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Research Note: Histopathology of *Puccinia philippinensis* Syd. & P. Syd, A Fungus that Causes Leaf Rust Disease to *Cyperus rotundus* L.

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ABSTRACT

Cyperus rotundus L., popularly known as purple nutsedge, mutha or barsanga, is a weed problem in most crop productions due to its prolific underground parts that permit rapid production of multiple young sprouts in the soil. Yield losses in many crops due to competition by *C. rotundus* range from 35 to 90%. *Puccinia philippinensis*, on the other hand, is a potential biological control against *C. rotundus*. Its anatomy of infection inside tissues of the weed, however, is still very much unexplored. This study was conducted to determine the histopathology of leaf rust disease caused by *P. philippinensis* inside leaf tissues of *C. rotundus*. Methods such as staining, embedding, and mounting of leaves with structures of the rust fungus were conducted using the standard staining equipment, glasswares, and chemicals. Microscopic examination revealed that the histopathology of rust disease in leaves of *C. rotundus* started with swelling due to the development of *P. philippinensis*'s uredinium in the lower epidermis. The event was followed by the development of numerous urediniospores that came from the uredinia. Lower epidermis of *C. rotundus* was ruptured due to pressing and pushing out of the urediniospores. Urediniospores of *P. philippinensis* were sub-globose to globose in shape measuring 13 x 16μ spores^{-1}.

Keywords: Histopathology, *Cyperus rotundus* L., *Puccinia philippinensis*, leaf rust, fungi, uredinia, urediniospores

INTRODUCTION

Purple nutsedge (*Cyperus rotundus* L.) is one of the world's worst weeds (Holm*et al.,* 1977). It occurs in many countries causing significant losses in crop yields by way of competition with the limited nutrients, light, water, and space (Donayre *et al.*, 2015; Baltazar *et al.,* 2004). The weed is very persistent in the soil causing application of chemicals ineffective due to escape of its tubers (Siriwardana and Nishimoto, 1987). Handweeding is not also effective against purple nutsedge because aside from being too laborious and expensive, it can

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enhance the population of the weed in the soil (Islam *et al.,* 2009). Significant yield losses in a wide range of crops due to competition by *C. rotundus* can range from 35 to 90% (Islam *et al.,* 2009, Baltazar *et al.,* 2004; Santos *et al.,* 1998; Keeley, 1987). In rice ecosystem, full competition of *C. rotundus* with upland and lowland rice plants can significantly reduce yield by 42 and 50%, respectively (Islam *et al.,* 2009; Okafor and de Datta, 1974). Histology, on the other hand, is the study of tissues and cells at the microscopic level. In plant pathology, it is mostly termed as histopathology which means the art and science of studying the anatomy of the disease and its causal organism in a tissue section of a particular plant (Hossain and Natural, 2003; Romero *et al.,* 2003; Kunwar *et al.*, 1986). Histopathology is one of the basic tools that is utilized not only in studying the development of plant pathogenic microorganisms inside the tissues of a particular plant but also, in vice versa, in determining the resistance and hypersensitive reactions of the hosts during invasion by the pathogens (Ma and Shang, 2009; Kloppers and Pretorius, 1995; Niks, 1981).

Purple nutsedge like any other cultivated plants is also beset by disease-causing microorganisms (Macedo *et al.*, 2008; Tangonan, 1999; Kadir and Charudattan, 1999; Ribeiro *et al.,* 1997). One of these is *Puccinia philippinensis* Syd. & P. Syd. causing leaf rust disease. *P. philippinensis* has the potential as biological control agent against purple nutsedge because the rust fungus is specific to the weed (Hiratsuka *et al.*, 1992; Gardner and Hodges, 1989). Explorations on utilizing *P. philippinensis* as biological control agent against purple nutsedge, however, have not yet been reported in the world. Reports on histopathology of *P. philippinensis* have not yet been done. Hence, this paper was conducted to determine the histopathology of leaf rust disease caused by *P. philippinensis* inside leaf tissues of *C. rotundus*.

MATERIALS AND METHODS

Collection of Infected Leaves

Rust-infected leaves of purple nutsedge were collected at the vegetable area of the Agronomy and Soil Science Cluster, College of Agriculture, University of the Philippines at Los Baños, College, Laguna. Leaf samples were placed inside paper bag and brought into the Plant Pathology Laboratory of the Crop Protection Cluster for tissue processing and microscopic examination. Histopathological procedures used in this study were based on the works of Hossain and Natural (2003).

Tissue Preparation and Staining

Portions of infected leaves with pustules of the pathogen were cut into 5mm pieces and placed in a vial previously filled with Rawlin's FAA #11 (100ml of 50% ethyl alcohol, 10ml formalin and 10 ml glacial acetic acid) as preservative. Cut sections then were transferred and immersed through a series of ethyl alcohol (ETOH) and tertiary butyl alcohol (TBA) solutions for dehydration [(a) 50ml distilled water + 40ml 95% ETOH+ 10ml TBA (2hr); (b) 30ml distilled water + 50ml 95% ETOH+ 20ml TBA (overnight); (c) 15ml distilled water + 50ml 95% ETOH+ 35ml TBA (1 hr); (d) 45ml 95% ETOH+ 55ml TBA (1 hr); (e) 75ml

TBA+ 25ml absolute alcohol (1 hr); (f) 100% TBA (TBA); (g) Pure TBA (TBA); (h) 100% TBA (overnight); and (i) 50ml TBA and 50ml paraffin oil (1 hr)].

