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Newly discovered basidiocarps of *Phellinus noxius* on 33 tree species with brown root rot disease in Taiwan and the basidiospore variations in growth rate

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ABSTRACT: Since 2007, a comprehensive survey of the occurrence of brown root rot disease has been conducted in Taiwan. The rare basidiocarps of the pathogen *Phellinus noxius* (Corner) Cunningham were also investigated throughout Taiwan and on outer islands. To 2018, a total of 2287 disease cases of brown root rot were recorded, and 164 cases were found with basidiocarps on diseased trees. These 164 cases included 33 newly discovered tree hosts on which were borne the fruiting body of *P. noxius*. Most of the basidiocarps were of the flat type which is inconspicuous, while some belong to bracket type. Generally, basidiocarps were found in most counties of Taiwan and on the outer islands, but they exhibited higher frequencies in the central and southern parts of Taiwan. They tended to occur more at elevations of 200~300 m in the hills, but gradually disappeared at the elevation increased to 800 m. To compare variations in growth rates, 34 single-basidiospore isolates were obtained and grown on both potato dextrose agar (PDA) and malt extract agar medium (MEA). Results showed three levels of significant growth differences on PDA, and four levels of differences on MEA, indicating growth rate variations among these sexual basidiospores. The abundance of *P. noxius* basidiocarps in nature suggests that genetically variable basidiospores are involved in long-distance dispersal of this fungus which is responsible for serious tree diseases.

KEY WORDS: Basidiocarp, Basidiospore, Brown root rot, *Phellinus noxius*, Taiwan.

INTRODUCTION

Brown root rot disease caused by *Phellinus noxius* (Corner) Cunningham is the most important tree disease in Taiwan, Hong-Kong, Macao, and southern China (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). This pathogenic fungus of plants is widespread in tropical and subtropical areas of Southeast and East Asia, Oceania, Africa, Central America, and the Caribbean (Ann *et al.*, 2002; Bolland, 1984; Chung et al, 2015; Chung et al, 2017). Based on previous reports, more than 200 agricultural and forest plant species are hosts of *P. noxius*; most are woody but some are herbaceous plant hosts (Ann *et al.*, 1999a; Ann *et al.*, 2002; Chang and Yang, 1998; Fu, 2005). Among the 200 host plant species, about half of them were reported for the first time from Taiwan (Ann *et al.*, 2002).

Phellinus noxius belongs to the family Hymenochaetaceae, order Hymenochaetales, and phylum Basidiomycota of the kingdom Fungi. It 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). The fungus causing brown root disease of trees was also described in Singapore by Corner in 1932 as *Fomes noxius* (Corner, 1932) and reclassified by Cunningham in 1965 as *Phellinus noxius* (Cunningham, 1965). It produces two types of fruiting bodies: a flat (resupinate) type and a bracket type (Bolland, 1984; Pegler and Waterson, 1968).

Identifying this fungus is based on cultural characteristic when grown on potato dextrose agar (PDA). It forms colonies that are initially white to yellowish-brown and are later decorated with irregularly shaped lines or dark-brown patches (Ann and Ko, 1992; Ann *et al.*, 2002; Chang, 1992). Characteristic trichocysts and arthrospores formed in culture are also used as identification references for this fungus, although no clamp connection was found (Ann and Ko, 1992; Ann *et al.*, 2002; Chang, 1992).

Infection by this disease usually begins in the roots and spreads to the tree collar. The pathogen can colonize lateral roots, tap roots, and basal stems, but usually is limited to a height of 1~2 m. The interior root or stem tissue first turns brown and then white and soft, usually with a network of dark brown lines in the tissue (Ann and Ko, 1992; Ann *et al.*, 2002; Chang, 1992). In the field, infected roots and basal stems are covered with dark-brown mycelial mats of *P. noxius* (Ann and Ko, 1992; Ann *et al.*, 2002; Chang, 1992). When the disease progresses, trees usually show symptoms of leaf chlorosis and defoliation due to root girdling by the fungus. Eventually, the tree may die in one or a few years or be felled by strong winds. In addition to the chronic type pattern, some hosts may show acute decline, causing death within a few months (Ann *et al.*, 1999b; Ann *et al.*, 2002).

