# Interspecific differences in phosphorus use and acquisition strategies of trees in Bornean lowland tropical rainforests

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## Chapter1. General introduction

### 1.1. Plant phosphorus-use and -acquisition strategies in tropical rainforests

In terrestrial ecosystems, P is derived mostly from rock weathering (Walker & Syers 1976). Bioavailable P (i.e., labile inorganic P (Pi) and organic P (Po)) in soils decreases with increasing soil age by leaching and/or the conversion to recalcitrant forms (e.g., occluded P), and consequently P is impoverished in old soils (Walker & Syers 1976; Crews et al. 1995; Turner et al. 2007). Most of lowland tropical rainforests are under P deficiency due to the intense weathering as described above. Despite this, these forests maintain huge biomass with 50-60 m high. How is huge biomass maintained in P-deficient tropical rain forests?

To answer this question, many studies using natural soil P gradient have been conducted (Kitayama and Aiba 2002; Aoki et al. 2012; Fujii et al. 2013; Ushio et al. 2015; Tsujii et al. 2017a). These series of studies have indicated that tropical trees may maintain high productivity under P-depleted conditions by enhancing P-use and -acquisition capacity. First, plants in such P-depleted conditions may increase their P-use efficiency by decreasing their P requirement, adjusting P allocation and increasing the residence time of P, for instance by re-translocating P from senescing organs, including leaves and the sapwood (Chapin 1980; Meerts 2002; Veneklaas et al. 2012). They may acquire Pi by investing in absorptive roots and acclimating their root architecture and morphology to improve their Pi-foraging capacity and efficiency (Lynch and Brown 2001; Lambers et al. 2006). They may enhance their root phosphatase activities to acquire P by degrading soil Po (Nannipieri et al. 2011; Kitayama 2013; Ushio et al. 2015). Moreover, plant roots under P-depleted conditions exudate organic acids, which can increase the availability of major soil nutrients (e.g., N and P) via enhancing soil microbial activity and mobilizing nutrients (Lambers et al. 2006, 2008; Aoki et al. 2012).

In Chapter 1, I focus on fertilization experiments as a method for clarifying patterns and mechanisms of nutrient limitation, and summarize the current knowledge on mechanisms of nutrient limitation in tropical rainforests by reviewing previous studies. Specifically, after reviewing studies in the Hawaiian Islands, the traditional fertilization experiments using model ecosystems, I point out that the pattern and mechanisms of P-limitation from the Hawaiian sites are not necessarily consistent in species-rich lowland tropical rainforests in Africa, Southeast Asia, and the neotropics (section 1.2). I also point out that P-restriction pattern of the biological

processes would differ among different species with different mycorrhizal types and successional status because of the differences in nutrient-use and -acquisition characteristics among these functional types (section 1.3). Lastly, I address the needs of research to assess the patterns and mechanisms of nutrient limitation in tropical lowland rainforests and the aim and question of the present thesis (section 1.4). Furthermore, I explain a description of the main research site for the present thesis and a field fertilization experiment site on the Malaysian Borneo (section 1.5).

### 1.2. N and P fertilization experiments

Fertilization experiment is a method to directly test patterns and mechanisms of nutrient limitation of biological processes. Operational definitions of nutrient limitation of biological processes contain positive responses to experimental fertilization (Vitousek and Howarth 1991; Vitousek et al. 2010). The limitation of plant growth by nutrients, such as N and P, was first studied by crop and soil researchers who developed an operational definition of nutrient limitation that pursed to maximize crop yield: if adding a nutrient increased crop yield, the crop was limited by that nutrient(Sprengel 1828 and Liebig 1840; cited in van der Ploeg et al. 1999). Crop scientists have been largely successful at overcoming the nutrient limitation, chiefly through widespread production and use of inorganic fertilizers (e.g., Tilman et al. 2002).

However, as noted by Chapin et al. (1986), applying a simple, yield-centric perspectives to nutrient limitation in natural ecosystems ignores many inherent complexities. For example, species within the same ecological community (or even individuals of different sizes of the same species) may differ in the extent and identity of nutrient limitation (Alvarez-Clare et al. 2013), the proximate limiting nutrient may change over multiple time scales (Harrington et al. 2001; Davidson et al. 2007), and different nutrients may limit different (but crucial) ecosystem processes (Wright et al. 2011). For these challenges, ecological researchers have developed definitions for natural ecosystems (Chapin 1980; Chapin et al. 1986; Vitousek et al. 2010): if an added nutrient stimulates an ecosystem process that is the net result of the community assemblage of plant species (such as net primary production (NPP)), that process is considered to be limited by the nutrient (Vitousek and Howarth 1991). More recently, the definition of nutrient limitation has developed to distinguish ‘‘proximate’’ nutrient limitation (an added nutrient that stimulates an ecosystem process) from ‘‘ultimate’’ nutrient limitation (a nutrient that fundamentally alters the availability of other nutrients and a community or ecosystem itself)

(Vitousek et al. 2010). However, in the end, the common denominator of most ecological definitions of nutrient limitation is a requirement for nutrient additions. Therefore, fertilization experiments remain widely viewed as the most robust method to assess nutrient limitation (e.g., Elser et al. 2007; Cleveland et al. 2011). On the other hand, the number of terrestrial fertilization experiments are fewer than that of aquatic ones, mainly because of the long biological turnover of terrestrial vascular plants and the need to continue fertilization for a long period to detect fertilizer responses (Sullivan et al. 2014). Furthermore, there are extremely few examples of field fertilization experiments, especially in tropical forests, due to the limited accessibility and poor logistics (Sullivan et al. 2014; Wright et al. 2018).

### 1.2.1. Responses of forest productivity to N and P fertilization in Hawaii

Classic fertilization experiments in forest ecosystems have tested the hypothesis that N and P limit Hawaiian forests growing on young vs. old soils, respectively (Harrington et al. 2001). These sites are montane rainforest at about 1000 m elevation and species-poor ecosystems dominated by one species, *Metrosideros polymorpha*. Although dominant vegetation, climate, parent material, and topography of all of the sites were similar, these sites are at different stages of soil development (300, 20,000, and 4,100,000 yr old), and therefore the effects of soil ages on the soil nutrient availability and ecosystem functions can be tested. As a results, P availability increased and decreased unimodally across the chronosequence (Crews et al. 1995). Specifically, P availability is low in the youngest and oldest sites and greatest at the intermediate-aged site (Crews et al. 1995). Moreover, N fertilization increased tree growth rates on the young substrate, P fertilization did so on the old one, and both N and P fertilization did so on the intermediate one (Vitousek et al. 1993; Herbert and Fownes 1995; Vitousek and Farrington 1997). These results of the fertilization experiment indicate that aboveground productivity is limited primarily by N in the youngest site, P in the oldest site, and neither N nor P independently in the relatively fertile 20000-yr-old site. These results suggest a developmental progression from N limitation in the youngest site, to combined limitation by N and P in the relatively nutrient-rich intermediate-aged site, to P limitation in the oldest site. The series of studies in Hawaii are consistent with the Walker & Syers' 1976 model that predicted a pattern of soil nutrient availability during long-term soil development, and have directly verified that biomass production is limited by P at sites with advanced soil weathering like lowland tropical rainforests.

### 1.2.2. Responses of forest productivity to N and P fertilization in lowland tropical

**rainforests**

Nevertheless, in more complex ecosystems such as species-rich tropical forests, patterns of the response of P fertilization is not consistent (Table 1-1; Wright et al. 2018). Meta-analyses on fertilization experiments in various terrestrial biomes have reported that strong and positive responses of primary productivity to N and P fertilization, suggesting that both N and P limit plant communities (Elser et al. 2007). On the other hand, the response to fertilization in old lowland tropical rainforests varies from site to site, and forest biomass, especially in most old-growth forests, is often not altered by P fertilization (Table 1-1; but see Cunha et al. 2022). In addition, simple fertilization responses such as those observed in Hawaiian forests dominated by a single tree species are not always found in lowland tropical rainforests (Wright at al. 2018). Therefore, the overall evidence for P limitation in the tropics would be largely inconclusive (Wright at al. 2018). Possible reasons why limited growth responses of tree species in tropical forests on severely P-depleted soils include that; some species are able to maintain productivity despite P deficiency (Turner et al. 2018), the fertilization period may not be long enough (Wright 2019), and P fertilization response varies depending on soil P concentrations and P fertilization response is observed at sites with very low P concentrations (Cunha et al. 2022). Moreover, meta-analyses have reported increases in above-ground productivity with multiple nutrient fertilizer applications in tropical forests (Wright et al. 2018), suggesting that in addition to P, N and other cations (calcium, potassium, magnesium, etc.) may cause complex nutrient limitation. Thus, the mechanisms of nutrient limitations in lowland tropical forests would be very complex and remains unclear.

### 1.2.3. Effects of N and P fertilization on plant traits

To understand the complex mechanisms of nutrient limitation in tropical forests, examining responses of tree functional traits such as leaf nutrient (a), root phosphatases (b), root exudation rates (c), and root morphology and chemistry (d), would be a valuable approach (Aoyagi et al. 2022). Previous studies on these functional traits suggest that tropical trees under P-depleted conditions would maintain their productivity by flexibly changing their P-use and -acquisition strategies (e.g., Aoyagi et al. 2024).

(a) Trees often use N and P efficiently by maintaining low leaf N and P concentrations or high NRE and PRE. The studies in the fertilization experimental sites in Hawaii have been reported that P fertilization increases leaf P concentrations and decreases PRE of

leaves under P-limitation, suggesting that low P environments may maintain low leaf P concentrations and high PRE (Vitousek 1998; Ostertag 2010). Similarly, Mayor et al. (2014) reported an increase in leaf P concentrations following P fertilization in a fertilization experiment in tropical lowland rainforests. However, this study was conducted on three climax-AM species and one palm species that are dominant in primary forests. Although there have been studies examining leaf N and P concentrations and/or NRE and PRE in experimental fertilization sites in subtropical plantations (Yang 2018; Wang et al. 2019; Song et al. 2022), there are no studies examining nutrient use characteristics in leaves for species with different mycorrhizal types and/or successional status in field fertilization experiments in tropical lowland rainforests.

(b) In the tropics, where soil Po is one of the most important P resources, it is crucial to evaluate the strategy of P acquisition from soil Po via root phosphatases. In the Hawaiian fertilization experimental site, Treseder and Vitousek (2001) studied the response of N or P fertilization on PME activities (that is a class of root phosphatases). They reported that phosphatase activity decreased with P fertilization and increased with N fertilization, especially at P-limited forest sites. The results suggest that trees under P-limitation allocate excess N to construction of extracellular phosphatases to acquire P (Treseder and Vitousek 2001). This phenomenon suggests that the construction process of phosphatases for P acquisition is limited by N, which would be one example of complex nutrient limitations. However, in tropical lowland rainforests, while P fertilization often decrease the phosphatase activities, N fertilization does not increase (Yokoyama et al. 2017; Lugli et al. 2021). This is because trees depend on different chemical forms of Po for P acquisition by secreting different phosphatases for different species and/or functional types in tropical lowland forests where diverse species coexist (Turner 2008). However, because most studies examine only PME activities at the plot levels, the strategy of P acquisition via phosphatases in tropical lowland rainforests remain unclear.

