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Leaf rot caused by *Rhizopus oryzae* on pak choy (*Brassica campestris* ssp. *chinensis*) in China

Samiah ARIF[1], Muhammad Farooq Hussain MUNIS[2,3], Fiza LIAQUAT[4], Iftikhar Hussain SHAH[1], Yidong ZHANG[1,*]

[1] *Department of Plant Sciences, School of Agriculture and Biology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, China*
[2] *Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan*
[3] *Department of Plant Pathology, University of California, Davis, 95616, United States of America*
[4] *School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, 200240, P. R. China*
*Corresponding author: zhyd@sjtu.edu.cn

Abstract. Pak choy (*Brassica campestris* ssp. *chinensis* L.) is the major vegetable crop cultivated in China. During December 2017, leaf rot was frequently observed on leaves of pak choy in greenhouses of Shanghai, China. Diseased leaf samples were plated onto acidified potato dextrose agar and fungal cultures were isolated and identified as *Rhizopus oryzae*, based on morphological features and molecular identification. Definitive identification as *R. oryzae* was based on the comparative molecular analysis of rRNA gene sequences. Blast analysis revealed 99% similarity with *R. oryzae*. Pathogenicity was determined by inoculating healthy pak choy leaves and plants with hyphal suspensions of *R. oryzae*. The fungus was re-isolated from developing similar lesions on the inoculated plants and identified as similar to the inoculated fungus, thus fulfilling Koch's postulates. This is the first report of fungal leaf rot caused by *R. oryzae* on pak choy in China.

Keywords. ITS sequence, Shanghai.

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INTRODUCTION

Pak choy (*Brassica campestris* subsp. *chinensis* L.) is a leafy green Chinese cabbage which does not form heads. It is a popular vegetable crop, with smooth dark green leaves and is grown widely in southern China and Southeast Asia (Khalid *et al*., 2017). Compared to other Brassicaceae members, pak choy does not have well-developed root systems, producing sessile leaves with a short stems. Due to high water and soil fertility requirements, this plant cannot tolerate dry conditions for long periods (Ajisaka *et al*., 2001). A 100 g portion of fresh pak choy supplies 13 calories and is rich in vitamins A, C and K (Noia, 2014).

Rhizopus oryzae is fungus in the Zygomycetes, and is a ubiquitous soil pathogen (Battaglia *et al*., 2011) causing worldwide losses in crop yields (Bassanezi *et al*., 2011). It causes diseases on many economically important crops, including citrus (Hakim *et al*., 2015), sweet potato (Wang *et al*., 2017), rice (Lanoiselet *et al*., 2007) and yellow oleander (Arif *et al*., 2017). *Rhizopus oryzae* is found globally but has wide distribution in tropical and sub-tropical regions. The fungus causes small water-soaked spots on plant tissues which become soft, pulpy and ultimately rot. Whitish mycelia with many spore producing sporangia are usually evident near infected tissues (Shtienberg 1997; Kwon *et al*., 2012a).

In the winter of 2017, a leaf rot disease of pak choy was observed in green houses in Shanghai, China. Early foliar symptoms were characterized by yellow to grey, irregular-shaped lesions on the affected leaves. As these spots increased in size, they developed to irregular round spots that turned to sunken whitish grey lesions surrounded by brownish borders. After approx. 2 weeks, the lesions expanded to cover entire leaves. Infected leaves turned dark brown and withered. Random disease distribution was observed on 79% of plant population. This disease was observed in more than 20 greenhouses in Shanghai area. The objective of the present study was to isolate and identify the causal agent of leaf rot of pak choy.

MATERIALS AND METHODS

For isolation of the disease causing pathogen, 3-5 diseased leaves were randomly collected from 12 different greenhouses. Small portions (<1 cm^2) of symptomatic leaf tissues were surface sterilized in 75% ethanol for 30 s, then rinsed three times with sterile distilled water, and were then were then placed on acidified potato dextrose agar (APDA) for incubation at 28°C in the dark. Single conidiospore cultures were made from 36 isolated colonies, and these were incubated on APDA.