Phellinus noxius is a high-temperature organism with optimal growth at about 30 °C (Ann *et al.*, 2002). The geographical distribution is related to the growth temperature range of *P. noxius*: 10~12 to 36 °C (Ann and Ko, 1992; Ann *et al.*, 1999b). In the past 20 years or longer, brown root rot disease has become a serious threat to many perennial fruit trees, ornamental and landscape trees, and shade trees in Taiwan (Ann *et al.*, 1999b; Ann *et al.*, 2002; Chung *et al.*, 2015) and in the Ryukyu and Ogasawara Islands of Japan (Akiba *et al.*, 2015).

The roles of basidiocarps and basidiospores of *P. noxius* have not been clear since this fungus was discovered (Ann and Ko, 1992; Chang, 1992; Chang, 1995a). Previously in Taiwan, it was 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), although in sawdust medium, it can produce thin-layered, flat basidiocarps that are similar to those found in nature (Ann and Ko, 1992; Ann *et al.*, 1999b; Chang, 1992; Chang, 1995a). In the field, this disease is principally transmitted by root-to-root contact (Ann *et al.*, 1999a; Ann *et al.*, 2002).

When Sawada first described the brown root rot disease on 16 hosts in Taiwan (Sawada, 1928), he found no fruiting bodies, but later in 1930 he observed some fruiting bodies on *Bauhinia* sp. and described their structure including the basidiospore (Sawada, 1942). In 1992 when Chang (1992) and Ann and Ko (1992) reported the new spread of this disease on dozens of tree hosts, they found no fruiting bodies on any hosts except on the longan tree (*Dimocarpus longan*), although the fungus produced fruiting bodies in sawdust medium. On the longan tree, a thin, flat, brown basidiocarp was observed in 1990 by Dr. Ko (Ann and Ko, 1992). Later, a few fruiting bodies of *P. noxius* were found in nature and reported. Chang and Yang (1998) reported that the basidiocarps were observed on three trees species, *Casuarina equisetifolia*, *Delonix regia,* and *Ficus microcarpa*. At about the same time, Ann *et al.* (1999b) found that during rainy periods, basidiocarps were growing on litchi (*Litchi chinensis*) and sugar-apple trees (*Anona squamosa*), but basidiospores were absent. Generally, most previous papers (Ann *et al.*, 1999a; 1999b; 2002; Chang, 1995) indicated that fruiting bodies are rare in the field.

Since 2007, we have participated in a serial project of tree brown root rot surveys in Taiwan. In addition to the comprehensive disease survey, the occurrence of basidiocarps of *P. noxius* was one of the important topics. We also have tried to elucidate the role of basidiocarps of *P. noxius* in nature. Results of those studies are presented in this paper.

MATERIALS AND METHODS

Survey and diagnosis of brown root rot disease of trees in Taiwan

Since 2007, we started to survey the occurrence and distribution of tree brown root rot disease in Taiwan. The suspected disease incidences were reported from local counties and communities. A Brown Root Rot Diagnosis and Control Information Center was also set up by the government to accept reports of any tree disease problems from the public.

After receipt of a report of a suspected case of brown root rot disease, personnel, including a plant pathologist or tree doctor, were sent into the field to make a symptoms diagnosis and take diseased samples back for further isolation of the pathogen. The typical symptoms including leaf chlorosis, defoliation, network of dark brown lines in rotted root or basal stem were examined. Additional signs including brown mycelial mat and in some cases the basidiocarp were also carefully examined and recorded. The diseased samples at least from three parts of the rotted root or basal stem of each tree were collected by a small ax and taken to laboratory for pathogen isolation. A selective medium developed by Chang (1995b) was used for isolating the fungi from the diseased root or butt samples. It consists of a base of 20 g/L malt-extract and 20 g/L agar, amended after autoclaving with four other chemicals (10 mg/L benomyl, 10 mg/L dicloran, 100 mg/L ampicillin, and 500 mg/L gallic acid), which we tentatively named MA+4. Colonies of *P. noxius* turn dark brown as induced by gallic acid when it is present in the medium. The suspect mycelium on MA+4 medium was further transferred to potato dextrose agar (PDA) for additional culturing and identification by cultural characteristics (Ann *et al.*, 2002). Based on field characteristic symptoms and signs such as brown mycelial mat, brown lines in rot root or wood, the successful isolation of the pathogen with the MA+4 selective medium, as well as cultural characteristic on PDA, each suspect disease was identified as a positive case.