(c) Root exudation is one of the most important P acquisition strategies as well as root phosphatases (Reichert et al. 2022). Root exudates enhance plant N and P availability by promoting degradation of organic matter via increases microbial activity, and solubilizing P adsorbed on mineral soils (Lambers et al. 2008). Although a study on natural soil P gradients suggested that ease root exudation rates increase with decreasing soil P

availability (Aoki et al. 2012), no studies have examined root exudation rates in field experimental fertilization sites in Hawaii or in tropical lowland rainforests. Therefore, the importance of nutrient acquisition via root exudation in lowland tropical rainforests remains unclear.

(d) In addition, root morphology and productivity, and nutrient concentrations in root tissue are also important to examine nutrient restriction processes of tropical trees. In the Hawaiian P-limited site, P fertilization increases fine root length per unit area and fine root productivity (Ostertag 2001; Treseder and Vitousek 2001). These results suggest that P limits fine root productivity. Similarly, previous studies in lowland tropical rainforests reported that an addition of N, P, and K together reduce fine root biomass, length, and tissue density, and increase specific root length (Wurzburger and Wright 2015), that P fertilization increase annual root productivity (Lugli et al. 2021). Furthermore, Lugli et al. (2021) also reported that P and cation fertilization increased their element concentrations in root tissues. However, because all these studies examined composite samples of root systems of diverse species with soil core sampling, the responses of N and P fertilization on root morphology and chemistry at the species levels were not evaluated in tropical lowland rainforests.

### 1.3. Fertilization responses may differ among tree functional types

Nutrient-use and -acquisition strategies may differ among functional types of different successional status. An ecological paradigm on the trade-off between growth rate and resource-use efficiency (Reich 2014) predicts that slow-growing climax species are generally conservative in nutrient-use and -acquisition (acquire fewer nutrients but use them more efficiently) whereas fast-growing pioneer species are luxurious (acquire more nutrients and use them inefficiently). Additionally, climax species, which dominate primary forests, may have a stronger ability to use recalcitrant Po than pioneer species because belowground competition for Po by plants will be greater in the later stages of forest succession, with the accumulation of soil nutrients in wood biomass during this process and reduced cycling rates of limiting nutrients (Tang et al. 2011; Huang et al. 2013).

Furthermore, nutrient-use and -acquisition strategies may also differ among functional types

of different mycorrhizal types. Trees that form associations with ectomycorrhizal (ECM) fungi may depend more on soil organic P and mineral-bond P via phosphatases and organic acids than arbuscular mycorrhizal (AM-associated) tree species (Phillips and Fahey 2006; Rosling et al. 2016). This is because ECM fungi have evolved from saprotrophs and are known to exploit not only inorganic P but also organic P and mineral-bond P via the exudation of phosphatase enzymes and low-molecular-weight organic acids, including citric, malonic and oxalic acids (Phillips and Fahey 2006; Smith and Read 2008), whereas AM fungi are thought to mainly increase the acquisition of inorganic P only (Dodd et al., 1987; Joner et al., 2000; Tarafdar & Marschner, 1994). In fact, it has often been suggested that nutrient acquisition strategies differ among species with different root-associated microbial symbionts (Nasto et al. 2017, 2019; Liu et al. 2018). In addition, ECM trees had higher nutrient resorption efficiency in leaves than AM trees in boreal and temperate forests (Zhang et al. 2018). This is because leaves of ECM trees often decay more slowly than leaves of AM trees (Cornelissen et al. 2001), nutrient cycling is slower in ECM-dominated ecosystems than in AM-tree-dominated ecosystems (Lin et al. 2017), and consequently ECM trees may have a more conservative strategy than AM trees (Reich et al. 2014).

However, differences in adaptation mechanisms to P deficiency among functional types with different successional status and mycorrhizal types have been completely unknown. There are three reasons for this: First, it is difficult to examine root traits at the species level in the tropics due to the complex root systems of diverse species in the tropical soil; Second, few studies have looked at both nutrient acquisition and utilization simultaneously due to the lack of studies on roots; and finally, most fertilization experimental sites in lowland tropical rainforests were conducted in the Neotropics, where AM species are dominant, and did not conducted both primary and secondary forests on a same site (Table 1-1). Previous studies using meta-analysis, pot experiments, and natural soil P gradient have suggested that P-use and -acquisition strategies differ among mycorrhizal types (Liu et al. 2018; Zhang et al. 2018; Jiang et al. 2022) or among successional status (Huang et al. 2013; Jiang et al. 2023). However, for the reasons mentioned above, few studies so far have directly tested differences in P-use and -acquisition strategies among tree species with different functional types using field fertilization experiments in lowland tropical rainforests.

### 1.4. Synthesis and outline of this thesis

The objective of this study was to examine differences among functional types in P-use and -acquisition strategies in response to P deficiency in lowland, species-rich, tropical rainforests. I directly test the P-use and -acquisition strategies by examining leaf nutrients (Figure 1-1a; Chapter 3) and root phosphatases (Figure 1-1b; Chapter 4) and root exudates (Figure 1-1c; Chapter 5) in each functional type in my fertilization experimental plots.

In Chapter 2, I examined the relationship between leaf traits, including leaf nutrient concentrations, and leaf herbivory. Leaf herbivory potentially affects carbon (C) and nutrient cycling in forest ecosystems. Such herbivory diverts resources from the detrital pathway to the grazing pathway, which alters the C and nutrient cycling in forest ecosystem. In particular, mangrove forests have very low tree species diversity (Imai et al. 2006), making it easier to link the understanding of species characteristics to community and ecosystem functions. However, the determinants of leaf herbivory in mangrove forests are not well understood. By examining the determinants of leaf herbivory in the mangrove forests of Iriomote Island, I verified the factors driving grazing pathway in forest ecosystem while eliminating species-specific plant-herbivory interactions.

In Chapter 3, I examined the responses of leaf nutrients (Figure 1-1a) to long-term N and P fertilization in lowland tropical forests. Nutrient-use strategies (e.g., nutrient resorption efficiency) in leaves may differ among functional types of different successional status and mycorrhizal types. However, most previous studies have examined only limited functional types in the tropics (e.g., only AM and climax species) or along a soil P gradient. Therefore, in this study, I examined nutrient concentrations in fresh leaves and leaf litter of adult trees for each functional type in N and P fertilization experiments in Bornean tropical lowland forests to verify N and P resorption efficiency in leaves.

In Chapter 4, I examined the response of fine-root phosphatase activities (Figure 1-1b) to N and P fertilization in lowland tropical forests. Soil Po has various chemical forms with different degradation properties (e.g., monoester, diester, phytic acid). Plants acquire P from Po by secreting corresponding phosphatase enzymes {phosphomonoesterase (PME), phosphodiesterase (PDE), and phytase (PhT)} from the fine roots and degrading Po. Most previous studies have evaluated phosphatase activities at the plot scale and/or focused on only PME and PDE. However, no studies have directly tested all PME, PDE, and PhT activities at the

species level in the field fertilization experimental plots. Thus, differences in soil Po acquisition among functional types in response to P deficiency remain unclear. In this study, three fine root phosphatase activities (PME, PDE, and PhT), specific root length (SRL), fine root diameter, and tissue density were measured.

In chapter 5, I examined the response of root exudation rates (Figure 1-1c) of tropical trees to N and P fertilization. Plant roots exudate organic acids, which can increase the availability of major soil nutrients (e.g., N and P) via enhancing soil microbial activity and mobilizing nutrients. Although fertilization experiments have been conducted in the temperate zone, there has been no testing of root exudation in field fertilization experiments in the tropics. Thus, significance of this mechanism and its interspecific difference among tropical tree species on highly weathered low-P soils remain unclear. In this study, I measured root exudation rates, root morphological characteristics (SRL, fine root diameter, and tissue density) and chemistry (tissue N concentration and CN ratio) for each functional type.

In chapter 6, we summarized this study and how nutrients-use and -acquisition strategies differ among functional types with different successional status and mycorrhizal types. I showed the overall changes of nutrient-use and -acquisition traits following P fertilization for each functional group to identify important nutrient-use and -acquisition strategies for each functional type.

## 1.5. Study sites: N and P factorial fertilization experiments in Malaysian Borneo

### 1.5.1. Study sites

Study sites were located in Deramakot Forest Reserve and Tangkulap Forest Reserve in Sabah, Malaysian Borneo (5°14– 30′N, 117°11– 36′E) (Figure 1-2; Imai et al. 2009; 2010; 2012; Yokoyama et al. 2017). The climate is humid equatorial. The mean annual temperature is 25.2°C and the annual precipitation is 3098 mm (Ong et al. 2013). The soils are characterized as acrisols (Sabah Forestry Department 2005). The natural forests in the two reserves are composed largely of overlogged mixed lowland dipterocarp forests (Ong et al. 2013). Tangkulap Forest Reserve, in particular, has been highly damaged by intensive logging (Imai et al. 2012; Ong et al. 2013).

Twelve 0.12 ha (30 m × 40 m) plots were established in primary forest in Deramakot Forest Reserve and another 12 plots in secondary forest in Tangkulap Forest Reserve by N. Imai, unpublished data. The primary forests are dominated by climax species, such as those of the

family Dipterocarpaceae, whereas the secondary forests are dominated by pioneer species of the genus *Macaranga* (Euphorbiaceae). Aboveground biomass and species richness are higher in primary forests than in secondary forests (Table 1-2; Imai et al. 2012). Factorial N and P fertilization (control, +N, +P, +NP, n = 3 each) was initiated in December 2011 for each forest type. A total of 24 plots (2 forest types ×4 treatments ×3 replicates) were used for the experiment. Urea and triple superphosphate (TSP) were scattered by hand at rates of 100 kg N ha−1 and 50 kg P ha−1, respectively. Each 0.12 ha plot was divided into 12 subplots of 0.01 ha (10 m × 10 m), and a fixed amount of fertilizer was applied evenly to each subplot. Fertilization was continued annually. Environmental parameters and soil properties are shown in Table 1-3 and 1-4. Soil chemistry and fluxes of $CO_2$, $CH_4$, and $N_2O$ in the fertilized plots were determined by Yokoyama et al. (2017), Mori et al. (2017), Mori et al. (2018), Hirano et al. (2022), and Mori et al. (2023).

### 1.5.2. Target species

I selected nine evergreen broad-leaved species, representing a broad range of the taxa of the commonest woody species in my study sites (Table 1-S1). Seven of the nine species (*Shorea multiflora*, *Shorea obscura*, *Dipterocarpus acutangulus*, *Gluta wallichii*, *Knema latericia*, *Mallotus penangensis*, and *Sindora irpicina*) are defined as climax species because these species are common in my primary forest sites. *Shorea multiflora*, *S. obscura*, and *D. acutangulus* are canopy-dominant dipterocarp species. *Gluta wallichii* (Anacardiaceae), *K. latericia* (Myristicaceae), *M. penangensis* (Euphorbiaceae), and *S. irpicina* (Leguminosae) are sub-canopy species. Two of the nine species (*Macaranga pearsonii* and *M. gigantea*) are pioneer species of Euphorbiaceae, which dominate secondary forests. Symbiotic fungi are AM for most tree species in lowland tropical rainforests (Alexander, 1989). Although recent study has indicated that dipterocarp species may be Dual-mycorrhizal plants, which form symbiotic associations with both ECM and AM fungi (Teste et al. 2020), I defined dipterocarp species as trees forming symbiotic associations with ECM fungi in this study (Alexander and Lee, 2005). *Sindora* is a genus of the family Leguminosae, but it is a non-nodulating (non-$N_2$-fixing) genus (Afkhami et al. 2018). Therefore, *S. multiflora*, *S. obscura*, and *D. acutangulus* are ECM-associated species, whereas *G. wallichii*, *K. latericia*, *M. penangensis*, *S. irpicina*, *M. pearsonii*, and *M. gigantea* are AM-associated species. For seven of these species (*S. multiflora*, *D. acutangulus*, *G. wallichii*, *K. latericia*, *S. irpicina, M. pearsonii*, and *M. gigantea*), I sampled

root systems and measured root phosphatase activities, root exudation rates and root morphological and chemical traits (Figure 1-S1).