Five minutes before the 9th solution (50ml TBA and 50ml paraffin oil), the vial was filled with ¾ of melted paraffin and was allowed to partially solidify. Samples were placed on top of solidified paraffin and simultaneously covered with TBA-paraffin mixture for embedding. The vials with the samples were placed in the oven for a period of 1 hr to allow the tissues to sink into the bottom. Pure melted paraffin solutions were then poured at different times (2, 24, and 1 hours).

Tissue Mat Embedding

Leaf tissue samples from the oven were placed in embedding dishes at 4 to 6 samples per dish previously coated with glycerine and melted paraffin. A warm needle was used to facilitate proper orientation of the tissues in such a way that individual pieces can be cut easily in the finished block. Embedding dishes with tissue samples were then placed immediately on top of the surface of ice and later transferred in refrigerator to enhance solidification of the melted paraffin. After solidification, hardened paraffin with individual leaf tissue sample in it was removed from the dish by cutting with a sharp scalpel and mounted on a wooden block by heating the bottom of the paraffin and the top of the wooden blocks. Finished blocks with leaf tissue samples were placed back inside the refrigerator for rehardening of the paraffin.

Rotary Microtome Sectioning

Finished block was carefully placed near the edge and parallel with the blade of the microtome machine. To obtain a good ribbon, the razor blade was angled at 8^0 vertical enough to touch and clean the block with cotton (previously moistened with xylene), and do even strokes. Using a dissecting needle and widened-width knife, formed ribbons at desired length were transferred and laid in black lint free paper.

Ribbon Mounting and Slide Staining

Ribbons in the black lint free paper were individually cut and transferred to slides previously applied, immersed, and flooded with Mayer's adhesive, ETOH, and 4% formalin, respectively. Afterwhich, each slide with ribbon was transferred to slide warmer for overnight incubation at 40^0C. After 24hr, each slide was immersed simultaneously in xylene (5min), xylene-100% alcohol (5min), 100% alcohol (5min), 95% alcohol (5min), 70% alcohol (5 min), 50% alcohol (5 min), 1% Safranin in 50% alcohol (60 min), tap water (10 sec), 50% alcohol (3min), 70% alcohol (3 min), 95% alcohol (3 min), 0.5% fast green from 50% clove oil + 50% alcohol (3 sec), 100% alcohol, xylene -100% alcohol (3 min), and xylene (5 min). Stained tissues in slides were smeared with Canada balsam then covered separately with cover slides.

Microscopic Examination

Structures of the rust fungus inside the tissue in each slide was examined and measured at 1000x magnification using the microscope digital camera (Olympus DP72-BSW).

RESULTS AND DISCUSSION

Symptomatology

Distinguishing symptoms of leaf rust disease of purple nutsedge caused by *P. philippinensis* had yellowing around the infected leaf surface area. In the center of infected area were the big, ellipsoidal, orange to brown pustules of the pathogen arranged in longitudinal pattern with the leaf veins of the weed (Figure 1). Under severe infection, pustules of the pathogen were scattered in the entire leaf surfaces of the weed.

Histopathology

Microscopic examination revealed that the anatomy of rust disease caused by *P. philippinensis* in leaf tissues of *C. rotundus* started with swelling due to series of uredinium development in the sub-epidermal part (lower epidermis) (Figure 2b). Simultaneous swelling events were followed by the development of numerous urediniospores (Figure 2c-d). Each urediniospore was singly attached in stalk called pedicel. Lower epidermis was ruptured due to pressing and pushing out of the urediniospores that came from the uredinia. At closer examination, the urediniospores of *P. philippinensis* were sub-globose to globose in shape measuring 13 x 16µ spore^{-1} (Figure 3). According to Alexopoulos and Mims (1979), uredinia (sing. uredinium) are structures of rust fungi where urediniospores are borne. Webster and Weber (2007) also emphasized that urediniospores that are attached in stalk-like structure called pedicel are the ones that break-off at maturity and are disseminated by wind to re-infect the host.

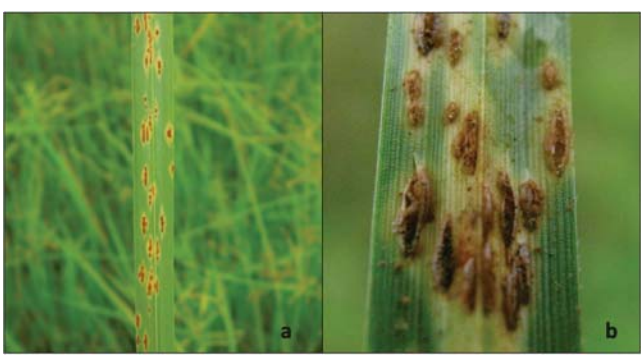

Figure 1. Leaf rust disease of *Cyperus rotundus* caused by *Puccinia philippinensis*. a) Yellow symptoms around infected leaf surfaces (n=1x), and b) close-up of orange to brown pustules (n=10x).