Investigation of the occurrence of basidiocarps of P. noxius in the field in Taiwan

In each confirmed brown root rot disease case as mentioned above, the occurrence of basidiocarps of *P. noxius* was also carefully examined by the authors. According to previous publications (Ann *et al.*, 2002; Bolland, 1984; Pegler and Waterson, 1968), the probable flat or bracket types of fruiting bodies producing on the diseased basal stem or exposed roots were examined and recorded. Based on previous experiences, usually the fruiting bodies were present at shaded and humid sites. Therefore, for each case these favorable sites were especially investigated.

All suspected basidiocarps were further identified by culturing on MA+4 selective medium and cultural characteristic on PDA. The same cultural characteristics on PDA (Ann *et al.*, 2002) were used for a positive identification as mentioned above. In some cases, the basidiospores were collected from fresh basidiocarps for growth studies or a pathogenicity test (Hsiao *et al.*, 2019).

Comparison of growth rates of P. noxius among isolates from single basidiospores

In order to demonstrate growth variations among offspring of a *P. noxius* basidiocarp, basidiospores were collected from a fresh basidiocarp growing on a small-leafed banyan (*Ficus microcarpa*) in Nantou County. Sterile distilled water was used to rinse out the basidiospores, which were then observed under a light microscope, counted, and diluted to adequate concentrations. Spores were plated out on MA+4 selective medium at 24 °C. Two to 3 days later when spores had germinated, each single spore with its mycelium tip was selected, cut with a needle, and transferred onto PDA for further use. In total, 34 isolates each from a single basidiospore were obtained.

To compare growth rates of these 34 single-basidiospore isolates, both PDA and malt extract agar (MEA) media were prepared. A sterilized 5-mm borer was used to cut mycelium discs from the active colony margin of each single-basidiospore culture. The agar mycelium disc was placed at the center of each PDA or MEA plate and incubated at 30 °C for 3 days. Each isolated culture was replicated on five plates, and the experiment was repeated once. The colony diameter of each isolate on the plate was measured, and the growth rate was counted in units of mm/day.

RESULTS

Survey and diagnosis of brown root rot disease of trees in Taiwan

The suspect *Phellinus noxius* can grow on MA+4 selective medium with characteristic mycelium observed under a light microscope. When the mycelium on MA+4 was further transferred to potato dextrose agar (PDA), they are initially white to yellowish-brown and later decorated with irregularly shaped lines or dark-brown patches. Based on successful isolation with MA+4 selective medium, cultural characteristics on PDA, and field symptoms and signs such as a brown mycelial mat, brown lines in rotted roots or wood, each suspect disease was identified as a positive case. From 2007 to 2018, a total of 2287 confirmed brown root rot cases were obtained in this survey study.

The geographic distribution of these disease cases is demonstrated in Table 1. The distribution is similar to that reviewed by Ann *et al.* (2002). Basically the disease was found in every county of Taiwan, but not in on the Penghu Islands in the Taiwan Strait. However, the occurrence of the brown root rot disease as shown in Table 1 seemed to be more abundant in southern, central and northern region of Taiwan, each with 28.8, 28.7 and 27.5%, respectively.

Table 1. Number and percentage of brown root rot cases in Taiwan recorded from 2007 to 2018.

Locality (County/City)	Cases (percentage)	Cases (percentage) with basidiocarps
Northern part		
Keelung	68 (3.0)	0 (0)
Taipei	92 (4.0)	10 (6.1)
New Taipei	116 (5.1)	15 (9.2)
Taoyuan	89 (3.9)	0 (0)
Hsinchu	168 (7.3)	2 (1.2)
Miaoli	95 (4.2)	4 (2.4)
Central part		
Taichung	233 (10.2)	15 (9.2)
Changhua	116 (5.1)	4 (2.4)
Nantou	188 (8.2)	30 (18.3)
Yunlin	118 (5.2)	5 (3.0)
Southern part		
Chiayi	226 (9.9)	28 (17.1)
Tainan	221 (9.7)	17 (10.4)
Kaohsiung	115 (5.0)	15 (9.2)
Pingtung	96 (4.2)	4 (2.4)
Eastern part		
Yilan	96 (4.2)	2 (1.2)
Hualien	108 (4.7)	10 (6.1)
Taitung	86 (3.8)	2 (1.2)
Outer islands		
Penghu	0 (0)	0 (0)
Kinmen	56 (2.5)	1 (0.6)
Total	2287 (100)	164 (100)