**Table 1-1.** Summary of fertilization experiments conducted in lowland tropical rainforests (<400 m elevation).

| Forest type | Site | Region | Soils | Fertilizer | Years fertilizer applied | Plot size (m) [and number] | Forest age (yrs) | Statistically Significant Effects | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Tissue nutrient | Litter production | Tree/biomass growth | |
| Old growth | El Verde, Perto Rico | Neotropics | Oxisols & Ultisols | complete | 4 | 20 by 20 [8] | - | Not studied | Increases with complete fertilizer | Insignificant | Walker et al. 1996; Li et al. 2006 |
| Old growth | La Selva, Costa Rica | Neotropics | Ultisols(Saplings) | complete | 2.5 | Individual saplings | - | Not studied | Not studied | Increases in high light with complete fertilizer. | Chou et al. 2017 |
| Old growth | Iguazú, Argentina | Neotropics | Ultisols(Forest gap saplings) | N and P togather | 5 | 15 by 15 [10] | - | Not studied | Not studied | Increases in high light with +NP. | Villagra et al. 2013 |
| Old growth | Korup, Cameroon | Neotropics | No soil type (Dominant trees ectomycorrhizal) | P | 2 | 50 by 50 [14] | - | +P increases foliar and litter P. | Insignificant | Insignificant | Newbery et al. 2002 |
| Old growth | Tombopata, Peru | Neotropics | Alluvial terrace | Factorial N and P | 4 | Individual trees | - | Insignificant | Not studied | Insignificant† | Fisher et al. 2013 |
| Old growth | Limón, Costa Rica | Neotropics | Clayey, volcanic origin | Factorial N and P | 3 | 30 by 30 [24] | - | +N (+P) increases foliar N (P) in selected species. | Insignificant | Increases with +P for small trees. Trees > 100 mm DBH unaffected. | Alvarez-Clare et al. 2013, 2015 |
| Old growth | BCNM, Panama | Neotropics | Oxisols & Inceprisols | Factorial N, P & K | 15 | 40 by 40 [32] | - | +P increases foliar & litter P. | Increases with +P | Insignificant | Kaspari et al. 2008; Wright et al. 2011; Mayor et al. 2014; Wright et al. 2018 |
| Old growth | Manaus, Brazil | Neotropics | Oxisols | Factorial N, P & cations | 2 | 50 by 50 [32] | - | +P increases root P. | Increases with +P | Total productivity increases with +P. Fine root biomass increases with +P. | Cunha et al. 2022; Lugli et al. 2021 |
| Old growth | Kalimantan, Borneo | Southeast asia | Yellow, sandy | Factorial N and P | 4 | 50 by 50 [20] | - | +N, +P & +NP increase litter P & N. | Increases with +N, +P & +NP | Insignificant | Mirmanto et al. 1999 |
| Secondary | Guanacaste, Costa Rica | Neotropics | Andic and Typic Haplustepts (Alfaro et al. 2001) | Factorial N and P | 2.75 | 25 by 25 [16] | 30 | Not studied | Not studied | Belowground biomass increased with +P. | Waring et al. 2019 |
| Secondary | San Carlos de Rio Negro, Venezuela | Neotropics | Oxisol | NPK | 0.33 | 1.5 by 1.5 [8] | 0 | Not studied | Not studied | Increases with +NPK | Uhl 1987 |
| Secondary | Igarapé Açu, Pará, Brazil | Neotropics | Typic Kandiudult ~ 70% sand by mass | Complete fertilizer combined with -1 treatments | 2 | 6 by 7 [80] | 0 | +P increases foliar P. Litter not studied. | Not studied | Increases with +P | Gehring et al. 1999 |
| Secondary | Yucatan, Mexico | Neotropics | Shallow, organic rich directly over limestone | Factorial N and P | 3 | 12 by 12 [16] | 10 | +P increases foliar & litter P. | Increases with +NP | Increases with +N, +P, & +NP | Campo & Dirzo 2003; Campo & Vázquez-Yanes 2004; Campo et al. 2007 |
| | | | | | | 12 by 12 [16] | 60 | +NP increases foliar & litter P. | Increases with +NP | Increases with +N, +P, & +NP | |
| Secondary | Paragominas, Pará, Brazil | Neotropics | Kaolinitic yellow Latosols | Factorial N and P | 2 | 20 by 20 [12] | 6 | +P (+N) increases foliar P (N). Litter not studied. | Not studied | Increases with +N | Davidson et al. 2004 |
| Secondary | Paragominas, Pará, Brazil | Neotropics | Oxisol | P | 2 | 20 by 20 [6] | 24 | Not studied | Not studied | Insignificant | Markewitz et al. 2012 |

**Table 1-2.** Aboveground forest structure and tree species diversity of one 2-ha plot in primary and secondary forest. All data from Imai et al. (2012). Parentheses indicate values for trees > 5 cm dbh. Estimated number species (per 850 stems in 2-ha plots) were calculated by interpolation from the species-individual curves. Pioneer species: five *Macaranga* species (*M. conifera*, *M. gigantea*, *M. hypoleuca*, *M. pearsonii*, *M. bancana*) and two *Croton* species (*C. argyratus*, *C. oblongus*) of Euphorbiaceae, and two Rubiaceae species (*Neolamarckia cadamba*, *Neonauclea* sp.)

| | Primary | Secondary |
|---|---|---|
| *Forest structure* | | |
| Stem density (/ha) | 607 | 428 |
| Above ground biomass (Mg/ha) | 378 | 216 |
| Maximum dbh (cm) | 129 | 92 |
| Basal area ($m^2$/ha) | | |
| Total | 34.2 | 23.3 |
| Dipterocarp | 17.6 | 8.5 |
| Pioneer | 0.4 | 4.2 |
| *Tree species diversity* | | |
| Obsereved no. families | 52 (52) | 48(51) |
| Obsereved no. genera | 135 (144) | 121 (131) |
| Obsereved no. species | 296 (319) | 243 (263) |
| Estimated no. species | 257 | 243 |

**Table 1-3.** Environmental parameters of N and P fertilization plots in primary and secondary forests. All data from Mori et al. (2017). Values are mean (SD). WFPS = water filled pore space.

| Forest type | Treatment | Air temperature (°C) | Soil temperature (°C) | WFPS (%) |
|---|---|---|---|---|
| Primary | Control | 26.0 (1.7) | 24.7 (0.4) | 25.3 (2.4) |
| | +N | 25.7 (0.7) | 24.5 (0.2) | 24.6 (4.1) |
| | +P | 26.3 (0.7) | 24.5 (0.2) | 30.9 (8.5) |
| | +NP | 26.0 (1.1) | 24.8 (0.3) | 28.5 (4.1) |
| Secondary | Control | 25.8 (1.3) | 25.0 (0.3) | 36.3 (7.8) |
| | +N | 27.5 (0.4) | 25.4 (0.3) | 30.1 (5.2) |
| | +P | 26.6 (2.2) | 25.1 (0.3) | 30.1 (4.0) |
| | +NP | 26.9 (1.6) | 25.3 (0.3) | 33.7 (4.3) |

**Table 1-4.** Soil properties of N and P fertilization plots in primary and secondary forests. [a] Data from Yokoyama et al. (2017). Bray-I-method was used to extract P from soil. [b] Data from Mori et al. (2023). Values are means with standard deviations in parentheses (n = 3). All soil samples (0–5 cm) were taken from the same study plots as the present study. All values are means with standard errors in parentheses. DOC = dissolved organic carbon, DON = dissolved organic nitrogen.

| Forest type | Treatment | pH ($H_2O$)[a] | $NO_3^-$ (μg N g $soil^{-1}$)[b] | $NH_4^+$ (μg N g $soil^{-1}$)[b] | DOC (μg C g $soil^{-1}$)[b] | DON (μg N g $soil^{-1}$)[b] | Bray P (μg P g $soil^{-1}$)[a] |
|---|---|---|---|---|---|---|---|
| Primary | Control | 4.3 (0.1) | 1.8 (0.8) | 27.7 (2.7) | 242.5 (8.7) | 27.3 (0.5) | 1.5 (0.4) |
| | +N | 4.2 (0.1) | 3.6 (2.0) | 28.8 (2.4) | 246.9 (6.1) | 30.1 (2.0) | 1.1 (2.3) |
| | +P | 4.5 (0.1) | 4.8 (1.7) | 26.7 (3.0) | 247.2 (10.9) | 29.4 (1.2) | 16.1 (10.0) |
| | +NP | 4.3 (0.1) | 4.8 (2.6) | 26.7 (1.8) | 239.5 (9.2) | 28.9 (1.7) | 15.8 (6.6) |
| Secondary | Control | 4.6 (0.1) | 1.5 (0.8) | 29.2 (2.7) | 289 (11.8) | 32.1 (0.1) | 1.0 (5.7) |
| | +N | 4.4 (0.1) | 3.7 (1.5) | 24 (0.4) | 261.2 (10.6) | 29.2 (1.2) | 1.8 (4.9) |
| | +P | 4.5 (0.0) | 3.1 (1.0) | 21.5 (4.3) | 259.7 (17.0) | 28.9 (2.7) | 22.0 (14.9) |
| | +NP | 4.5 (0.1) | 3.1 (1.6) | 21.6 (5.2) | 233.9 (25.9) | 24.9 (3.9) | 27.1 (0.4) |

**Table 1-S1.** List of tree species collected in this study in Malaysian Borneo. C: control, N: N fertilization, P: P fertilization, NP: N and P fertilization together. *For these species, only green and senescent leaves were sampled.