To perform the morphological observations of fungal isolates, samples from 7-d-old cultures of all 36 isolates were mounted with water on glass slides and examined under a light microscope (Leica DM750, Leica Microsystems,) to observe culture and fungus morphological characters. As all isolates had similar form one isolate was randomly selected and used for molecular identification and pathogenicity testing.

Genomic DNA was extracted from mycelia of the isolate using the CTAB method (Cullings 1992). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd, and the resulting products were sequenced. The PCR was carried out sequentially in a volume of 25 µL containing Master Mix (10 µL), 10 mM forward primer (1 µL), 10 mM reverse primer (1 µL), DNA template (1 µL) and sterile distilled water (12 µL). The cycling parameters were 94 °C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and final extension of 72°C for 1 min. The PCR product was sequenced by TSINGKE Biological Technology and analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast). Nucleotide sequences were aligned with MEGA version 6.0 (Tamura *et al*., 2013). Phylogenetic analysis was performed using the maximum likelihood with 1,000 bootstrap replications.

Pathogenicity tests were carried out on detached, young pak choy leaves. Twenty non-infected detached 20-d-old were surface-sterilized and each inoculated by placing 10 µL of conidium suspension (10^6 conidia mL^{-1}) of the isolated fungus using either non-wound/drop or wound/drop methods. The same inoculation pattern was used for control leaves while distilled water was used instead of conidium suspension (Ford, 2004). The infected leaves were incubated at 28°C and 80 to 85% humidity with a 12 h photoperiod. The experiment was repeated twice. Koch's postulates were assessed using 20-day-old healthy pak choy plants. Ten plants were sprayed with spore suspensions (10^6 conidia mL^{-1}), while plants sprayed with water served as negative controls. The plants were then covered with plastic bags for 48 h and kept in a greenhouse. After symptom development, the pathogen was re-isolated from the inoculation induced lesions, and was morphologically identical to the original isolates obtained from diseased pak choy plants.

RESULTS AND DISCUSSION

Thirty-six isolates of similar morphology were obtained from 40 diseased leaf samples (Figure 1a), and the frequency of isolation from diseased leaves was 90%. After incubation at 28°C on APDA, white mycelium initially appeared then became heavily speckled with sporangia and finally became blackish-gray and spread rapidly with stolons attached at various points to the substrate by rhizoids (Figure 1b). The reverse sides of Petri plate colonies were light brown (Figure 1c). The mycelial appearance, colour and growth pattern was similar to previously described features of *Rhizopus oryzae* (Zhang *et al*., 2013). Sporangiophores were mostly erect, subhyaline to brown, single or in groups, and 180 to 1400 µm long and 8 to 14 µm wide (Figure 1d). Sporangia were globose to subglobose, greyish brown to black, and 60

