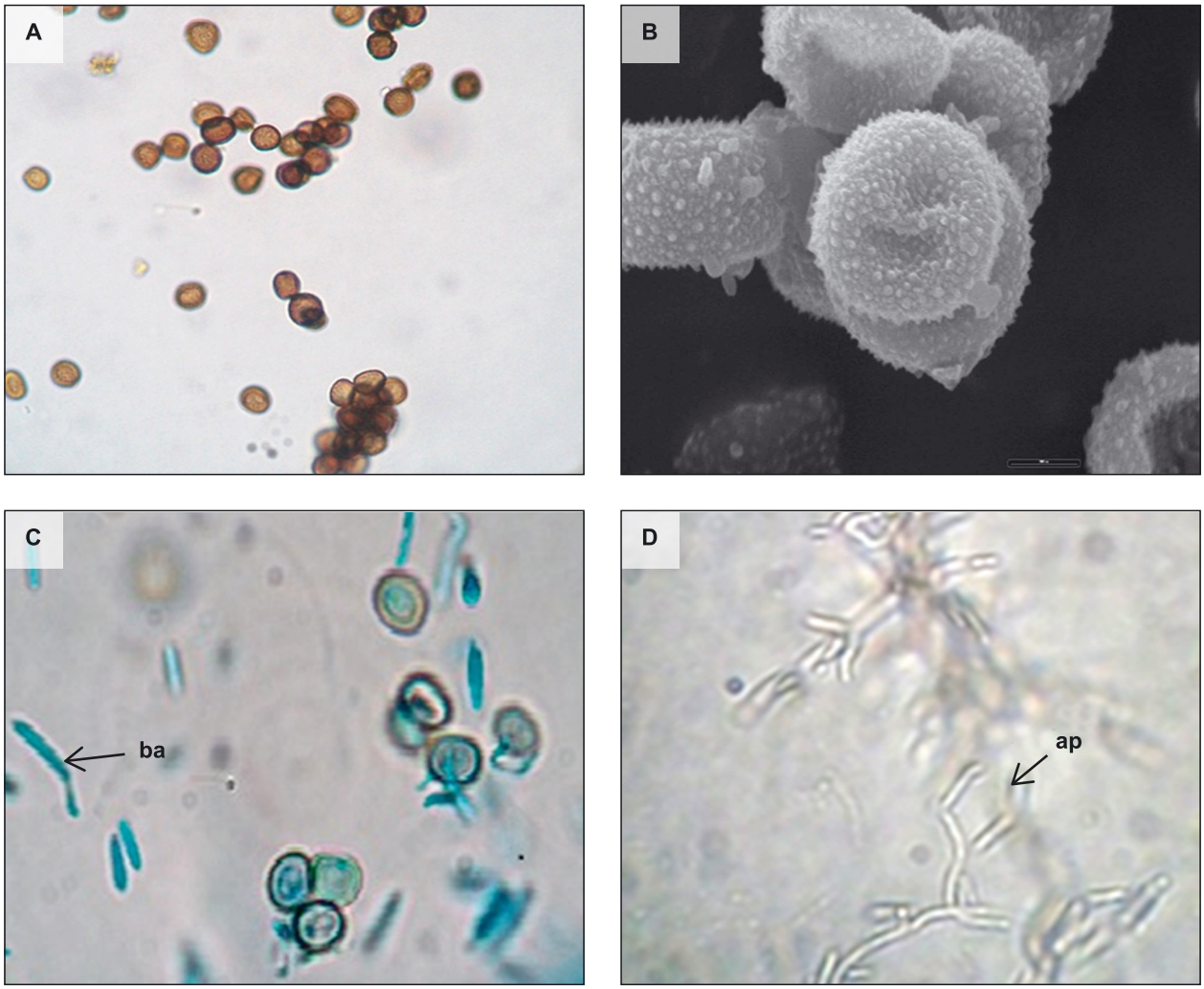

Fig. 2. Reproductive structures observed by light microscope (LM) and scanning electron microscope (SEM): A – teliospores of *Ustilago filiformis* (LM), scale bar = 14 μ; B – teliospores of *U. filiformis* (SEM), scale bar = 22 μ; C, D – yeast-type teliospore germination showing basidiospores (ba) and basidiospores mating (ap) on PDA