| Species (Family) | Treatment | N | DBH (cm) | |
|---|---|---|---|---|
| | | | Average | Range |
| *Dipterocarpus acutangulus* (Dipterocarpaceae) | C | 3 | 12.5 | 11.5–13.1 |
| | P | 3 | 38.5 | 11.4–60.5 |
| | N | 3 | 46.3 | 6.7–81.7 |
| | NP | 4 | 15.9 | 12.5–17.5 |
| *Shorea multiflora* (Dipterocarpaceae) | C | 6 | 49.7 | 12.4–87.8 |
| | P | 3 | 23.0 | 17.3–32.6 |
| | N | 5 | 21.6 | 11.9–30.3 |
| | NP | 3 | 27.5 | 17.2–43.0 |
| *Shorea obscura* (Dipterocarpaceae)* | C | 3 | 22.3 | 18.0–30.3 |
| | P | 3 | 15.1 | 10.7–21.5 |
| | N | 3 | 23.0 | 17.6–27.8 |
| | NP | 3 | 34.7 | 13.1–45.7 |
| *Gluta wallichii* (Anacardiaceae) | C | 3 | 16.8 | 11.9–20.9 |
| | P | 3 | 19.4 | 11.0–32.4 |
| | N | 3 | 14.1 | 11.0–15.9 |
| | NP | 3 | 13.8 | 11.7–15.1 |
| *Knema latericia* (Myristicaceae) | C | 4 | 12.2 | 10.3–17.3 |
| | P | 3 | 13.8 | 11.6–17.0 |
| | N | 3 | 12.5 | 11.7–13.9 |
| | NP | 3 | 12.9 | 11.3–15.2 |
| *Mallotus penangensis* (Euphorbiaceae)* | C | 3 | 12.0 | 11.1–13.0 |
| | P | 3 | 17.5 | 14.8–19.2 |
| | N | 3 | 13.0 | 11.3–14.5 |
| | NP | 3 | 16.1 | 11.6–18.4 |
| *Sindora irpicina* (Leguminosae) | C | 3 | 32.0 | 8.0–65.7 |
| | P | 2 | 12.0 | 10.2–13.8 |
| | N | 2 | 13.0 | 8.8–17.3 |
| | NP | 2 | 14.9 | 8.8–21.1 |
| *Macaranga pearsonii* (Euphorbiaceae) | C | 4 | 25.4 | 20.9–30.6 |
| | P | 3 | 15.2 | 12.6–16.7 |
| | N | 3 | 33.3 | 28.2–41.9 |
| | NP | 3 | 23.7 | 14.4–31.4 |
| *Macaranga gigantea* (Euphorbiaceae) | C | 4 | 13.6 | 11.8–16.5 |
| | P | 4 | 17.5 | 13.4–26.3 |
| | N | 3 | 24.2 | 21.2–28.0 |
| | NP | 3 | 24.0 | 20.4–26.2 |

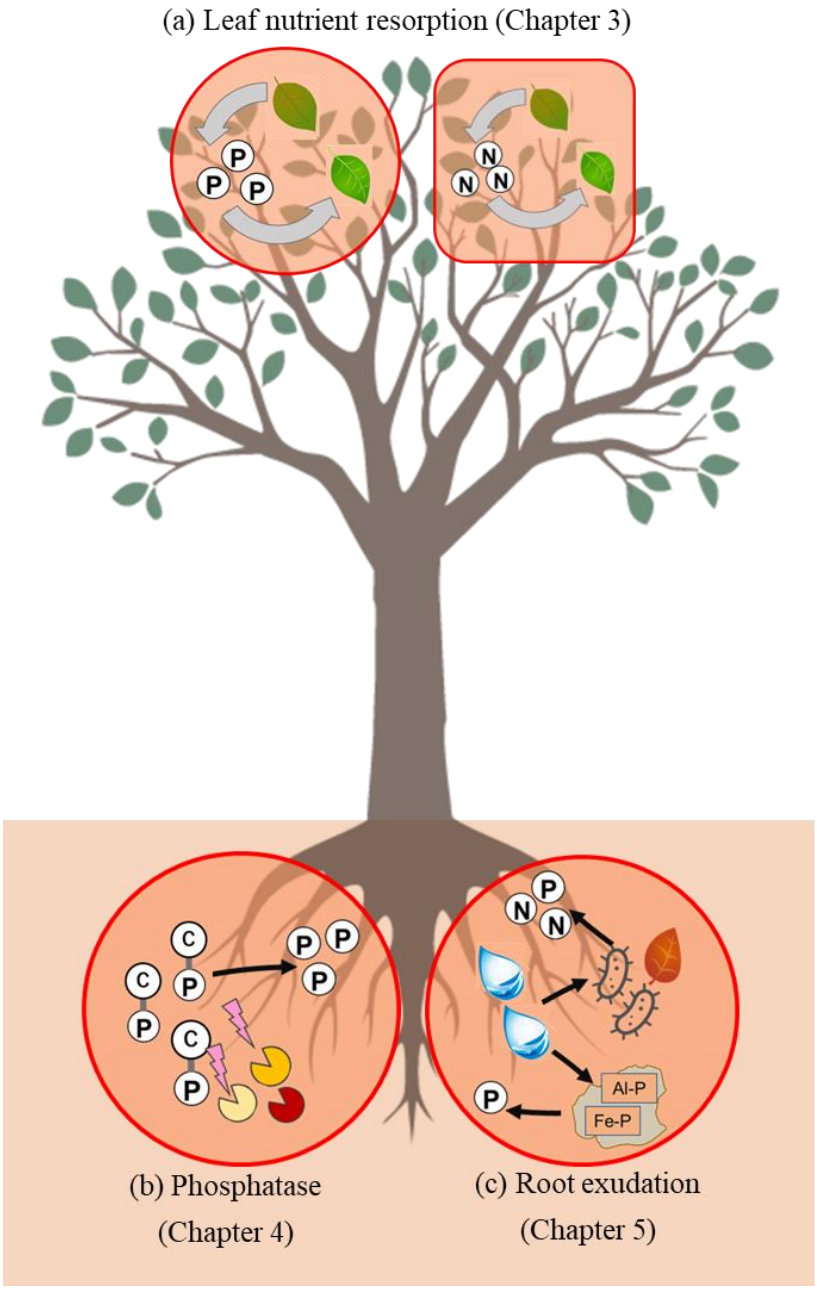


**Figure 1-1.** Illustration of the nutrient-use and -acquisition strategies of trees. (a) Leaf P or N resorption is an indicator for plant P or N use; P and N are remobilized from senescing leaves before they fall and is reused in sink organs. (b) Phosphatases (yellow or brown) are enzymes that degrading Po. (c) Root exudation (blue waterdrop), including low-molecular-weight organic acids, can increase the availability of major soil nutrients (e.g., N and P) via enhancing soil microbial activity and mobilizing nutrients.

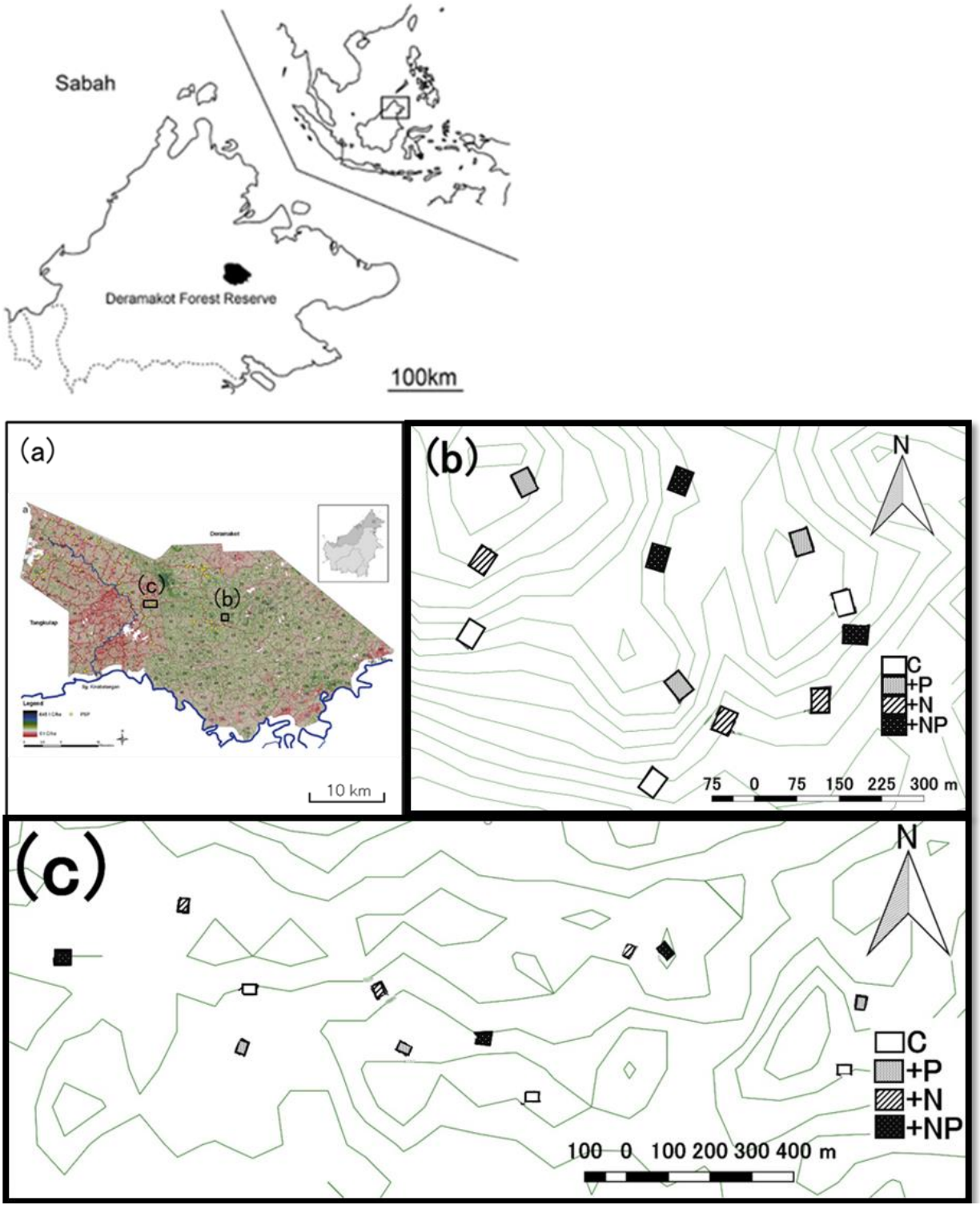


**Figure 1-2.** Location map of the study sites. For Fig. 1b and c, C, +N, +P, +NP are control, phosphorus-, nitrogen-, nitrogen and phosphorus- fertilized plot, respectively.