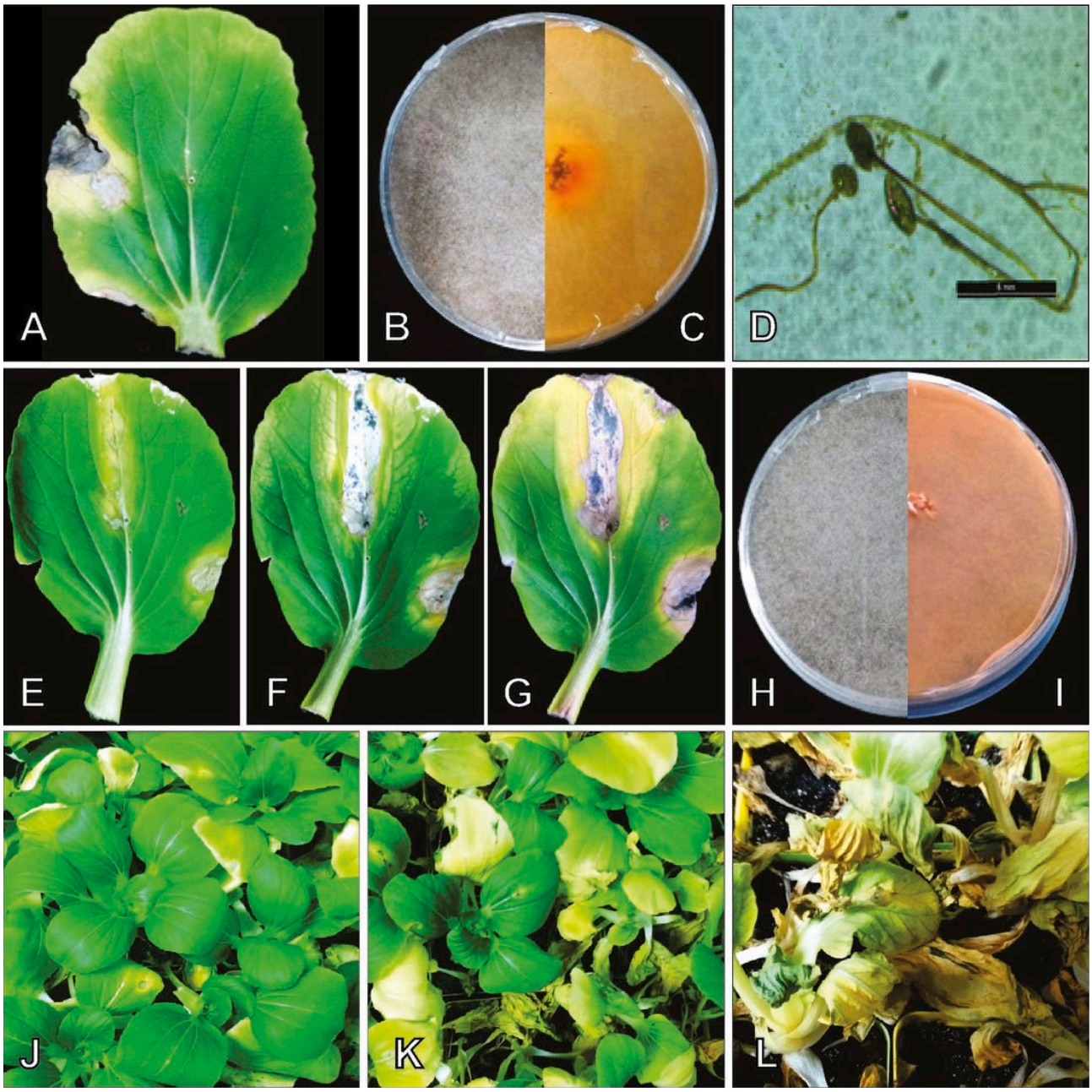

Figure 1. Disease symptoms on a leaf of pak choy (A). Disease causing pathogen was isolated on APDA and observed from front side (B) and back side (C) of Petri plates. Fungus morphology was observed under a compound microscope (D). Typical disease symptoms were observed after 4 d (E), 6 d (F) or 8 d (G) after inoculation. The inoculated pathogen was isolated from diseased leaves (H and I). After foliar inoculation of fresh leaves (J), lesions appeared in the 1st first week after innoculation (K) and completely damaged plants in the 3rd week (L).

to 180 µm in diameter (Figure 1d). Columella were subglobose to ovoid, subhyaline to greyish brown, and 35 to 100 × 50 to 120 µm. Sporangiospores were ovoid, angular, striate, pale greyish to brown, and 5 to 8 × 3 to 5 µm (Figure 1d). Similar features have been described for *R. oryzae* by Park *et al*., (2014).

Whitish grey lesions surrounded by brownish borders, similar to those observed on naturally infected pak choy leaves in the commercial greenhouses, appeared on all inoculated leaves, then rotted within 7 d. No lesions developed on the control leaves (Figure 1; e, f and g). All the inoculated pak choy plants also showed the typi-

