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The pathogenicity of basidiospores of *Phellinus noxius* which causes brown root rot disease in Taiwan

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ABSTRACT: The role of basidiospores of *Phellinus noxius* in nature has not been clearly elucidated since brown root rot disease was discovered. Previously, it was reported that *P. noxius* rarely produces basidiocarps in nature, while a total of 33 new tree host species bearing basidiocarps were recently found in Taiwan. The more-abundant basidiospores were collected for germination and infection studies in this paper. Fresh basidiospores were collected from live basidiocarps growing on a small-leafed banyan (*Ficus microcarpa*), and the basidiospores were rinsed out. The germination experiment revealed that they could germinate in all kinds of liquids including rainwater, with germination rates usually exceeding 50%. The direct inoculation of basidiospore suspensions at concentration of 10^5~10^6 spores/mL on four tree species was consistently unsuccessful. The same spore suspension was used to inoculate wood chips from Taiwan zelkova (*Zelkova serrata*) sapwood. After 2 months, some of the wood chips had been colonized by basidiospores, showing dark-brown line or network symptoms. Using these colonized wood chips, we successfully inoculated wounded roots of Taiwan zelkova seedlings within 3 months, proving the virulence and role of basidiospores in nature. Since 2007, 13 cases of twig or stem infection by *P. noxius* were found in the field, confirming transmission by basidiospores. In Taiwan, due to the high frequency of typhoon damage to trees, abundant stem wounds should act as important sites for basidiospore deposition and infection. This could facilitate the long-distance dispersal of this disease in nature.

KEY WORDS: Basidiospore, Brown root rot, Long-distance dispersal, Pathogenicity test, *Phellinus noxius*, Taiwan.

INTRODUCTION

Brown root rot disease caused by *Phellinus noxius* (Corner) Cunningham is now widespread in Taiwan, Hong-Kong, Macao, southern Japan, and southern China (Akiba *et al.*, 2015; Ann and Ko, 1992; Ann *et al.*, 1999a; Ann *et al.*, 1999b; Ann *et al.*, 2002; Chang, 1992; Chang, 1995a; Chang and Yang, 1998; Chung *et al.*, 2015; Fu, 2005). Based on previous reports, more than 200 agricultural and forest plant species serve as hosts of *P. noxius* (Ann *et al.*, 1999a; Ann *et al.*, 2002; Chang and Yang, 1998; Fu, 2005). *Phellinus noxius* is a member of the family Hymenochaetaceae, and can produce two types of fruiting bodies: a flat (resupinate) type and a bracket type (Bolland, 1984; Pegler and Waterson, 1968). This species was first reported by Sawada in Taiwan in 1928, but at that time the fungus was identified as *Fomes lamaensis* (Sawada, 1928). Later, it was considered a synonym of *Fomes noxius* (Sawada, 1942). Brown root disease of trees was also reported in Singapore by Corner in 1932 as *Fomes noxius* (Corner, 1932) and reclassified by Cunningham in 1965 as *Phellinus noxius*.

Identifying this fungus is based on symptoms and cultural characteristic when grown on potato dextrose agar (PDA) (Ann and Ko, 1992; Ann *et al.*, 2002; Chang, 1992). In the field, this disease is principally transferred by root-to-root contact (Ann *et al.*, 1999a; Ann *et al.*, 2002). The role of basidiocarps and basidiospores of *P. noxius* has not been clarified since this fungus was discovered (Ann and Ko, 1992; Chang, 1992; Chang, 1995a). Previously, scientists concluded that the fungus rarely produces basidiocarps on diseased trees in the field (Ann and Ko, 1992; Ann *et al.*, 1999a; Chang, 1992; Chang, 1995a). Recently, the same authors of this paper discovered a total of 33 new host tree species with fruiting bodies on diseased trees (Hsiao *et al.*, submitted to *Taiwania*), indicating that the occurrence of basidiocarps in nature is not rare. Both types of fruiting bodies, including the flat and bracket types, were frequently found in the field in Taiwan.