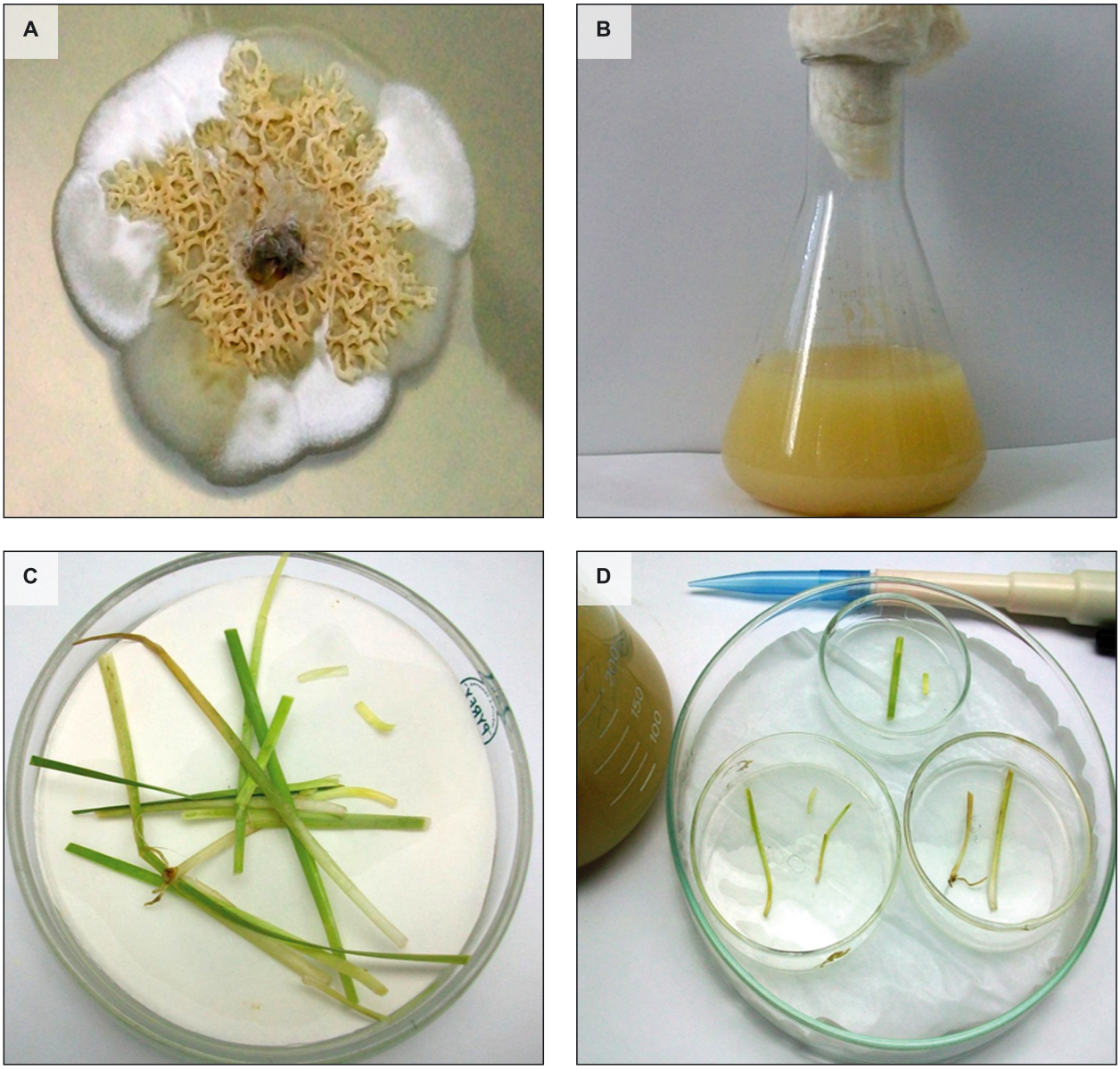

Fig. 3. A – Colony of *Ustilago filiformis* on Potato Dextrose Agar (PDA); B – Growth of *U. filiformis* on Potato Broth (PB) media to be used in inoculations; C – Samples of *Glyceria* plants to be inoculated with teliospores and basidiospores of the fungus, in pathogenicity assays; D – Moist chamber