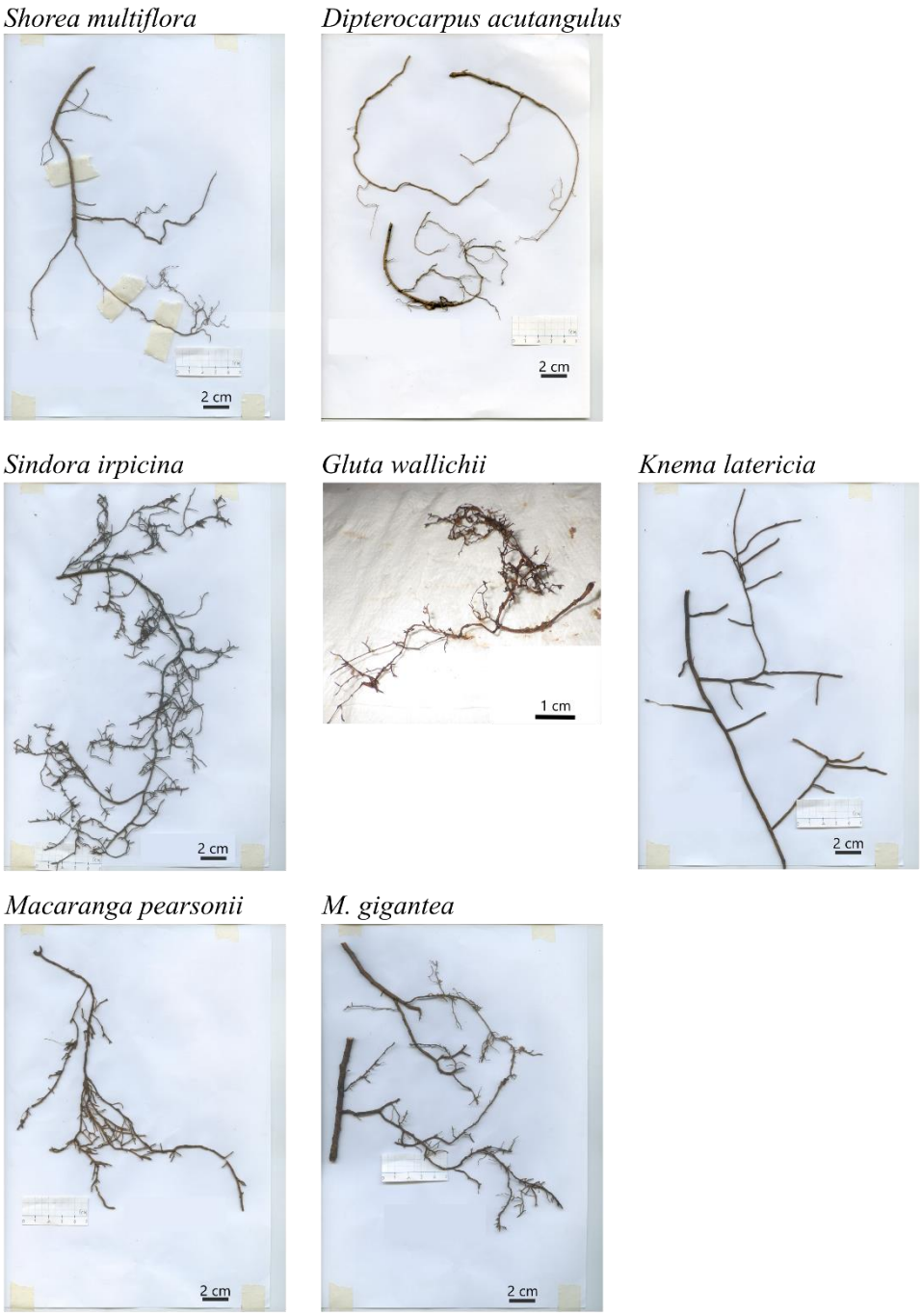


**Figure 1-S1.** Representative specimens of the fine root systems of the seven tree species studied in my study sites in Malaysian Borneo. The specimens were washed free of soil, and their images (except for *Gluta wallichii*) were digitized using a flatbed scanner.

## Chapter 2. Relationship between herbivory and leaf traits in mangroves on Iriomote Island

### 2.1. Introduction

Plants maintain their huge biomass through photosynthesis by allocating nutrients such as N and P in their leaves. In general, leaf N and P concentrations of fast-growing pioneer tree species are greater than those of slow-growing climax tree species. In addition, studies on the leaf economic spectrum (Reich et al. 1992, 1997, 1999; Wright et al. 2004) suggested that there is a trade-off between productivity and persistence among sun leaves of different plant species: Inexpensive short-lived leaves exhibit faster photosynthetic rate (i.e., slow C return) whereas costly long-lived leaves exhibit slower photosynthetic rate. Thus, leaf functional traits including leaf lifespan, photosynthetic capacity, chemical and physical/mechanical properties vary along with leaf nutrients (Wright et al. 2004).

Leaf herbivory potentially affects C and nutrient cycling in forest ecosystem. Leaves generally contribute to high productivity of forest ecosystems through photosynthetic carbon assimilation (Bouillon et al. 2008), and they often suffer high levels of herbivory damage. Such herbivory diverts resources from the detrital pathway to the grazing pathway, which alters the C and nutrient cycling patterns in ecosystems (Huntly 1991; Feeley and Terborgh 2005; Metcalfe et al. 2014).

Leaf nutrients and other leaf functional traits influence herbivore behaviour and anti-herbivore defence strategies (Crone and Jones 1999; Mundim et al. 2009), which consequently can be factors driving leaf herbivory. For example, leaves with higher nutrient concentrations and lower C-to-nitrogen ratios can be more palatable to herbivores and experience intensive herbivory (Yamasaki and Kikuzawa 2003; Marquis et al. 2012). In contrast, leaves with higher levels of chemical and physical/mechanical defences can be protected from herbivory. For example, the content of condensed tannins, major chemical defence compounds, plays a role in defending leaves from herbivores by forming protein complexes (Schofield et al. 2001). The physical and mechanical traits of leaves, such as the leaf dry matter content (LDMC), leaf mass per area (LMA), leaf thickness, and the force required to punch or tear the leaf, may also contribute to protect leaves from herbivores that are inadequately adapted to overcome these physical barriers (Hanley et al. 2007; Pérez-Harguindeguy et al. 2013; Caldwell et al. 2016).

Although relationships between leaf traits and leaf herbivory has been reported as described above, it remains unclear if these relationships exist in tropical and subtropical areas where tree species diversity is high (Coley 1983; Kurokawa and Nakashizuka 2008). Kurokawa and Nakashizuka (2008) who studied in a Malaysian tropical forest have found that most leaf chemical and physical/mechanical traits are not correlated with leaf herbivory rates, and that the only one leaf trait that was correlated with herbivory rates, leaf toughness, has a weak effect. This would be probably because the high diversity of plant and herbivores in lowland tropical rainforests promotes specialization of plant-herbivore interactions. Such specialization of plant-herbivore relationships would have resulted in a lack of patterns between leaf traits and leaf herbivory rates across tree species in lowland tropical rainforests (Kurokawa and Nakashizeka 2008).

In mangrove forests, which have low tree species diversity and simple forest structure despite being established in tropical/ subtropical regions (Imai et al. 2006), the relationship between leaf herbivory and leaf traits may be clearer than in lowland tropical rainforests because the plant-herbivore interaction may be simpler than in lowland tropical rainforests. To reveal this, it is necessary to take into account the various leaf functional traits including physical and chemical properties in relation to leaf herbivory. However, most studies of mangrove forests that have investigated herbivory and leaf traits have focused only on leaf morphology (Feller and Chamberlain 2007) or chemistry, including nutrient and/or tannin concentrations (Anderson and Lee 1995; Erickson et al. 2004; Tong et al. 2006; Balakrishnan et al. 2016; Trisnawati et al. 2019a, b). Therefore, the relationship between herbivory and various leaf traits, including physical/mechanical traits, in mangrove forests remain unclear.

Herbivory levels may also differ among leaf phenotypes (sun leaves vs shade leaves), leaf ages (young leaves vs old leaves), and seasons (summer vs winter). In general, primary productivity tends to be higher in sun leaves than in shade leaves (Martin et al. 2020), and the concentrations of some defensive compounds, such as tannins, and the physical and mechanical strength of sun leaves are also often higher in sun leaves (Aide and Zimmerman 1990; Dudt and Shure 1994; Henrikson et al. 2003; Stoepler and Rehill 2012). Therefore, the levels of herbivory on sun leaves can be lower than on shade leaves because their defensive levels are higher (Dudt and Shure 1994; Stoepler and Rehill 2012). Similarly, herbivore levels may be lower on old leaves than on young leaves (Aide and Zimmerman 1990; Tong et al. 2006; Trisnawati et al.

2019b) because old leaves are typically tougher and often have lower nutrient concentrations (Kursar and Coley 1991; Mundim et al. 2009). Furthermore, herbivory rates often show considerable seasonal variation owing to factors such as the productive periods of young leaves, which are susceptible to herbivory (Tong et al. 2006; Balakrishnan et al. 2016). However, most studies of herbivory in mangrove forests have focused only on leaf age (Farnsworth and Ellison 1991; Feller and Chamberlain 2007) and/or season in 1–3 species (Faraco and Da Cunha Lana 2004; Tong et al. 2006; Balakrishnan et al. 2016). No study has examined leaf herbivory on multiple species along the tidal gradient in terms of leaf age, phenotype, and season.

I examined the herbivory and leaf traits at each leaf age stage and for each phenotype of the dominant species with different growth rates in each of the six communities extending from the seaward fringe to inland on Iriomote Island, Okinawa, Japan. The aims of this study are: (1) to identify the differences in herbivory among species, leaf phenotypes, age, and seasons; and (2) to investigate the relationships between herbivory and leaf traits in mangrove forests.

## 2.2. Materials and methods

*Study site*

This study was performed in the subtropical mangrove forests on the Shiira River (24°20′N, 123°50′E), which are located in the southeastern part of Iriomote Island, Ryukyu Islands, southern Japan (Figure 2-1). The climate is subtropical coastal, the annual precipitation is 2304.9 mm, and the mean annual temperature is 23.7 °C. The tidal regime on Iriomote Island is semi-diurnal with a range of ~1.5 m (Nanjo et al. 2011). In this study area, the typical zonation pattern includes the *Avicennia marina* zone, *Sonneratia alba* zone, *Rhizophora stylosa* zone, *Bruguiera gymnorhiza* zone (hereafter, *Bruguiera*-L), and *Heritiera littoralis* zone along the intertidal gradient from the seaward fringe to the inland (Table 1). Furthermore, a dense *B. gymnorhiza* stand of trees with slender trunk is present along the riverside (hereafter, *Bruguiera*-R).

*Plot establishment and tree measurement*

In August 2016, three 10 × 10 m plots in each of the six vegetation zones were established (18 plots in total; Imai et al. unpublished). Each plot was subdivided into four 5 × 5 m sub-plots. After I was carefully levelled using a digital level (Leica Sprinter 150M, Leica Geosystems AG, Switzerland), the frequency of tidal inundation in each zone was calculated from a tide table and

expressed as the number of days per year (d $yr^{-1}$) on which the tide reached each community. The difference in ground level between the lowest *Avicennia* zone and the highest *Heritiera* zone was 113 cm (Table 1). The inundation frequency decreased from the seaward fringe to the inland (Table 1).

All trees of ≥5 cm diameter at breast height (dbh) were tagged, identified, and the stem dbh measured, avoiding protrusions. The dbh of all *R. apiculata* was measured at least 50 cm above the highest prop root. Because most *A. marina* were dwarf (<5 cm dbh), their trunk diameters were measured at the 1/10 height of the target tree. The height of the tallest individual tree in each plot was measured using a measuring tape in the *Avicennia* zone and a digital hypsometer (Vertex Ⅳ, Haglöf, Sweden) in the other zones. I optically measured the leaf area index (LAI) in each plot with a portable spectral LAI analyser (MIJ-15, Environmental Measurement Japan, Fukuoka, Japan). I took a total of nine LAI readings systematically at a height of 1.7 m at the corners of the 5 × 5 m sub-plots. In the dwarf *Avicennia* zone, I randomly took four readings under the canopies of three *A. marina* trees at a height of 0.3 m (12 readings in total).

*Herbivory*

In the period August 28–September 8, 2018 (summer), and December 1–5, 2020 (winter), trees of each of the seven dominant species (*A. marina*, *S. alba*, *R. stylosa*, *B. gymnorhiza*, *H. littoralis*, *Excoecaria agallocha*, and *Rhaphiolepis indica*) were chosen in the zone in which each species was dominant. In general, arboreal crabs (e.g., *Aratus pisonii*) and Lepidoptera are the main leaf-feeding herbivores in mangrove forests (Beever et al. 1979; Murphy 1990; Emmerson and Ndenze 2007). Moreover, there are generally no large annual variations in herbivory rates in mangrove forests (Johnstone 1981; Tong et al. 2006). In addition, no mass defoliation by herbivores (such as that reported by Whitten and Damanik 1986) was observed during the study period. Therefore, I tested the seasonality of herbivory by comparing the herbivory rates between summer in 2018 and winter in 2020. I selected one dominant tree species in each zone, except in the *Heritiera* zone, where three species (*H. littoralis*, *E. agallocha*, and *R. indica*) dominated. *Bruguiera gymnorhiza* was sampled in both the landward and riverine *Bruguiera* zones (*Bruguiera*-L and -R, respectively).