In Queensland, Australia, fruiting bodies of *P. noxius*, were consistently observed on hoop pine by Bolland (1984), who even tried to inoculate pine stumps with a basidiospore suspension to verify their pathogenicity. Bolland collected basidiospores and arthrospores of this fungus, and inoculated 30 freshly cut pine stumps with them. The inoculation results showed that only two stumps developed brown rot symptoms on the stumps, but no results were seen with arthrospore treatment. However, a further re-isolation study showed that only one of the two stumps could the pathogen be successfully re-isolated (Bolland, 1984). As the success rate was very low, the role of basidiospores in long-distance dissemination was still in doubt.

Recent molecular and genetic studies were also joined to elucidate the role of basidiospores in long-distance dissemination of brown root rot. Chung *et al.*

(2015) reported that population genetic studies based on simple sequence repeat (SSR) markers revealed very high genotype diversity within its populations in Taiwan, suggesting that genetically variable basidiospores are involved in long−distance dispersal of this fungus. They also found nearly identical genotypes within infected foci, indicating that the spread over short distances is via root − to − root contact. A similar phenomenon was reported by Akiba *et al.* (2015) in Japan. They collected 128 isolates from 12 of the Ryukyu Islands and three of the Ogasawara Islands, and found that all isolates had unique genotypes. This suggests that basidiospores should play an important role in dissemination by forming new disease foci (Akiba *et al.*, 2015).

Another approach to demonstrate the role of basidiospores is based on variations of the virulence of different isolates. Sahashi *et al.* (2010) conducted cross-inoculation tests using nine isolates obtained from nine different tree species. Results showed significant variations in virulence among these nine isolates, suggesting that basidiospores play a role in increasing genetic diversity.

Since 2007, we have participated in a serial project of tree brown root rot surveys in Taiwan. In addition to the comprehensive disease survey, we also attempted to reveal the role of its basidiospores in long-distance transmission. Results of pathogenicity studies are presented herein.

MATERIALS AND METHODS

Collection of P. noxius basidiospores and measurement of their germination rates

Basidiospores were collected from fresh basidiocarps growing on small-leafed banyan (*Ficus microcarpa*) in Nantou County, central Taiwan. Sterile distilled water was used to rinse out the basidiospores, which were then observed under a light microscope, counted, and adjusted to adequate concentrations. In some cases, a clean plastic Petri dish was placed under a basidiocarp to collect the basidiospores, which were then rinsed with tap water or rainwater, and directly used for germination tests.

Spores were transferred onto four kinds of medium, including: (1) water agar (2% agar autoclaved), (2) potato dextrose agar (PDA), (3) malt extract agar (MEA), and (4) MA+4 selective medium developed by Chang (1995b) (20 g/L malt-extract and 20 g/L agar, amended with four other chemicals after being autoclaved: 10 mg/L benomyl, 10 mg/L dicloran, 100 mg/L ampicillin, and 500 mg/L gallic acid). They were kept at 28 °C for 24 h. Then, spore germination was examined under a light microscope. Successful germination was defined as outgrowth of mycelium longer than the spore length itself. For each treatment, 100 basidiospores were examined, and the germination rate (%) was calculated.

Direct inoculation with P. noxius basidiospores on tree seedlings

Direct inoculations with a *P. noxius* basidiospore suspension were conducted on seedlings of four tree species at the age of about 2 years. They were Formosan ash (*Fraxinus formosana* Hay.), Formosan sweet gum (*Liquidamber formosana* Hance), common elaeocarpus (*Elaeocarpus sylvestris* (Lour,) Poir), and red cedar (*Bischofia javanica* Blume). All potted seedlings were grown in a greenhouse.

Basidiospores were collected from fresh basidiocarps growing on small-leafed banyan in Nantou County. Sterile distilled water was used to rinse out the basidiospores, and then the spores were observed under a light microscope, counted, and diluted to 10^5~10^6 spores/mL. The basal stem of each tree seedling was cut with a sterile scalpel to make a wound after surface disinfection with 75% alcohol; then 1 mL of the spore suspension was injected into the wound, and the wound was wrapped with a layer of parafilm. In each experiment, four seedlings of each species were inoculated, and the experiment was repeated two times. Another four seedlings of each species were inoculated with sterile distilled water instead as the control. After inoculation, all seedlings were incubated in a growth chamber at a temperature 28 °C and a diurnal light/dark cycle of 8/16 h. They were inspected periodically for symptoms development and all other responses.

