Summary

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Title	Research and Development of Early Detection and Control Methods for Anthracnose Agent (植物炭疽病菌の早期検出と防除に関する研究)

Key words: Cashew; Anthracnose; *Colletotrichum gloeosporioides*; Diagnosis; Management.

This PhD thesis dissertation is comprised in one abstract and six independent chapters. The first chapter provides a general introduction to this work, a literature review of relative aspects presented in the document and the research objectives. The chapter two reports on my personal immersion in the field of biotechnology given my natural resources/forestry management background. That preparation step consists in a field survey for on-field recognition of the disease in cashew plantations, the collection, isolation, identification and culture of the pathogen in laboratory settings. Also, it describes the conventional DNA extraction methods, PCR and LAMP detection methods for the target pathogen. The chapter three reports on the research and fabrication of a biosensor adapted for on-field use and rapid detection of *Colletotrichum gloeosporioides*. The chapter four presents the investigation and application of copper nanomaterials as control measure of the pathogen and the mechanism involved was further studied in chapter five, using model membrane systems and Fourier Infrared spectroscopy (FTIR) analysis. Finally, chapter six contains a summary of the results, conclusions and perspectives of this dissertation.

Chapter I: General introduction

In this first chapter, we introduced the background of the study by reviewing related studies. A general overview of anthracnose disease, its causal agent, and current detection and control methods was provided, indicating the importance of the present research. In addition, the complexity of the phytopathogens and their impacts in agriculture, economy and food security were shown. Current understandings suggest that a rapid and accurate detection of the fungus responsible of the pathology is

a key in the diagnosis process, which will significantly affect the effectiveness of the disease management methods, whether existing or proposed in this work. However, multiple challenges such as accessibility for developing and underdeveloped areas in the world, accuracy and effectiveness of the existing diagnosis and control methods are described. Based on this background, the objectives of the study were proposed.

Chapter II: Cashew Anthracnose Diagnosis with Pathogen Collection, Isolation, Identification and Culture

Infection control measures depend on proper identification of diseases and the causal agents. Therefore, proper and quick diagnosis is vital in order to avoid waste of resources such as time, money and farm inputs in diseases control.

In the present chapter, we report on the recognition and collection of *Colletotrichum gloeosporioides* in cashew plantations. Methods for the pathogen's isolation, identification and culture were then described. After extraction of the pathogen's DNA, nucleic acid amplification assays were performed for the detection of the pathogen. We successfully demonstrated that Polymerase Chain Reaction (PCR) and Loop-mediated isothermal amplification (LAMP) were both sensitive and effective for detecting anthracnose causal agent in cashew settings.

Chapter III: Immuno-Dipstick for *Colletotrichum gloeosporioides* Detection: Towards On-Farm Application

Early and quick detection of pathogens is crucial in managing spread of infections in biomedical, biosafety, food and agriculture fields. While molecular diagnostics can offer the specificity and reliability in acute infectious diseases, detection of pathogens is often slowed down by present benchtop molecular diagnosis, which are time consuming, labor intensive, and lack the mobility for application at point-of-need. In this chapter, we developed a complete on-farm-use detection protocol for a plant devastating anthracnose agent *Colletotrichum gloeosporioides*. Our methods combined a very simplified DNA extraction on paper that is compatible with loop-mediated isothermal amplification (LAMP), coupled with a paper-based immunoassay lateral flow sensing. Our results offer

simple, quick, easy, and minimally-instrumented toolkit for *Colletotrichum gloeosporioides* detection. This scalable and adaptable platform is a valuable alternative to traditional sensing systems towards on-the-go pathogen detection in food and agriculture, biomedical and other fields.