I selected 3–6 (usually three) individuals of each species in the *Heritiera* zone and three

individuals in each plot in the other zones. For each individual, 3–4 (usually three) shoots were selected from a sunlit position in the upper part of the canopy, and three shoots were also selected from a shaded position in the lower part of the canopy, which was covered with leaves from the upper layer and/or surrounding trees. I sampled one from the apical, one from the basal, and three from the middle leaves as the youngest, oldest, and middle-aged leaves, respectively. The leaf lifespan at each position may vary among mangrove tree species (Imai et al. 2009b), and the leaf age at each position may also vary among seasons (Lee 1991). However, in this study, I tested the effect of leaf age on the herbivory rates by sampling leaves from the apical branch to the basal branch in the same manner for all species and seasons. In total, the herbivory rates on 3365 leaves were analysed as follows: 2 seasons × 5 zones × 3 plots × 3 individuals × 2 layers × 3 shoots × 5 leaves = 2700 leaves, plus 665 leaves in the *Heritiera* zone (*Heritiera*: 2 seasons × 3 plots × 1–2 individuals × 2 layers × 3–4 shoots × 5 leaves (*Rhaphiolepis* and *Excoecaria*: 2 seasons × 3–6 individuals × 2 layers × 3–4 shoots × 5 leaves).

Immediately after leaf sampling, I took digital images of the leaves with a flatbed scanner (CanoScan Lide 220, Canon, Japan). The leaf images were then analysed using the ImageJ v1.53e software (https://imagej.nih.gov/ij/) to measure the leaf area. I estimated the leaf herbivory rates according to Suzuki et al. (2013), with some modifications. There are four patterns of leaf damage, each of which was reconstructed using the following methods to estimate the original leaf area. If damage occurred somewhere on the edge of a leaf blade, the original area was estimated by connecting the two outer edges of the missing portion with a straight line on a scanning image with the Paint 3D v.6.2203.1037.0 computer software (1). If the tip of a leaf blade was missing, the original area was estimated using straight lines to connect each of the two edges to the tip of the original leaf (2); the tip point was estimated by a central line projected from the midrib and two tangential lines projected from the two edges. If one of two tangential lines did not intersect the central line, only one side of the leaf blade was used and the original area was estimated by doubling that area, under the assumption of leaf symmetry (3). In the rare case in which both of the two tangential lines did not intersect the central line, I used the average leaf area of the leaves of that species in that layer and position as the value of the original leaf area (4). Herbivory rate was calculated as: herbivory area/original leaf area × 100.

*Leaf traits*

Immediately after leaf sampling, I used a SPAD-502Plus chlorophyll meter (Konica Minolta, Japan) for the non-destructive estimation of chlorophyll content. Three SPAD readings were evenly distributed over the whole leaf area and averaged for each leaf. I measured leaf thickness using a screw-gauge micrometer, and the fresh leaf mass as the fully saturated leaf mass (Mf). After the leaf mechanical traits were measured (see below), I oven-dried the leaves at 65 °C for 72 h to determine the leaf dry mass (Md). LDMC (%) was calculated as 100 × (Md/Mf) (Pérez-Harguindeguy et al. 2013). LMA (g $m^{-2}$) was calculated as the ratio of the leaf dry mass to the leaf area.

I measured the force required to tear and to punch a leaf using a digital force gauge (DST-50N, Imada, Aichi, Japan), as described by Pérez-Harguindeguy et al. (2013). I cut a longitudinal strip up to 5 mm wide from one side of the leaf to avoid the midrib and margins, with a length 10 times the strip width to avoid the effects of necking (Vincent 1990). The maximum force per unit width of the leaf specimen was defined as the 'force to tear' (N $mm^{-1}$) (Pérez-Harguindeguy et al. 2013). I then used a penetrometer test to measure the maximum force required for a punch rod to penetrate the leaf. These measurements were made on leaf laminas (excluding the midribs), and the diameter of the flat-end punch rod was 2.0 mm. The maximum force per fraction circumference along the leaf lamina surface was defined as the 'force to punch' (N $mm^{-1}$) (Pérez-Harguindeguy et al. 2013).

The leaf samples that I used to measure the physiological, physical, and mechanical traits of the leaves were analysed for their contents of nutrients (total C, nitrogen [N], phosphorus [P], potassium [K], calcium [Ca], magnesium [Mg], sodium [Na], iron [Fe], and zinc [Zn]) and condensed tannins. The oven-dried leaf samples were ground before their chemical analysis. The chemical properties were determined separately for each sun and shade leaf, and for each apical, basal, and middle leaf. The C and N concentrations were determined by combustion coupled to gas chromatography using a nitrogen and carbon analyser (Sumigraph NC-220F, Sumika Chemical Analysis Service, Japan). To measure the other elements, the dried ground samples were digested with concentrated $HNO_3$ in an acid decomposition system in heat blocks (DigiPROBE, GL Science, Japan). The concentrations of K, Ca, Mg, Na, Fe, and Zn were determined by inductively coupled plasma optic emission spectrometry (ICPS-8100, Shimadzu Co., Japan). Because the quantities of leaf sample were small, the concentration of P was determined colorimetrically on a spectrophotometer at 720 nm using the Truog and Meyer

method (Truog and Meyer 1929). The concentrations of condensed tannins were determined colorimetrically on a spectrophotometer at 500 nm using the vanillin–HCL method (Broadhurst and Jones 1978) with catechin as the standard.

Considering the effect of herbivore attacks on inducing changes in leaf traits (Karban and Baldwin 1997; Nabeshima et al. 2001), I measured all leaf traits during winter, when the herbivory rate is expected to be relatively low (Tong et al. 2006). Therefore, I used only the herbivory rates in winter for the downstream statistical analyses of the relationships between herbivory rates and leaf traits.

*Statistical analysis*

The free statistical environment R 4.2.2 (R Core Team 2022) was used for all analyses. To test the effect of the overall differences in leaf nutrient concentrations on herbivory, the major leaf nutrient concentrations (N, P, K, Ca, Mg, Na, Fe, and Zn) were subjected to a principal components analysis (PCA). I then used the scores on axis 1 of the PCA (hereafter, Chemi. PCA1) for the downstream statistical analyses.

I used linear mixed models (LMMs) for each species to assess the significant effects of season and the leaf phenotype and position on the herbivory rate using the lmer function in the lmerTest package (Kuznetsova et al. 2017). I treated the individual tree as a random effect, and species, season, and leaf phenotype or position were treated as fixed effects in the LMMs.

The interspecific differences in the leaf herbivory rates and leaf traits were also analysed with LMMs. I treated leaf phenotype and leaf position as random effects in the LMMs of leaf traits, and leaf phenotype, leaf position, and season were treated as random effects in the LMMs of leaf herbivory rates, whereas species was treated as a fixed effect. Post hoc tests were performed using the glht function in the multcomp package (Hothorn et al. 2008) using Tukey's contrast for multiple comparisons to assess interspecific differences. In addition, I used two-way ANOVA to test for differences in each leaf trait among the leaf phenotypes and positions within each species.

Finally, I constructed a linear model and conducted a model selection analysis to identify the factors that influence the herbivory rates. In the linear model, I treated the herbivory rate in winter as the response variable and species, leaf phenotype, leaf age, and all the leaf traits as the explanatory variables. To address multi-collinearity, I developed a correlation matrix for

all coefficient estimates and eliminated any combinations of variables that correlated strongly (absolute value of correlation coefficients > 0.7; Dormann et al. 2013), leaving a total of nine explanatory variables (including species, leaf phenotype, and leaf age). I performed the model selection based on the corrected Akaike's information criterion (AICc) using the MuMIn package (Barton 2022) and assessed the significance of the explanatory variables using ANOVA and F tests for the best model. I then used LMMs to assess the significant effects of each leaf trait on the herbivory rate. I treated tree species as a random effect and the leaf traits as fixed effects in the LMMs.

In all statistical analyses, the variables were ln (x + 1)-transformed when necessary to meet the assumption of normality.

## 2.3. Results

*Vegetation*

Only one species dominated each of the vegetation zones and accounted for 85%–100% of the relative number of trees, except in the *Heritiera* zone (Table 2-1). In the *Heritiera* zone, several dominant canopy species each accounted for ≥10% of the trees, including *H. littoralis*, *E. agallocha*, and *R. indica*. Tree density tended to be lower in the two seaward zones (*Avicennia* and *Sonneratia* zones), higher in the three rhizophoraceous zones (*Rhizophora* and the two *Bruguiera* zones), and intermediate in the inland zone (*Heritiera* zone). The basal area and canopy height increased from the seaward to inland zones. LAI tended to be lower in the *Avicennia* zone and higher in the other five zones.

*Herbivory*

The mean herbivory rates at the species level ranged from 1.20% to 12.5% (Figures 2-1). Overall, the herbivory rates decreased from the seaward fringe to the inland, excluding *Heritiera*. The herbivory rates were higher in summer than in winter for the two seaward species (*Avicennia* and *Sonneratia*) and *Bruguiera*-L (Figure 2-3a,b,d), whereas there was no clear trend in the other species. The herbivory rates were higher on sun leaves than on shade leaves in the two seaward species but lower for *Rhizophora* (Figure 2-3a–c), whereas there was no consistent pattern for other species. The herbivory rates were higher on the middle and/or basal leaves than on the apical leaves only for *Bruguiera*-L and -R (Figure 2-3l,m; leaf position is a proxy of leaf age),

whereas there was no consistent pattern for the other species. There were significant differences in the herbivory rates of *Excoecaria* between the seasons, phenotypes, and positions (Figure 2-3h,p), which was attributed to the high herbivory rates on the apical leaves among the sun leaves in winter (Figure 2-3p).

*Leaf traits*

The total leaf C showed no clear pattern across species along the tidal gradient (Figure 2-4a). Leaf N, P, and Chemi. PCA 1 decreased from the seaward to the inland species, except for *Excoecaria* (Figure 2-4b–d). The concentration of condensed tannins, the C:N ratio, and the tannins:N ratio showed the opposite pattern (Figure 2-4e–g). There was no overall pattern in SPAD, a physiological trait, but LDMC and LMA were lower for the two seaward species and *Excoecaria* than for the other species (Figure 2-4h–j). LDMC increased from the seaward to inland species, except for *Excoecaria* (Figure 2-4i). There was no clear pattern in leaf thickness, force to tear, or force to punch (Figure 2-4k–m).

Two inland species, *Heritiera* and *Excoecaria*, have unique leaf traits. Despite the highest herbivory rate, *Heritiera* showed the highest condensed tannin concentration, LDMC, force to tear, and force to punch (Figure 2-2; Figure 2-4e,i,l,m). Conversely, despite the lowest herbivory rate, *Excoecaria* had relatively high nutrient concentrations, but the lowest level of condensed tannins, LMA, thickness, force to tear, and force to punch (Figure 2-2; Figure 2-4b–e,j–m).