Indirect inoculation with P. noxius basidiospores on wood chips then on tree seedlings

Indirect inoculations with a *P. noxius* basidiospore suspension were first conducted on sapwood chips from Taiwan zelkova (*Zelkova serrata*) to test their colonization ability. The size of the sapwood chips was 3 × 3 × 0.5 cm (L × W × H). Basidiospores were also collected from fresh basidiocarps growing on small-leafed banyan in Nantou County as mentioned above, and diluted to three concentration levels of 10^3~10^4, 10^4~10^5, and 10^5~10^6 spores/mL. Each freshly cut sapwood chip was placed in a 500-mL glass jar and inoculated with 2 mL of a spore suspension. These were loosely covered and kept at room temperature for observation. In each experiment, three sapwood chips were inoculated, and the experiment was repeated once. Each time, another three sapwood chips were inoculated with sterile distilled water instead as the control. Colonization of wood chips by germinated basidiospore was determined 2 months later. When the wood chips showed a brownish discoloration with dark-brown lines or a network, they were re-isolated for *P. noxius* with MA+4 medium to verify successful inoculation.

After the sapwood chips from Taiwan zelkova were successfully inoculated by the basidiospore suspension, each colonized chip was used to inoculate a Taiwan zelkova seedling of about 3 years old. Half of the root system of a seedling was exposed and washed with tap water to remove soil particles, and then the chip inoculum was adhered to the wounded main root at the bottom of the pot. In each experiment, three seedlings were inoculated, and the experiment was repeated once. Another set of the seedlings was inoculated with healthy wood chips instead as a control. After inoculation, all seedlings were incubated in a growth chamber at a temperature of 28 °C and under a diurnal light/dark cycle of 8/16 h. They were inspected periodically for symptoms development and all other responses.

Investigation of twig or stem infection by P. noxius brown rot in Taiwan

Since 2007, we have participated in a project of tree brown root rot surveys in Taiwan as recently reported (Hsiao *et al.*, unpublished manuscript). The brown rot symptoms found to begin on a twig or above-ground stem were investigated. If there was no brown root rot accompanying the above-ground brown rot, then it was recognized as a twig-infection or stem-infection case of the same pathogen, *P. noxius*.

All suspected twig- or stem-infection cases were further sampled and identified by culturing on MA+4 selective medium and PDA. The same cultural characteristics were used for a positive identification as mentioned previously (Hsiao *et al.*, unpublished manuscript).

RESULTS

Collection of basidiospores and measurement of their germination rates

Basidiospores were easily collected from fresh basidiocarps growing on small-leafed banyan in Nantou County. They had an oval or broadly ellipsoidal shape with a globosely nuclear-like or yolk-like content as shown in Figure 1(A).

Germination rates of *P. noxius* basidiospores collected from fresh basidiocarps on four different media and three types of liquid are shown in Table 1. The highest germination rate of 82% was found on MEA medium, but over 50% germination rates were generally achieved on every kind of test medium or liquid. Although rainwater is acidic in Taiwan, that did not affect spore germination compared to distilled or tap water. Generally higher than 50% germination rates seemed to be very beneficial for this pathogen to achieve long-distance transmission in the field.

Direct inoculation with P. noxius basidiospores on tree seedlings

We tried to directly inoculate the basal stem of seedlings of four tree species with a *P. noxius* basidiospore suspension for more than 2 years. Basidiospores were collected from fresh basidiocarps growing on small-leafed banyan in Nantou County. A spore suspension of 1 mL with a concentration of 10^5~10^6 spores/mL was inoculated onto the wounded basal stem of seedlings. Results showed that none of the inoculated seedlings expressed disease symptoms on the wounded stem after 1 year. They remained as healthy as that of the healthy control. These results are consistent with the reported dissemination pattern of root-to-root contact. Until now there were no cases proven of direct transmission by *P. noxius* basidiospores.

Table 1. Germination rates of ***Phellinus noxius*** basidiospores collected from fresh basidiocarps on different media and liquids.