Investigation of occurrence of basidiocarps of P. noxius in the field in Taiwan

To 2018, a total of 164 brown root rot cases with fruiting bodies present were found in the field as shown in Table 1. All these 164 cases were identified as brown root rot cases by successful isolation with MA+4 selective medium, cultural characteristics on PDA, and field symptoms and signs such as a brown mycelial mat, brown lines in rotted roots or wood. Usually the basidiocarp was produced on an infected butt or tap root in an advanced stage of infection, especially at shady and humid sites. Two types of fruiting bodies were frequently found in the field: a flat (resupinate) and a bracket type. The more-common one was the perennial flat type as shown in Fig. 1A. The bracket type also occurred in perennial stage, and is shown in Fig. 1B. All the investigated basidiocarps can be isolated and grown on MA+4 selective medium and then on PDA with colonies that are initially white to yellowish-brown and later decorated with irregularly shaped lines or dark-brown patches, identical to the characteristics of *P. noxius.*

In addition to those tree hosts on which fruiting bodies were previously found in nature in Taiwan (Ann and Ko, 1992; Ann *et al.*, 1999b; 2002; Chang and Yang, 1998; Sawada, 1942), we have newly discovered 33 tree

Fig. 1. Two types of ***Phellinus noxius*** basidiocarp produced on diseased trees in the field. **A**. Typical flat type as on white bark fig (***Ficus benjamina***). **B**. Typical bracket type as on kassod tree (***Cassia siamea***).

hosts bearing fruiting bodies in the field. They are listed in Appendix.

The distribution of these 164 brown root rot cases producing fruiting bodies based on locality is shown in Table 1. Generally, basidiocarps existed in most counties in Taiwan. However, basidiocarps seemed to be more abundant in central and southern Taiwan, both with 32.9 and 39.1%, respectively. This might be due to the higher disease incidence and favorable conditions in these areas.

The distribution of these 164 brown root rot cases producing fruiting bodies based on elevation is shown in Figure 2. Their occurrence seemed to have a peak at elevations of 200~300 m, and decreased as the elevation increased. The reason for the greater abundance in low-elevation hills of 200~300 m might be due to the higher humidity in hill areas, while the decline at high elevations over 800 m is coincident with the disease distribution pattern as reported previously that this pathogen is a high-temperature organism.

A distribution of these 164 brown root rot cases producing fruiting bodies based on the discovery month of the past 12 years is shown in Figure 3. The discovery time had a peak in summer, and decreased toward the winter season. This may be due to that brown root rot fungus cannot tolerate cold weather. However, basidiocarps of brown root rot fungus are perennial, and exist for several years on diseased trees. Therefore, the seasonal occurrence of basidiocarps may require further investigation in the future.

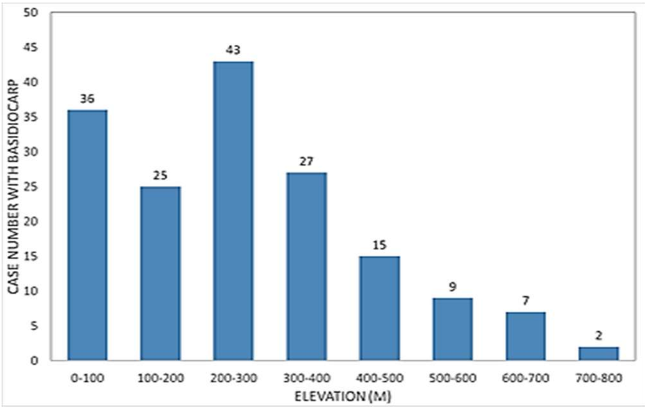

Fig. 2. Geographic distribution of basidiocarps of ***Phellinus noxius*** based on elevation in Taiwan.

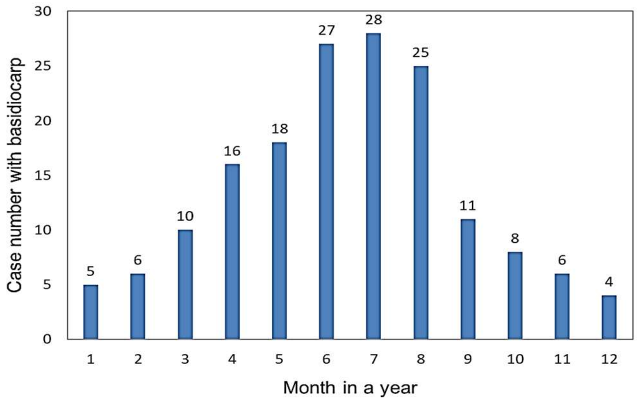

Fig. 3. Monthly distribution of basidiocarps of ***Phellinus noxius*** in Taiwan in this survey based on the discovery time.