In *Sonneratia* and the rhizophoraceous species, leaf N, P, and Chemi.PCA1 were often higher in the apical position than in the middle and/or basal positions (Figure 2-S1b–d). In contrast, the C:N ratio, SPAD, LMA, thickness, force to tear, and force to punch showed the opposite pattern (Figure 2-S1f,h,j–l). For most species, the chemical and physiological traits (C and nutrient concentrations, condensed tannins, C:N ratio, tannins:N ratio, and SPAD) did not differ among leaf phenotypes (Figure 2-S1a–h), whereas most physical and mechanical traits (LDMC, LMA, and thickness) were higher in sun leaves than in shade leaves (Figure 2-S1i–k). The force to tear and/or force to punch was also higher in the sun leaves than in the shade leaves of rhizophoraceous species (Figure 2-S1l,m).

*Relationships between herbivory rates and leaf traits*

When all data for herbivory rates in winter and leaf traits were analysed together, the linear models showed that species and condensed tannins significantly affected the variation in herbivory rates (Table 2-2; Table 2-3). In the LMMs for each leaf trait, the herbivory rates decreased significantly with increasing concentrations of condensed tannins (Figure 2-5e). The tannins:N ratio also correlated with the herbivory rate when the data for *Excoecaria*, which has toxic sap (Johnstone 1981), were excluded (Figure 2-5h). Unexpectedly, the herbivory rates increased with increasing forces required to tear and punch (Figure 2-5m,o). In contrast, these mechanical traits did not correlate with the herbivory rate when the data for *Heritiera*, which had extremely high punch and tear forces, were excluded (Figure 2-5n,p).

**2.4. Discussion**

The values for the herbivory rates at my mangrove site (ranging from 1.20% to 12.5%; Figure 1) are within the range of values for multiple mangrove tree species in western New Guinea (range 0.24%–14.2%; Johnstone 1981) and Australia (range 0.3%–35%; Robertson and Duke 1987). Although annual herbivory rates, a meta-analysis has reported that in terrestrial tropical rainforests, the herbivory rates in tree species range from 3% to 20% (Coley and Aide 1991). Therefore, the levels of herbivory in mangrove forests would be similar to the rates in other tropical forests.

Species and condensed tannins significantly affected the variation in the herbivory rate (Table 2-2; Table 2-3). Specifically, herbivory rate decreased from seaward to landward at the species level (Figures 2-1, 2-2). A decline in the herbivory rates from seaward to landward has also been reported in mangrove forests in Central America (Farnsworth and Ellison 1991; Feller 1995). The herbivory rates also decreased as the condensed tannin concentration and the tannins:N ratio increased across my mangrove tree species (Figure 2-5e,h). These negative correlations between herbivory and condensed tannin or the C:N ratio have also been reported in other mangrove forests (Erickson et al. 2004; Tong et al. 2006) because condensed tannins play a defensive role (Coley and Barone 1996). The condensed tannin concentration and/or tannins: N ratio were relatively lower in seaward species than in rhizophoraceous and landward species, except for *Excoecaria* (Figure 2-4e,g). Seaward mangrove species maintain high growth rates and short leaf lifespans, as do fast-growing pioneer species in inland forests, probably because they are adapted to their unique habitat, with its continuously renewed sediments and

frequent inundation, which would otherwise make them vulnerable to severe physical damage by tides (Imai et al. 2009). Species with high growth rates and short-lived leaves have low concentrations of immobile defences such as tannins (Coley 1988; Endara and Coley 2011). Therefore, the leaves of seaward species have lower concentrations of condensed tannins, reflecting their higher growth rates and shorter leaf lifespans, leading to a decline in the herbivory rate from seaward to landward in my mangrove forest.

The other leaf traits (e.g., leaf N, P, Chemi. PCA1, and physical/mechanical traits) did not correlate with herbivory rate (Table 2-1; Table 2-2; Figure 2-5). Previous studies have also reported a lack of correlation between leaf nutrients and herbivory rates (Erickson et al. 2004; Tong et al. 2006) and between herbivory and LMA (Feller and Chamberlain 2007) or toughness in mangroves (Feller 1995). This contrasts with the situation in other terrestrial tropical forests, in which herbivory often decreases with decreasing nutrient concentrations and increasing physical strength (Coley and Aide 1991; Coley and Barone 1996). The nutrient concentrations in mangrove leaves are lower than or near the lower limits of those reported in other tropical forests (Alongi 2009). Because the overall nutrient concentrations in mangrove leaves are low, herbivores feed on leaves with low nutrient concentrations, which may preclude a relationship between leaf nutrients and herbivory rate. Moreover, when Feller (1995) reported that although the sclerophylly of mangrove leaves decreased after P fertilization, the herbivory rate did not change, suggesting that the strong physical/mechanical leaves of mangrove trees are an adaptive mechanism related to a nutrient conservation strategy in P-deficient soils (Beadle 1966) rather than an adaptation to herbivory. For this reason, the physical/mechanical traits of leaves do not correlate negatively with herbivory rate, unlike the condensed tannin concentration in leaves, which was a main driver of herbivory in my mangrove forests.

The herbivory rates on the seaward mangroves and *Bruguiera* were higher in summer than in winter (Figure 2-3a,b,d). This is possibly because young leaves, which are produced more frequently in summer than in other seasons, are more frequently attacked by insect herbivores (Lee 1991; Tong et al. 2006). It may also be attributable to the fact that herbivorous insects are generally more active in summer; i.e., during the growing season (Bale et al. 2002).

The herbivory rates in mangrove forests may differ among leaf phenotypes in some species. *Rhizophora* showed a lower herbivory rate on sun leaves than on shade leaves (Figure 2-3c). This results of *Rhizophora* may reflect the stronger physical and mechanical traits (i.e.,

physically defensive traits) of sun leaves than of shade leaves (Figure 2-S1i–l). In general, this phenotypic difference in herbivory has often been reported (Maiorana 1981; Niesenbaum 1992). On the other hand, the two seaward species showed the opposite effect of leaf phenotype, despite the stronger physical and mechanical traits in their sun leaves than in their shade leaves (Figure 2-3a,b; Figure 2-S1i–k). In previous studies, habitat, rather than leaf quality or leaf phenotype, often influenced herbivory rate (Chacón and Armesto 2006; Stoepler and Rehill 2012). Because the frequency of tidal inundation is higher in the seaward mangroves than in other vegetation zones (Table 2-1), the shade leaves of these mangroves are soaked in seawater for longer periods and are more susceptible to disturbance by tides and waves. Therefore, sun leaves in the seaward mangroves are more suitable habitats for herbivores than shade leaves, which might be a reason why herbivory rate was greater on sun leaves. Future studies must examine herbivore communities on sun and shade leaves in mangrove forests for the better understanding of these plant–herbivory interactions.

In general, older leaves are less susceptible to herbivores because their chemical and physical/mechanical defensive properties are stronger and their nutrient concentrations are lower (Kursar and Coley 1991). Nonetheless, in my study, there was no relationship between herbivory and leaf position (which was a surrogate for leaf age in this study) for any species except *Bruguiera* (Figure 2-3i–p). Certainly, higher nutrient concentrations and lower levels of physical defences were observed in the apical leaves than in the middle and/or basal leaves (Figure 2-S1b–d,f,j–m). However, as mentioned above, no relationship was observed between herbivory rates and nutrient concentrations or physical/mechanical traits in my mangrove forest (Figure 2-5a–d,f,j–p). Although it is possible that the herbivory rates of old leaves may be affected the feeding attack that the leaf suffered when it was young due to the methodology of this study, considering the relationship between leaf traits and leaf herbivory rates as described above, there may be no differences in the herbivory rate among different leaf ages in most target species. In contrast, the basal and/or middle leaves of *Bruguiera*-L and -R showed higher herbivory rates than the apical leaves (Figure 2-3l,m). These results may be related to the fact that *Bruguiera* has a longer leaf lifespan than other species (Imai et al. 2009b), which leads to the apparent accumulation of leaf herbivory with increasing leaf age (Feller and Chamberlain 2007). Moreover, although a previous work confirmed that the position of a leaf on a branch can be used as an index of the relative leaf age (Imai et al. 2009b), in some species, such as *Heritiera*

species, there may be no relationship between leaf position and leaf age because the leaf emergence pattern is not sequential.

Species-specific characteristics may also affect the herbivory on mangrove species. *Excoecaria* showed the lowest herbivory rate, despite the lowest concentration of condensed tannins (Figure 2-2; Figure 2-4e). *Excoecaria* (Euphorbiaceae) has toxic sap and, therefore, experiences less absolute and relative leaf damage than any other mangrove species (Johnstone 1981). By contrast, *Heritiera* showed the highest herbivory, despite its high concentration of condensed tannins and the greatest physical strength (Figure 2-2; Figures 2-3e,l,m). *Heritiera* leaves have the lowest C:N ratio of most mangrove species (Rao et al. 1994), resulting in high herbivory rates (Johnstone 1981; Robertson and Duke 1987), including beetle attack by Scarabaeidae (such as *Adoretus gemmifer*) (Murphy 1990; Clough 2013), because its palatability is high. In fact, relatively low C:N ratios were also observed in *Heritiera* leaves in this study (Figure 2-4g), suggesting that such leaf traits may be a factor causing the high herbivory rates for *Hertiera* leaves. In this way, leaf herbivory on some mangrove species will be affected by the specific leaf characteristics.

My results suggest that the herbivory rates in mangrove forests are moderately driven by the concentration of condensed tannins in the leaves and decrease from seaward to landward. Overall, leaf traits other than condensed tannins (leaf nutrient concentrations and physical and mechanical traits), season, leaf phenotype, and leaf age have no effect on herbivory rates. However, in seaward and rhizophoraceous species, herbivory rates differ between seasons and leaf phenotypes. Understanding these mechanisms of grazing pathways is critical for a comprehensive appreciation of the high productivity of and nutrient cycling in mangrove and sub/ tropical forest ecosystems.

**Table 2-1.** Ground level, frequency of tidal inundation, and forest structural features in each vegetation zone. The species composition of the *Heritiera* zone included not only *Heritiera* but also *Rhaphiolepis* and *Excoecaria*.

| | | *Avicennia* | *Sonneratia* | *Rhizophora* | *Bruguiera* -L | *Bruguiera* -R | *Heritiera* |
|---|---|---|---|---|---|---|---|
| Ground level (cm) | | 0 | 3 | 19 | 45 | 47 | 113 |
| Frequency of tidal inundation (days year$^{-1}$) | | 79 | 70 | 33 | 4 | 3 | 0 |
| Number of species (n plot$^{-1}$) | Mean | 1.3 | 1.0 | 1.7 | 2.0 | 1.0 | 6.0 |
| | SD | 0.6 | 0.0 | 0.6 | 1.0 | 0.0 | 1.0 |
| Tree density (no. ha$^{-1}$) | Mean | 1067 | 1000 | 4533 | 3867 | 4567 | 3333 |
| | SD | 513 | 200 | 551 | 764 | 651 | 379 |
| Basal area (m$^2$ ha$^{-1}$) | Mean | 6.2 | 13.3 | 14.5 | 33.6 | 29.7 | 32.4 |
| | SD | 4.6 | 4.8 | 2.6 | 3.2 | 5.7 | 7.1 |
| Canopy height (m) | Mean | 2.1 | 5.2 | 4.6 | 7.1 | 7.4 | 9.1 |
| | SD | 0.4 | 0.3 | 0.5 | 0.5 | 1.2 | 0.2 |
| Leaf area index (m$^2$ ha$^{-2}$) | Mean | 1.6 | 2.1 | 2.0 | 2.2 | 1.9 | 2.0 |
| | SD | 0.2 | 0.4 | 0.2 | 0.7 | 0.8 | 0.3 |