Medium/liquid	Germination rate (%)*
Water agar	78
Potato dextrose agar	52
Malt extract agar	82
MA+4 selective medium	65
Distilled water	52
Tap water	56
Rainwater from Nantou County	58

* Data are the average of two replicates; germination was defined as the outgrowth of mycelium longer than the spore length itself.

Table 2. Colonization rates of wood chips from Taiwan zelkova after inoculation with ***Phellinus noxius*** basidiospores from fresh basidiocarps

Basidiospore concentration (spores/mL)	Successful colonization rate*		
	Experiment 1	Experiment 2	Average
10^3~10^4	1/3 (33%)	0/3 (0%)	17%
10^4~10^5	1/3 (33%)	1/3 (33%)	33%
10^5~10^6	2/3 (67%)	2/3 (67%)	67%

* Successful colonization was based on evidence of brownish discoloration with dark-brown lines or a network on wood chips and successful re-isolation.

Indirect inoculation with P. noxius basidiospores on wood chips then on tree seedlings

Despite the failure of direct inoculation with *P. noxius* basidiospores on tree seedlings, indirect inoculation on wood chips of Taiwan zelkova was successful. Some of the inoculated wood chips showed brownish discoloration with dark-brown lines or a network after incubation for 2 months, and the pathogen was re-isolated with MA+4 medium. None of the healthy control wood chips showed any signs or symptoms. After 6 months, successful colonization rates are shown in Table 2. At higher spore concentrations, the colonization rate reached 67% compared to 33% at lower spore concentrations.

After the sapwood chips from Taiwan zelkova were successfully colonized by basidiospores and their mycelium, they were used to inoculate the Taiwan zelkova seedlings at the exposed and wounded main root. Results as shown in Table 3 revealed that after 3 months, all of the seedlings (a total of six in two experiments) showed brown root rot symptoms on the main root and