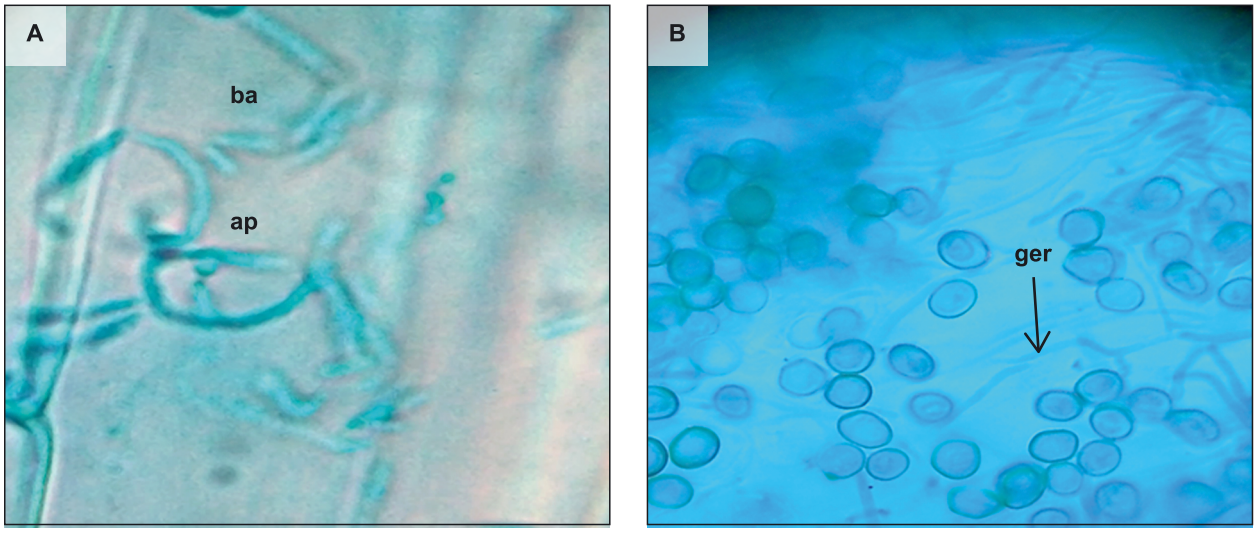

Fig. 4. Germination and infection of *Ustilago filiformis* on *Glyceria* leaves under *in vitro* conditions: A – inoculation with basidiospores (ba) and matings between compatible basidiospores (ap); B – teliospores inoculations where germination (ger) on the leaf can be seen

Fig. 5. Artificial inoculation with basidiospores of *Ustilago filiformis* developed on PC media, and observation of symptoms: A – inoculation at field conditions of *Glyceria* plants; B – first symptoms and signs of the disease (si); C – chlorosis and interveinal necrosis (ne) on the leaves; D – emergence of sterile panicles induced by the pathogen (pa); E, F – symptoms on the leaf; E – observation of striate with chlorosis (st-chl); F – striate with teliospores (st-e) and blistering (bl)

Ustilago filiformis life cycle

The infective process of the streak smut of *Glyceria* starts when teliospores from infected leaves are spread by wind and reach the healthy *Glyceria* plants in the wetlands (Fig. 8–1, 2). After a copulation process between cells which are sexually compatible, the dikaryotic teliospores (n + n) become diploid (2n) (Fig. 8–3). Later, haploid basidospores are produced and the infected mycelium (n + n) is produced (Fig. 8–4) by fusion. The infection of the host takes place when the parasitic mycelium forms the appressoria, and penetrates the leaf tissue by way of the stomata or by