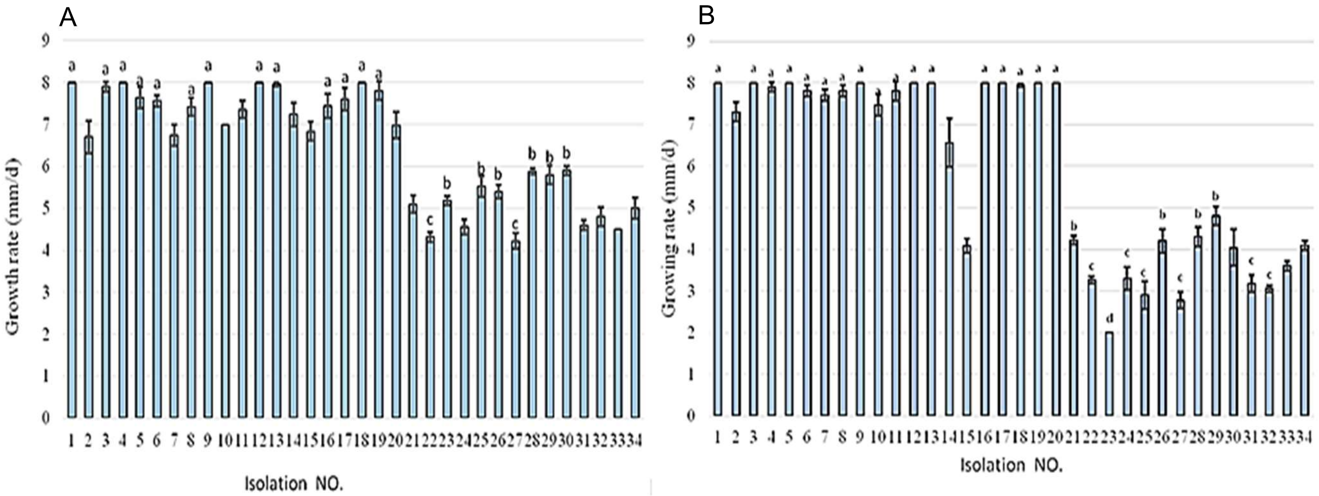

Fig. 4. Comparison of growth rates among 34 single-basidiospore isolates of *Phellinus noxius* growing on two different media. **A**. PDA. **B**. MEA. Error bars are the standard error of the mean (*n*=5). Means with different letters significantly differ at the 1% level by Fisher's protected LSD test.

Comparison of growth rates of P. noxius among isolates from a single basidiocarp

To compare growth variations among different basidiospores from a fruiting body, a total of 34 single-basidiospore isolates were obtained and grown on both PDA and MEA. Growth rates of these 34 isolates on PDA are shown in Fig. 4A, whereas growth rates on MEA are demonstrated in Fig. 4B.

Based on Fig. 4A and 4B, we found several levels of significant growth rate differences according to Fisher's protected least significant difference (LSD) test among these 34 isolates. There were three levels (a~c) of significant differences among them on PDA, while there were four levels (a~d) on MEA medium. These results indicated that individual basidiospores should have a specific genetic inheritance. When basidiospores are released and mate with each other at a remote new target or host, the new combination of a diverse genome could increase the genetic diversity of the offspring of *P. noxius*.

DISCUSSION

Previously, the roles of basidiocarps and basidiospores of *P. noxius* were not clear since this fungus and disease were discovered (Ann and Ko, 1992; Chang, 1992; Chang, 1995a). In Taiwan, it was indicated that the fungus rarely produced basidiocarps on diseased trees in the field (Ann and Ko, 1992; Ann *et al.*, 1999a; Chang, 1992; Chang, 1995a). However, from our investigation during the past 12 years, a total of 164 brown root rot cases on 33 new tree hosts were discovered with fruiting bodies on diseased trees. Both types of fruiting bodies were frequently found in the field, including the flat and bracket types. Most of the basidiocarps were of the flat type, which is inconspicuous, while some belong to bracket type. Because the dominant flat type of basidiocarp is not so conspicuous, and they usually develop in shady and humid sides of infested wood, people might not easily find them in the field. This might explain why they were not previously found and thought to be very rare in nature.