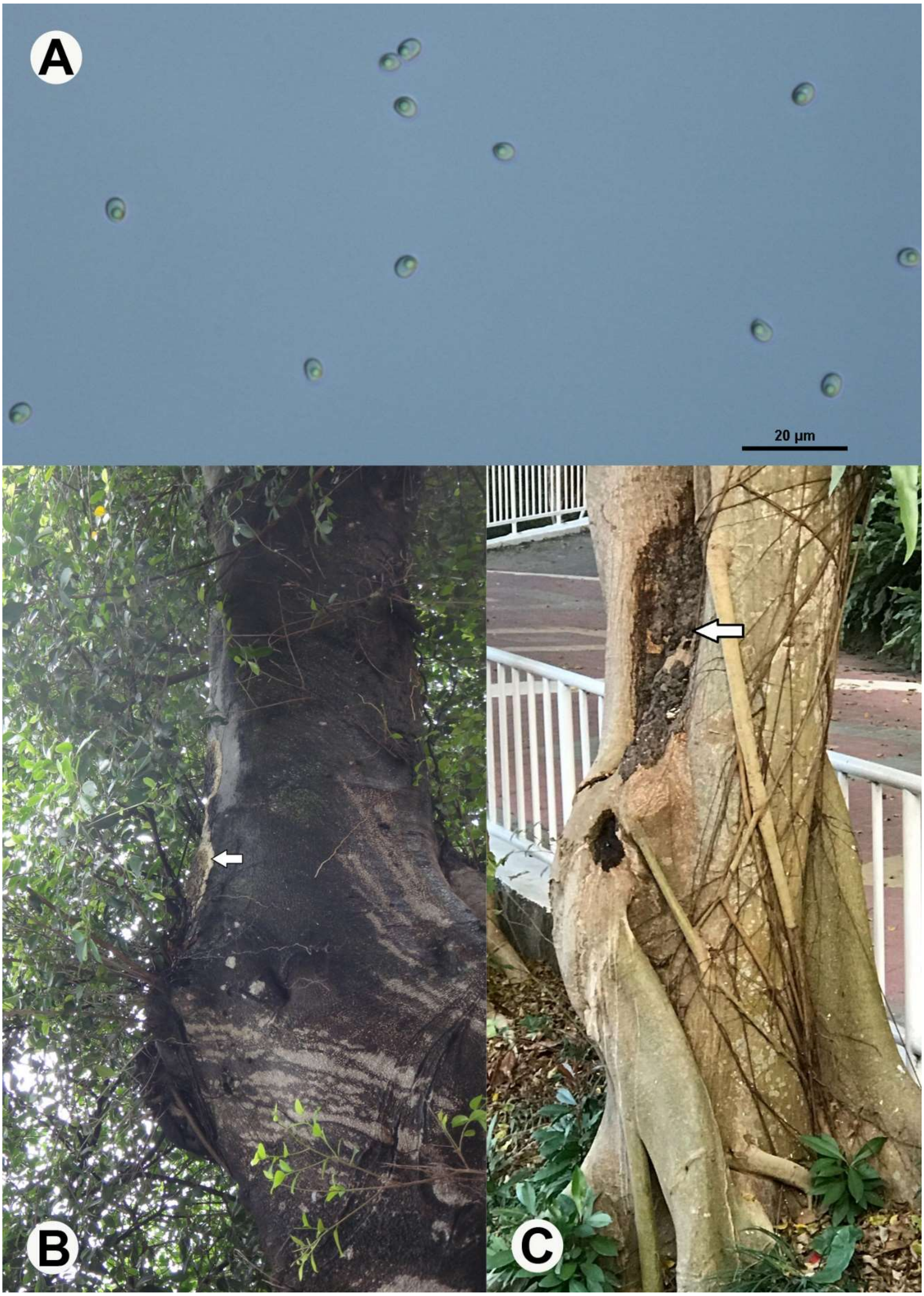

Fig. 1. (A) Morphology of basidiospores of ***Phellinus noxius*** used for germination tests and inoculation experiments in this study. (B). Typical stem infection by ***Phellinus noxius*** on a small-leafed banyan with a conspicuous brown mycelial mat (↑) on a higher part of the stem. (C). A stem infection by *Phellinus noxius* on a wounded part (↑) of a white-bark fig found in Taichung with no root or butt infection.

Table 3. Disease incidence of Taiwan zelkova seedlings after inoculation with ***Phellinus noxius***-colonized wood chips on exposed and wounded main roots

Inoculum	Disease incidence*		
	Experiment 1	Experiment 2	Average
P. noxius-colonized wood chips	3/3 (100%)	3/3 (100%)	100%
Healthy wood chips	0/3 (0%)	0/3 (0%)	0%

* Disease incidence was based on a root showing root rot symptoms and successful re-isolation.

basal stem. All healthy control seedlings remained healthy. The pathogen *P. noxius* was re-isolated from all inoculated and diseased seedlings, consistent with Koch's postulates.

Investigation of twig and stem infection by P. noxius brown rot in Taiwan

The authors have participated in a project of tree brown root rot surveys in Taiwan since 2007 and have also investigated twig and stem infection by *P. noxius* brown rot in the field. To 2018, a total of 13 twig- or stem-infection brown rot cases were found in Taiwan, including three cases in Taipei City, one case in Taichung, four cases in Nantou, two cases in Yunlin, two cases in Tainan, and one case in Kaohsiung. A typical stem infection on a small-leafed banyan is shown in Figure 1(B). The mycelial mat at about one-floor height on the stem was conspicuous, and the pathogen was successfully isolated. Another instance of a stem infection exhibited on a white-bark fig is shown in Figure 1(C). The infection was on a wounded part about 2 m height of the stem with no root or butt infection.

Compared to the total 2287 brown root rot cases we recorded from 2007 to 2018, the 13 cases identified as twig or stem infection comprised only about 0.57% of the population, indicating that its occurrence is very rare.