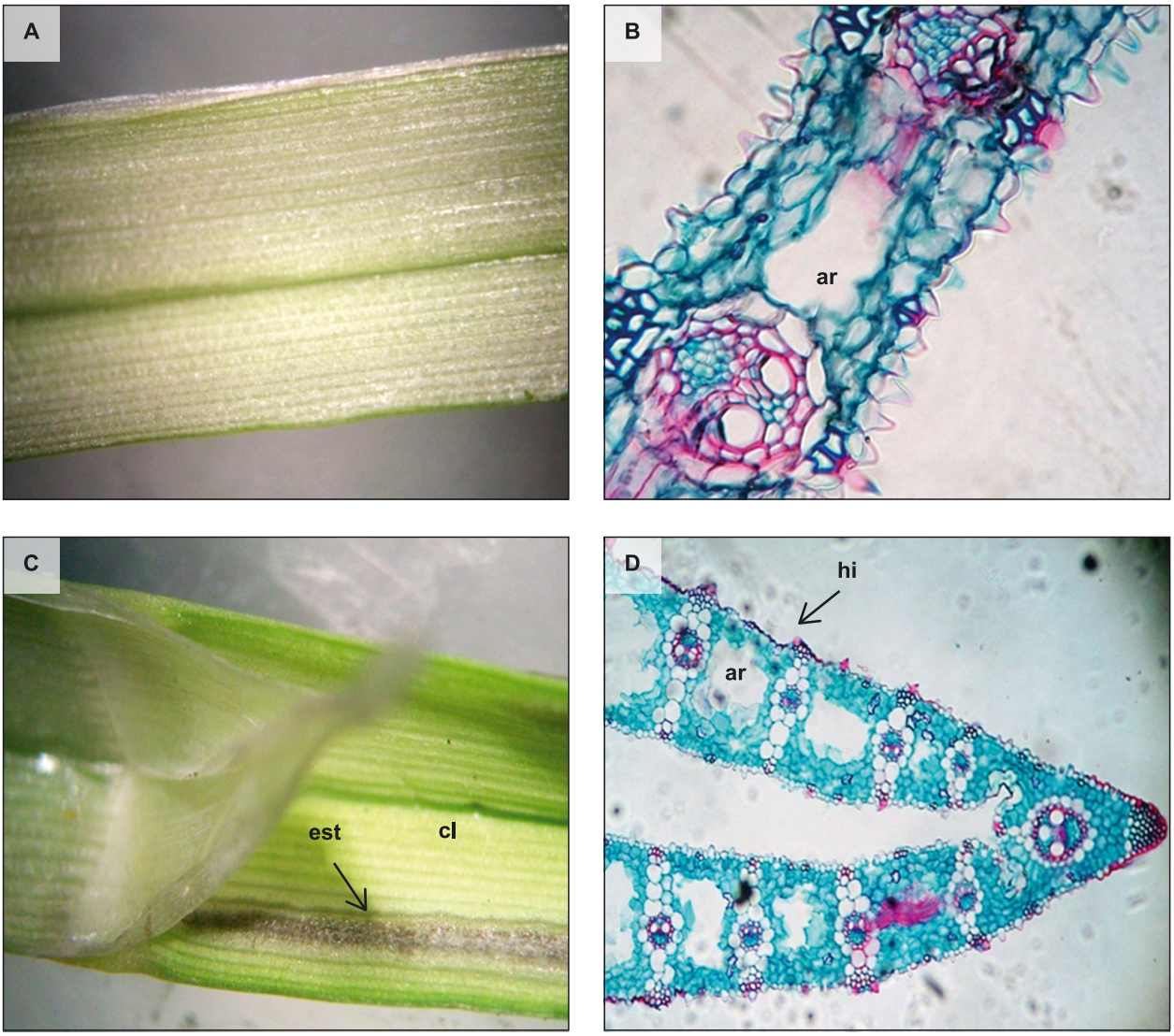

Fig. 6. *Glyceria* leaf from artificially inoculated plants and the control: A – healthy leaf section; B – cross section of *Glyceria* leaf showing healthy aerenquima (ar) by light microscope (LM); C – leaf symptoms in inoculated plants: chlorosis (cl) and striate (est); D – cross section of leaf showing fungus mycelium (hi) on the epidermis and aerenquima (ar) before being hypertrophied and the fungus infection