Compared to the total of 2287 brown root rot cases that we recorded from 2007 to 2018, those with fruiting bodies only comprised about 7.2%, indicating that the occurrence is still rare. Based on the elevation distribution pattern as shown in Fig. 2, their distribution is similar to that of the disease distribution as previously reported (Ann *et al.*, 2002; Chang and Yang, 1998). However, the occurrence seemed to have a peak on hills at elevations of 200~300 m, and decreased as the elevation increased. This might be due to the higher humidity of hill areas. Geographically, the occurrence of fruiting bodies seemed to be more abundant in central and southern Taiwan, both with 32.9 and 39.1%, respectively. This might also be attributed to favorable microclimates for inducing fruiting bodies.

The greater abundance of *P. noxius* basidiocarps in the field should benefit dispersal of the pathogen at greater distances. Actually in our survey, a few new brown root rot cases were found to have begun at above-ground points on twigs or stems, suggesting that they were not infected by the root-to-root contact pathway, and implying a spore transmission mechanism (Hsiao *et al.*, 2019).

Basidiospores of Basidiomycetes fungi theoretically are monokaryotic, and have more-diverse genomes. In our comparison of growth rates among 34 single-basidiospore isolates, there were significant differences among them. Four levels of significant differences existed in MEA culture, whereas three levels of significant differences were found in PDA culture. This is consistent with recent reports of molecular and genetic studies of this pathogen. Chung *et al.* (2015) reported that according to population genetic studies, this pathogen had very high genotype diversity within its populations in Taiwan. A similar finding was reported by Akiba *et al.* (2015) in Japan, who collected 128 isolates from 12 of the Ryukyu islands and three of the Ogasawara islands, and all had unique genotypes. If the basidiospores can successfully infect a remote host tree plant, they will greatly increase the genetic diversity within their population, and strengthen their survival and virulence ability in wider regions.

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Appendix: The checklist of new tree hosts producing fruiting bodies of ***Phellinus noxius*** in the field in Taiwan (each with scientific name, locality and year first found)

1. *Acacia confusa* Merr. Taichung, 2010
2. *Araucaria cunninghamii* Ait. ex Sweet Kaohsiung, 2010
3. *Artocarpus altilis* (Park.) Fosberg Taipei, 2010
4. *Bauhinia × blakeana* Dunn. Nantou, 2008
5. *Bauhinia variegata* Linn. Taichung, 2011
6. *Calocedrus formosana* (Florin) Florin Taichung, 2011
7. *Cassia fistula* Linn. Tainan, 2011
8. *Cassia siamea* Lam. Nantou, 2016
9. *Cinnamomum camphora* (L.) Sieb. Nantou, 2015
10. *Cinnamomum insularimontanum* Hay. Nantou, 2013
11. *Cinnamomum kanehirae* Hay. Nantou, 2017
12. *Chorisia speciosa* St. Hil. Tainan, 2010
13. *Chrysalidocarpus lutescens* (Bory) Wendl. Kaohsiung, 2010
14. *Distylium gracile* Nakai Tainan, 2010
15. *Eucalyptus robusta* Smith Tainan, 2010
16. *Ficus benjamina* Linn. Nantou, 2009
17. *Ficus elastica* Roxb. Taipei, 2007
18. *Ficus religiosa* Linn. Nantou, 2009
19. *Fraxinus formosana* Hay. Kaohsiung, 2012
20. *Koelreuteria henryi* Dummer Kaohsiung, 2011
21. *Liquidambar formosana* Hance Nantou, 2008
22. *Macaranga tanarius* (L.) Muell. -Arg. Nantou, 2016
23. *Mangifera indica* Linn. Chiayi, 2010
24. *Melia azedarach* Linn. Nantou, 2017
25. *Murraya paniculata* (L.) Jack. Hsinchu, 2010
26. *Pachira macrocarpa* Walp Changhua, 2016
27. *Spathodea campanulata* Beauv. Taitung, 2010
28. *Swietenia macrophylla* King Tainan, 2010
29. *Tamarindus indicus* Linn. Chiayi, 2011
30. *Tectona grandis* Linn. f. Tainan, 2011
31. *Tournefortia argentea* L. f. Taitung, 2011
32. *Trema orientalis* (L.) Blume Taichung, 2011
33. *Zelkova serrata* (Thunb.) Makino Hualien, 2010