DISCUSSION

Although the pathogen *P. noxius* of brown root rot disease can infect more than 200 tree species in tropical and subtropical areas (Ann and Ko, 1992; Ann *et al.*, 1999a; Ann *et al.*, 1999b; Ann *et al.*, 2002; Chang, 1992; Chang, 1995a; Chang and Yang, 1998; Chung *et al.*, 2015; Fu, 2005), the role of its fruiting body and basidiospores is not very well known. The disease distribution pattern usually suggests that brown root rot is transmitted from a center to adjacent trees by root-to-root contact (Ann *et al.*, 2002; Ann *et al.*, 1999a). Recently the same authors of this paper discovered a total of 33 new host tree species with fruiting bodies on diseased trees (Hsiao *et al.*, submitted to *Taiwania*), indicating that the occurrence of basidiocarps is not rare in nature. Both types of fruiting bodies, including the flat and bracket types, were frequently found in the field in Taiwan. The somewhat abundance of *P. noxius* basidiocarps in the field might be very conducive for the pathogen to spread to long-distance targets.

Basidiospores germination is also an important issue for the pathogen. Previously, no article had dealt with the germination rate of *P. noxius* basidiospores. Using a germination test in Table 1, we revealed that *P. noxius* basidiospores can germinate in many kinds of media and liquids, with germination rates exceeding 50% in nature. *Phellinus noxius* basidiospores had the highest germination rate of 82% on MEA medium, but they generally had over 50% germination rates in natural rainwater although it is acidic in Taiwan as reported by the Taiwan EPA (Taiwan Environmental Protection Administration, 2018), indicating that spores can adapt to an acidic environment. The high germination rate is quite beneficial for the pathogen to widely spread in the field and grow onto new targets. Therefore, the difficulty for long-distance dispersal does not seem to be due to weak basidiospores.

In spite of the high germination rate of basidiospores in the field, when we inoculated them directly on basal stems of four tree seedlings with cut wounds, none of the seedlings became infected. This result revealed the weakness of the mycelium grown from the basidiospores. This phenomenon is consistent with difficulty in virulence tests of *P. noxius* experienced by Bolland (1984) and reported by other authors (Ann *et al.*, 2002). To overcome this obstacle, Ko *et al.* (1986) developed a wheat-oat medium for growing the fungi, and with this high infection potential medium, inoculation was easier on longan or many other trees (Ann and Ko, 1992; Ann *et al.*, 2002). Chang (1995a) used a camphor tree twig segment about 1 cm in diameter and 5 cm long to grow the pathogen for 1 month after sterilization, with inoculation success rates of 35%~85%. Both wheat-oat medium and camphor tree twig segments seemed to provide stronger infection potential for the fungal mycelium to overcome environmental obstacles and successfully invade the tree. A third approach was conducted in Japan by Sahashi *et al.* (2010), who used enriched sawdust medium to grow the fungi, which also showed good inoculation results when inoculated on wounds of seedlings.

In order to provide stronger infection potential for mycelium grown from basidiospores, we selected Taiwan zelkova sapwood chips. Each freshly cut sapwood chip was placed in a glass jar, inoculated with a spore suspension, and loosely covered. With this better food and environment, the mycelium could grow well and finally colonized the wood chip, showing brownish discoloration with dark-brown lines or a network within 2 months. Successful colonization rates on wood chips were higher when the spore concentration increased, indicating a dose threshold for colonization by basidiospores.

A further indirect inoculation experiment using these colonized wood chips on Taiwan zelkova seedlings was also successful with a 100% infection rate. The increased infection potential for colonized wood chips to invade tree seedlings seemed to be consistent with that of camphor twig segments reported by Chang (1995a).

In our long-term survey from 2007 to 2018, a total 13 brown rot cases out of a population of 2287 cases (Hsiao *et al.*, unpublished manuscript), were found to begin at an above-ground twig or stem part, suggesting they were not infected by the root-to-root contact pathway, and supporting the spore transmission mechanism. Recent molecular and genetic studies also revealed very high genotype diversity within *P. noxius* populations in Taiwan and Japan, suggesting that genetically variable basidiospores are involved in long-distance dispersal of this fungus (Akiba *et al.*, 2015; Chung *et al.*, 2015; Chung *et al.*, 2017).

As in Taiwan and Southeast Asian regions, typhoons often cause serious damage to trees, producing a lot of wounds on tree stems and twigs, and these wounds inevitably supply abundant deposition sites for basidiospores of *P. noxius.* This can explain the occurrence of 13 twig or stem infections of this disease in Taiwan.

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