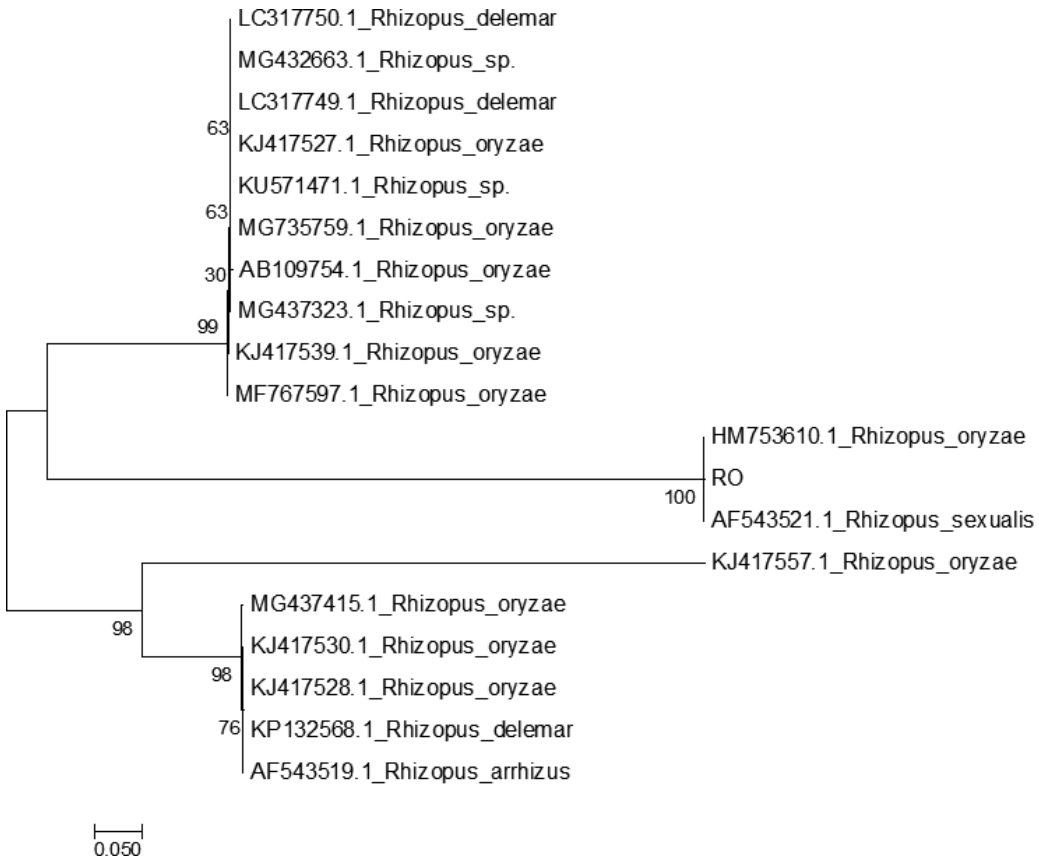

Figure 2. Phylogenetic analysis of a *Rhizopus oryzae* isolate from pak choy, with 18 related sequences from GenBank. Resulting from 1,000 replicates, bootstrap values are shown at the branch points.

cal symptoms within 10 d, whereas the control plants remained healthy (Figure 1; k, l and m). *Rhizopus oryzae* was re-isolated from the lesions of inoculated leaves, fulfilling Koch's postulates (Figure 1; h, i and j).

BLAST analysis indicated that the ITS sequence was 99% similar to *R. oryzae* (accession number HM753610). Phylogenetic analysis showed the isolated sample was conspecific with other members of *R. oryzae* (Figure 2).

Morphological, microscopic and molecular analyses, coupled with fulfillment of Koch's postulates, proved *R. oryzae* to be the casual pathogen of leaf rot disease of pak choy in this study. This fungus has been isolated from soil, decaying vegetation, vegetables, fruits, seeds and dung (Domsch *et al*., 1980). *Rhizopus oryzae* has been reported to cause infection and decay of tobacco leaves (Kortekamp 2006). Previous studies showed that the pathogen also causes soft or fruit rots of peach (Kwon *et al*., 2012a), jackfruit (Nelson 2005), and banana (Kwon *et al*., 2012b). However, there is no prior report of *R. oryzae* causing leaf rot on pak choy. To our knowledge, this is the first report of *R. oryzae* causing leaf rot on pak choy in China, and elsewhere in the world. Our future research will be focused primarily on management of this disease.