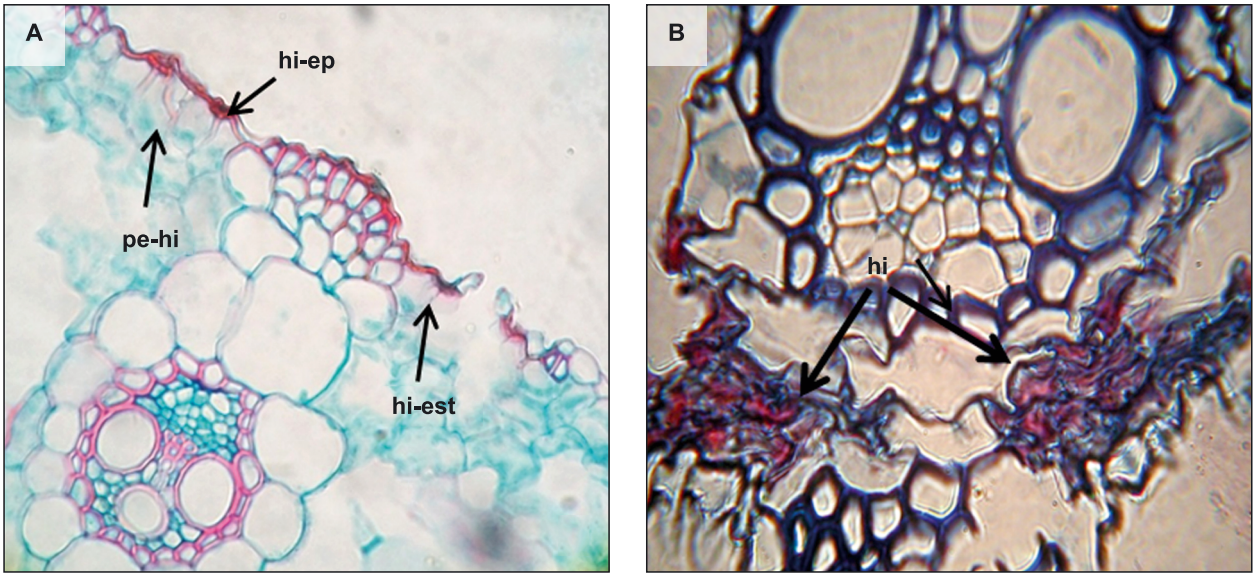

Fig. 7. Light microscope (LM) cross-sectional cuttings of the *Glyceria* infected leaf: A – hyphae on stoma (hi-est) and epidermis (hi-ep); penetration of the hyphae in/on the leaf (pe-hi); B – tissue colonized by the hyphae (hi) of the fungus close to the vascular bundles

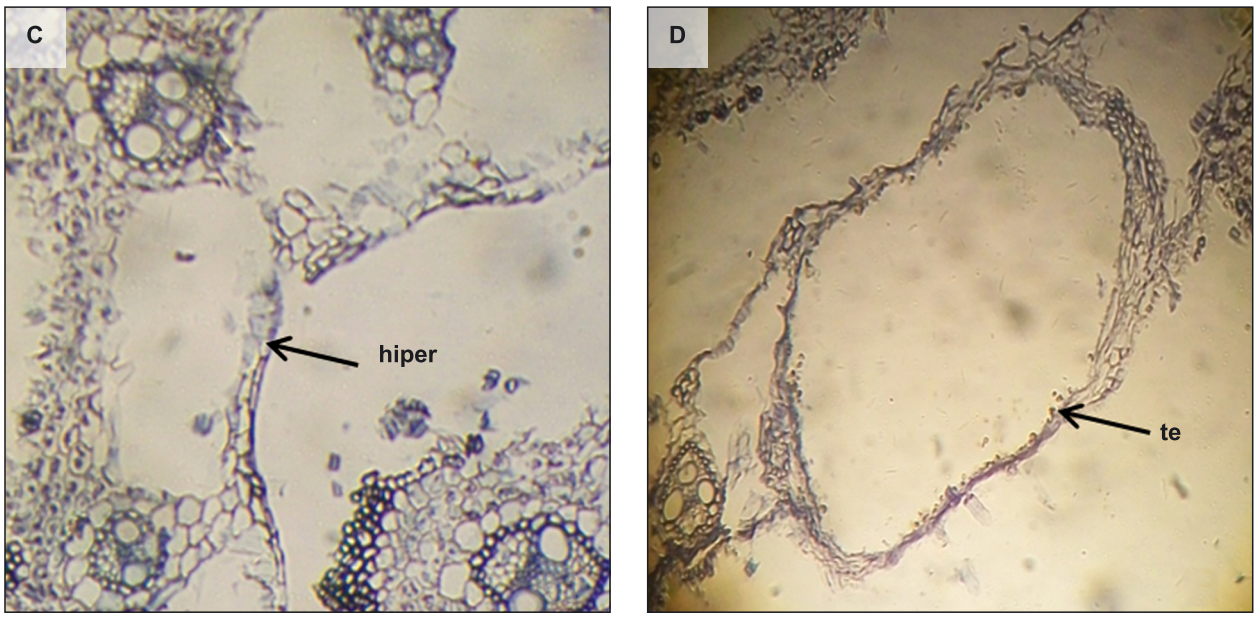

Fig. 7. Light microscope (LM) cross-sectional cuttings of the *Glyceria* infected leaf (*continuation*): C, D – hypertrophy (hiper) of tissue between the vascular bundles. Aerenchyma where teliospores (te) can be observed