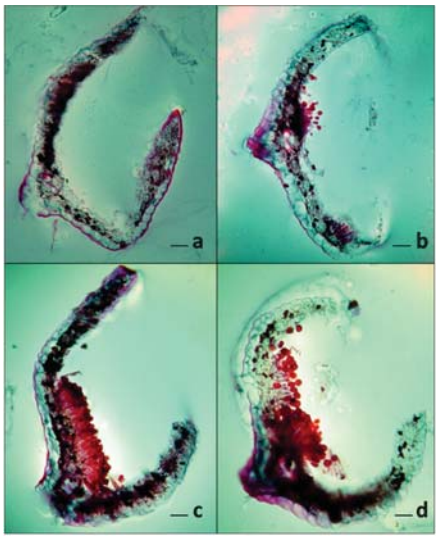

Figure 2. Cross-section of infected leaf tissues of *Cyperus rotundus* showing the anatomical structures of *Puccinia philippinensis*. a) no infection, b) surface swelling with early fungal development, and c-d) rupturing of epidermal tissue due to full development of uredinium and urediniospores; a-d= 45µ.

In studying rust disease in a weed, Hiratsuka *et al.* (1992) described similarly the morphological structures of *P. philippinensis* on leaves of purple nutsedge. They described the urediniospores of *P. philippinensis* as globose with light brown walls, and echinulate measuring 14-19 x 13-17µ while the uredinia were hypophyllous, scattered, ellipsoid measuring 1mm long with light brown color. According to Gardner and Hodges (1989), *P. philippinensis* is sometimes misidentified as *P. canaliculata* (Shw.) Lagerh. which was also reported infecting other members of Cyperaceae family.

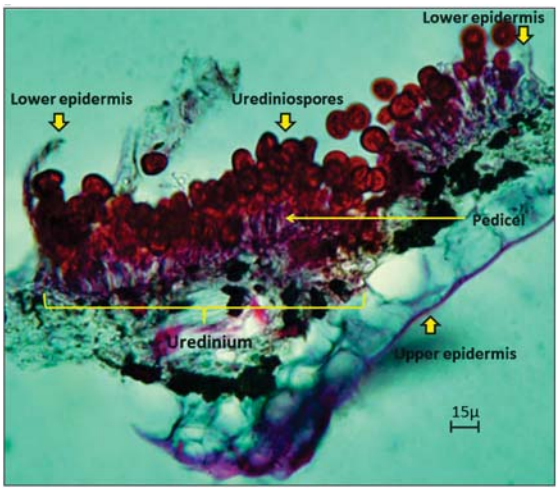

Figure 3. Cross-section of an infected leaf tissue of *Cyperus rotundus* showing the uredinium and urediniospores of *Puccinia philippinensis*.

Hiratsuka *et al.* (1992) emphasized, however, that urediniospores of *P. canaliculata* were larger in size and less spherical (obovoid) in shape than *P. philippinensis*. In the works of Hosain and Natural (2003) on histopathology of soybean rust caused by *Phakopsora pachyrhizi* Sydow and anthurium leaf blight caused by *Xanthomonas campestris* pv. *diffenbachiae*, they also observed that the tissues of the hosts were ruptured and disintegrated due to infection of the pathogens. They also observed that the epidermal part of the soybean leaf was ruptured due to rising of urediniospore of *P. pachyrhizi*. They added that the urediniospores of *P. pachyrhizi* inside tissues of soybean leaf were sub-globose in shape, hyaline to yellowish brown while the uredinia were yellow and short. Meanwhile in studying the histology of infection and development of four avirulent pathotypes of rust fungus, *Puccinia recondita* f.sp. *tritici*, in wheat line (RL6081) with gene *LR37*, Kloppers and Pletorius (1995) observed significant arrest of fungal structures in RL6081 at early infection stages inside adaxial flag leaf surfaces. Lr37 significantly decreased the rate of uredinial appearances of all four pathotypes. Compared with Thatcher, fewer uredinia of smaller dimensions developed on flag leaves of Rl6081.

Elucidating the histopathology of rust disease caused by *P. philippinensis* in leaf tissues of *C. rotundus* is a great help in explaining the simultaneous events and mechanisms involved during the infection process by the rust fungus unto its host. It also helps explain the pathogenicity of *P. philippinensis* as well as the occurrence of its visible pustules underneath the leaves of an infected *C. rotundus* plant. Furthermore, the histopathological information that had been obtained in this study can be utilized to promote the potential of *P. philippinensis* as biological control against *C. rotundus*.

CONCLUSION

The anatomy of infection of *Puccinia philippinensis* inside leaf tissues of purple nutsedge started with swelling in the lower epidermis due to the development of uredinia. The event was followed by rupturing of the lower epidermis due to production and eruption of numerous urediniospores that came from uredinia. Lower epidermis of *C. rotundus* was ruptured due to pressing and pushing out of the urediniospores of *P. philippinensis*.

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