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LITERATURE CITED

Ajisaka H., Kuginuki Y., Yui S., Enomoto S., Hirai M., 2001. Identification and mapping of a quantitative trait locus controlling extreme late bolting in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* syn. *campestris* L.) using bulked segregant analysis. *Euphytica* 118: 75.

Arif S., Liaquat F., Khizar M., Shah I.H., Inam W., … MUNIS M.F.H., 2017. First report of *Rhizopus ory-*

zae causing fruit rot of yellow oleander in Pakistan. *Canadian Journal of Plant Pathology* 39: 361–364.

Bassanezi R.B., Montesino L.H., Gasparoto M.C.G., Bergamin F.A., Amorim L., 2011. Yield loss caused by huanglongbing in different sweet orange cultivars. *European Journal of Plant Pathology* 130: 577–586.

Battaglia E., Benoit I., van den Brink J., Wiebenga A., Coutinho P.M., de Vries R.P., 2011. Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate degradation depicted at genome level. *BMC Genomics* 12: 38.

Cullings K.W., 1992. Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology* 1: 233–240

Domsch K.H., Gams W., Anderson T.H., 1980. *Compendium of Soil Fungi*, Academic Press, London, UK, 1: 1–860.

Ford R., Banniza S., Photita W., Taylor P.W.J., 2004. Morphological and molecular discrimination of *Colletotrichum truncatum* causing anthracnose on lentil in Canada. *Australasian Plant Pathology* 33: 559–569.

Hakim, Naz S., Gul S., Chaudhary H.J., Munis M.F.H., 2015. First report of *Rhizopus oryzae* causing fruit rot of *Citrus medica* L. in Pakistan. *Journal of Plant Pathology* 97: 215.

Khalid M., Hassani D., Bilal M., Liao J., Huang D., 2017. Elevation of secondary metabolites synthesis in *Brassica campestris* ssp. *chinensis* L. via exogenous inoculation of *Piriformospora indica* with appropriate fertilizer. *PloS One* 12: 0177185.

Kortekamp A., 2006. Effectiveness of calcium salts, hydrogen peroxide, azoxystrobin, and antagonistic bacteria to control post-harvest rot on tobacco caused by *Rhizopus oryzae*. *International Journal of Pest Management* 52: 109–115.

Kwon J.H., Kang D.W., Ha J.S., Kim J.W., Kwak Y.S., 2012a. Soft rot on peach caused by *Rhizopus oryzae* in Korea. *Korean Journal of Mycology* 40: 65–68.

Kwon J.H., Ryu J.S., Chi T.T.P., Shen S.S., Choi O., 2012b. Soft rot of *Rhizopus oryzae* as a postharvest pathogen of banana fruit in Korea. *Mycobiology* 40: 214–216

Lanoiselet V., Cother E., Ash G., 2007. Aggregate sheath spot and sheath spot of rice. *Crop Protection* 26: 799–808.

Nelson S., 2005. Rhizopus rot of jackfruit. *Plant Disease* 29: 1–2.

Noia J.D., 2014. Defining powerhouse fruits and vegetables: a nutrient density approach. *Preventing Chronic Disease* 11: 1–5

Park J.H., Cho S.E., Kim B.S., Shin H.D., 2014. First report of postharvest root rot caused by *Rhizopus oryzae* on *Codonopsis lanceolata*. *Australasian Plant Disease Notes* 9: 135.

Shtienberg D., 1997. Rhizopus head rot of confectionery sunflower: effects on yield quantity and quality and implications for disease management. *Phytopathology* 87: 1226–1232.

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* 30: 2725–2729

Wang R.Y., Gao B., Chen S.L., Ma J., Li X.H., Xue C.X., 2017. First report of *Rhizopus oryzae* causing soft rot on storage roots of sweet potato in China. *Plant Disease* 101: 1039.

Zhang X., Yao Z., Zhao S., Xie H., Yang M., 2013. Rhizopus stem rot of *Orobanche aegyptiaca* caused by *Rhizopus oryzae* in China. *Journal of Phytopathology* 161: 745–748.