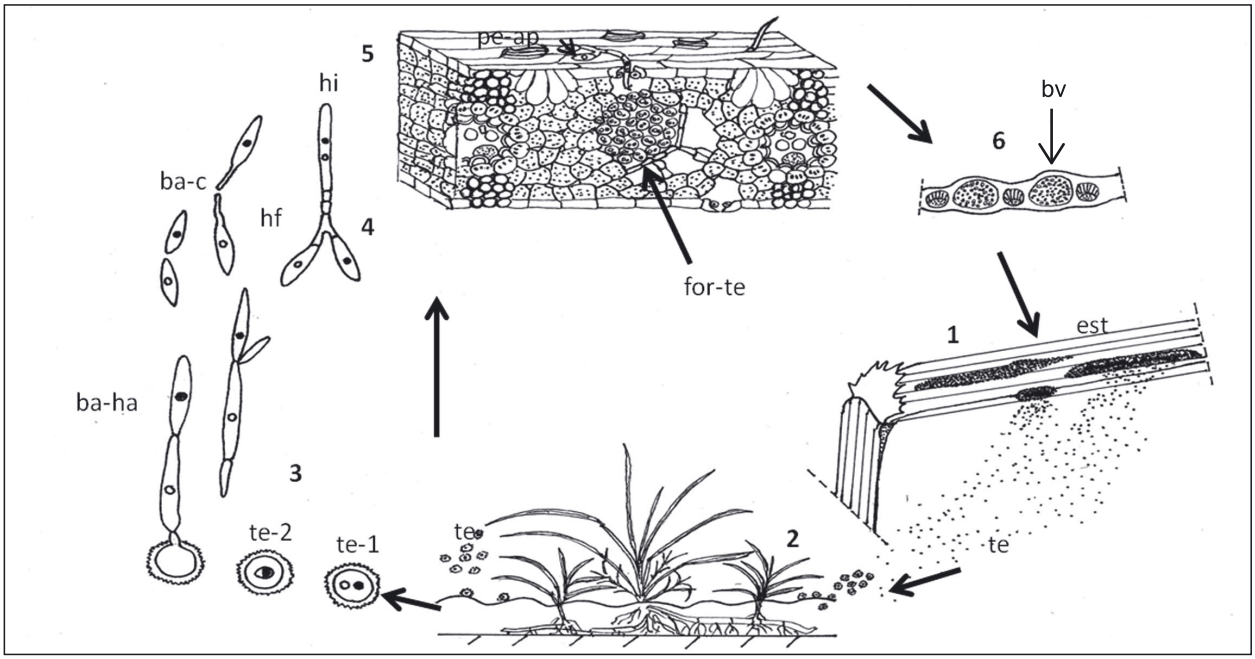

Fig. 8. Life cycle of *Ustilago filiformis*: 1 – *Glyceria* leaf infected with teliospores (te) spread by the wind; 2 – *Glyceria* plants in their natural habitat – wetlands – with teliospores reaching the surface of the water and approaching the leaves; 3 – teliospores (te-1) and mature teliospores n + n (te-2); 4 – formation of haploid basiodiospores (ba-ha) and conjugating of basidiospores (ba-c), infective hypha formation (hi) by fusion of cells (hf); 5 – cross-section of *Glyceria* leaf showing the pathogen infection. *Ustilago filiformis* can develop an appressorium and penetration (pe-ap) of the infective hyphae in the tissue by softening the walls and/or stomata, teliospore formation (for-te) in the aerenchyma of the mesophyll of *Glyceria* leaves; 6 – fungal infection between the vascular bundles (bv)

softening the cell walls. Then, the colonisation of the *Glyceria* leaves takes place. The fungus moves towards the vascular bundles producing internerval blisters. The teliospores are formed in the aerenquima (Figs. 8–5, 6). Blisters, previously formed in the leaf tissue, burst and release teliospores which are later spread by means of wind and/or water. Survival of the pathogen is possible by means of teliospores in the soil and/or infected plant debris (Astiz Gassó *et al*. 2012).

Conclusions

The histopathological process of infection of *U. filiformis* on *Glyceria* plants, and the life cycle of the fungus on the host, is recorded for the first time in this paper. It must be pointed out that the methodology applied using both teliospores and basidiospores can be equally used to produce the infection and thus, the typical symptoms of the

disease. The results recorded in this paper are valuable for understanding the epidemiology of the disease and the biology of the fungus.

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