Chapter IV: Antifungal Potential of Nanostructure Crystalline Copper and its Oxide Forms

Copper has been used as an antimicrobial agent for over a century and is now being added to commercial fungicides. Nanomaterials have attracted much attention due to the special properties they have over their bulk form. We studied nanostructured copper (Cu-NPs), investigating the potential for improved antifungal properties derived from its special properties and studied any effect that the oxidation of copper (CuO-NPs) may have. We conducted this research against *Colletotrichum gloeoesporioides*, a devastating pathogen to plants/crops worldwide. Research on the effects of copper on this fungus are limited. Our studies showed that nanoforms of copper had significant antifungal activities, with Cu-NPs offering the most sustainable efficacy and was more effective than its oxidative form (CuO-NPs). Scanning Electron Microscopy (SEM) images of the treated pathogen show that the hyphae had a swollen appearance, lost their filamentous structure, and the mycelia had a powder-like structure, indicating the probable destruction of the hyphal tubular cell wall. X-ray Diffractogram (XRD) outputs showed substantial changes in the physical characteristics of the Cu-NPs after interaction with the fungus. This is the first report to demonstrate chemo-physical changes in the metal compounds, opening new insights for further studies on the mechanism of copper's antifungal properties.

Chapter V: Understanding Antifungal Properties of Nanostructured Copper and its Oxide Derivative

To back up the antifungal properties of Cu and its oxide forms demonstrated in control *Colletotrichum gloeosporioides*, the causal agent of anthracnose, the molecular mechanisms underlying anti-fungal activities of nanostructured copper (Cu-NPs) and copper oxide (CuO-NPs) was investigated. Using biomimetic model membrane systems, we studied in real-time, spatio-temporal membrane dynamics induced by the copper nanoforms. We observed dynamics, with a dominance of large

fluctuations, some of which culminated into membrane transformations. Exo-bud /-cytosis was the major membrane change induced by both copper nanoforms. Chemical characterization of the functional groups using FTIR reveal that both studied nanoparticles interfered/interacted with functional groups in all major biomolecules (lipids, proteins and carbohydrates) of the pathogen, with CuO-NPs inducing more changes. Even though, those new findings were in concordance with some of our previous work, the clear translation of the cause to effect relation is not yet elucidated.

Chapter VI: General Conclusions

The spreading of pathogens has become an ever-growing issue of concern for the agricultural sector as a result of climate change and the increase in the large-scale global transport of plant products. As soon as the symptoms of a pathogen have become visible, visual assessment can be a quick and easy diagnostic method. Unfortunately, symptoms are not always visible or obvious and can vary extensively depending on the circumstances. The correct and quick diagnosis of suspect plant material at the production location is necessary to guarantee the production of healthy vegetative plant material and seeds. A quick intervention will also result in cost savings and a reduction in loss of revenue.

Anthracnose is one of the most economically damaging plant diseases affecting major crops such as cashew, strawberry, mango. And *Colletotrichum gloeosporioides* has become our target, being the primary causal agent of anthracnose. A key challenge in controlling anthracnose is detecting the pathogen on asymptomatic plants. To meet this end:

- We have successfully developed a paper-based immunoassay lateral flow biosensor for the detection of *Colletotrichum gloeosporioides,* which we believe is the first;
- A Loop mediated isothermal amplification (LAMP) assay, that incorporated a very simplified DNA extraction method, was developed and successfully tested for the detection of *Colletotrichum gloeosporioides;*
- We demonstrated the inhibiting effect of copper/ copper oxides metals and their nanoforms against the proliferation of *Colletotrichum gloeosporioides;*

- and finally, the molecular mechanism underlying the anti-fungal activities of nanostructured copper and copper oxide was deeply investigated.

We now have a full platform, that is simple, quick, easy, and made of minimally-instrumented kits, for *Colletotrichum gloeosporioides* detection. This scalable and adaptable set-up represents a significant step in the development of sensitive, portable and low-cost technologies for the detection of the pathogen at point-of-care. Also, we proposed based on our findings that copper nanoparticles could be a great alternative to the chemical treatments used to manage the phytopathology.