**Table 2-2.** Model selection for herbivory rates in mangrove forests on Iriomote Island, southern Japan; only the top five models (1–5) are shown. Models were sorted according to the increasing values of the corrected Akaike's information criterion (AICc, AIC corrected for small sample sizes) and delta AIC, which represents the difference in AICc between the current and most appropriate models.

| Model No. | Fixed term | AICc | Delta AICc |
|---|---|---|---|
| 1 | Species, Total C, Condensed tannins | 345.4 | 0.00 |
| 2 | Species, Total C, N, Condensed tannins | 345.5 | 0.11 |
| 3 | Species, leaf phenotypes, Total C, Condensed tannins | 346.8 | 1.37 |
| 4 | Species, leaf phenotypes, Total C, N, Condensed tannins | 347.0 | 1.53 |
| 5 | Species, Total C, Condensed tannins, LDMC | 347.2 | 1.76 |

**Table 2-3.** ANOVA table of the corrected Akaike's information criterion (AICc)- best model (model 1 in table 2-2) for herbivory rates.

| Fixed term | Df | *F* value | *P* |
|---|---|---|---|
| Species | 7 | 12.23 | < **0.001** |
| Total C content | 1 | 2.00 | 0.159 |
| Condensed tannins | 1 | 10.08 | < **0.01** |

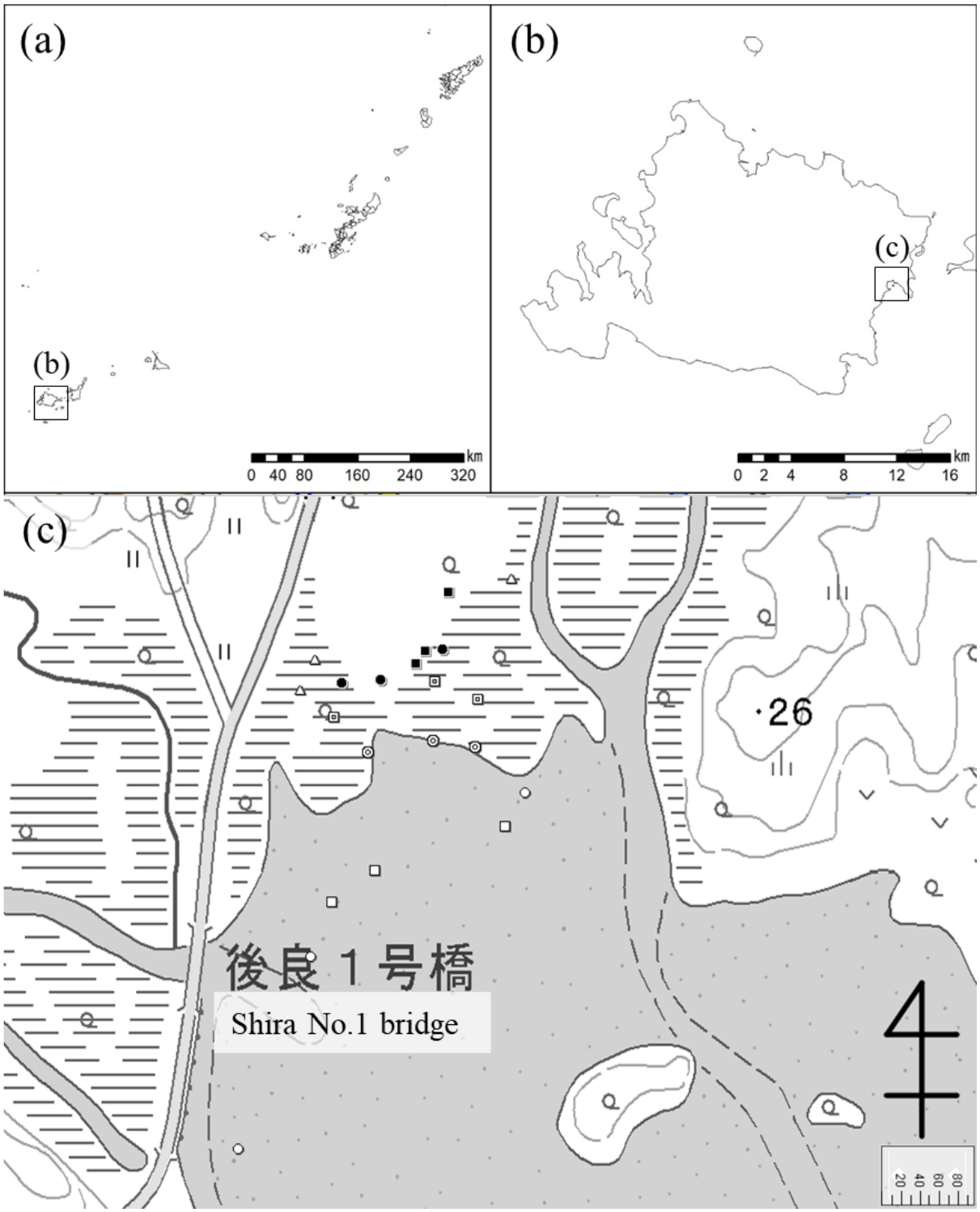


**Figure 2-1.** Location map of the study sites. The map shows the area around Sakishima Islands (the square frame in the figure indicates Iriomote Island) (a), the entire Iriomote Island (the square frame in the figure indicates the Shira river estuary) (b), and the Shira river estuary (c). Symbols in Figure c indicate plots: ◯ *Avicennia*, □ *Sonneratia*, ◎*Rhizophora*, ◎*Rhizophora* and *Brugiera* mixed, ● *Brugiera*-L, ■*Heritiera*, △*Brugiera*-R. The species composition of the Heritiera zone included not only *Heritiera* but also *Rhaphiolepis* and *Excoecaria*.

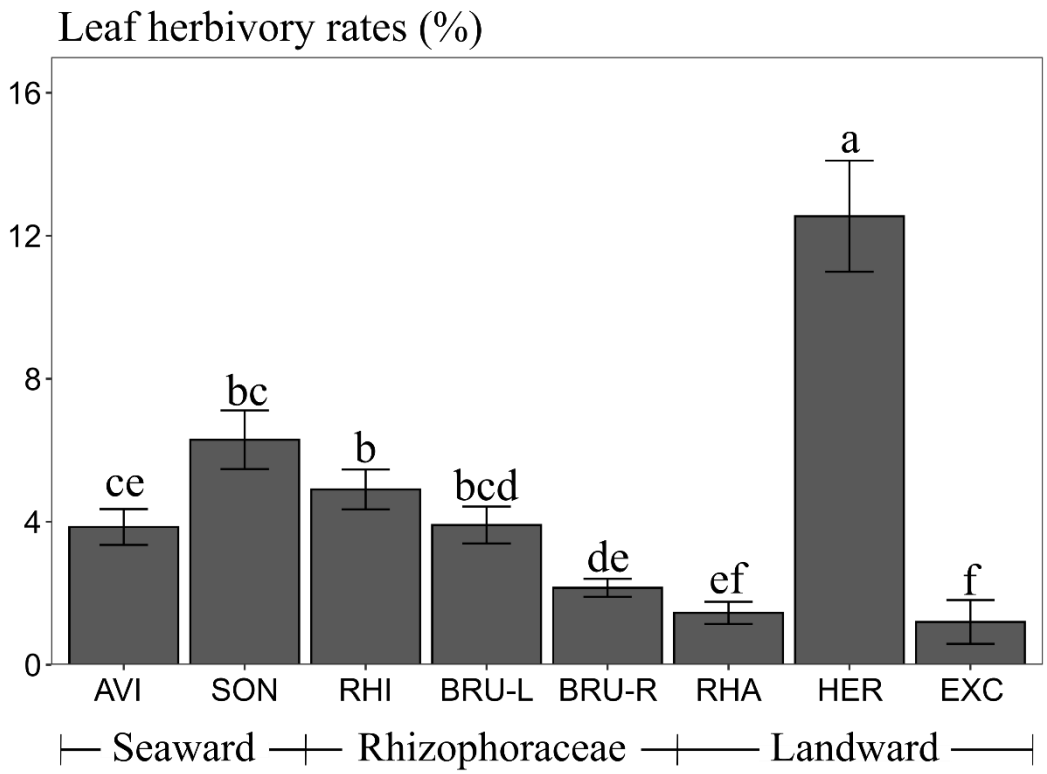


**Figure 2-2.** Leaf herbivory rates for seven mangrove tree species. AVI = *Avicennia*, SON = *Sonneratia*, RHI = *Rhizophora*, BRU-L = *Bruguiera*-L, BRU-R = *Bruguiera*-R, RHA = *Rhaphiolepis*, HER = *Heritiera*, and EXC = *Excoecaria*. Different letters in the panels indicate significant differences among species, according to Tukey's post hoc test ($P < 0.05$).

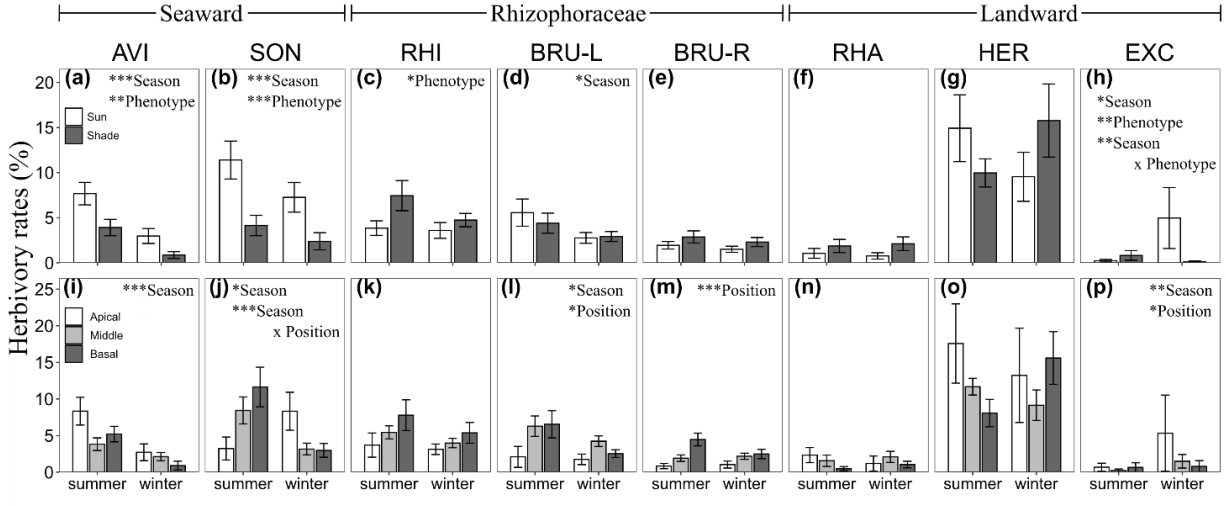


**Figure 2-3.** Herbivory rates for seven mangrove tree species with each leaf phenotype (a – h) and each leaf position (i – p). Bars represent geometric means ± SE. AVI = *Avicennia*, SON = *Sonneratia*, RHI = *Rhizophora*, BRU-L = *Bruguiera*-L, BRU-R = *Bruguiera*-R, RHA = *Rhaphiolepis*, HER = *Heritiera*, and EXC = *Excoecaria*. Significant effects are briefly described in the figures, followed by linear mixed model *P* values. *P < 0.05, **P < 0.01, ***P < 0.001.

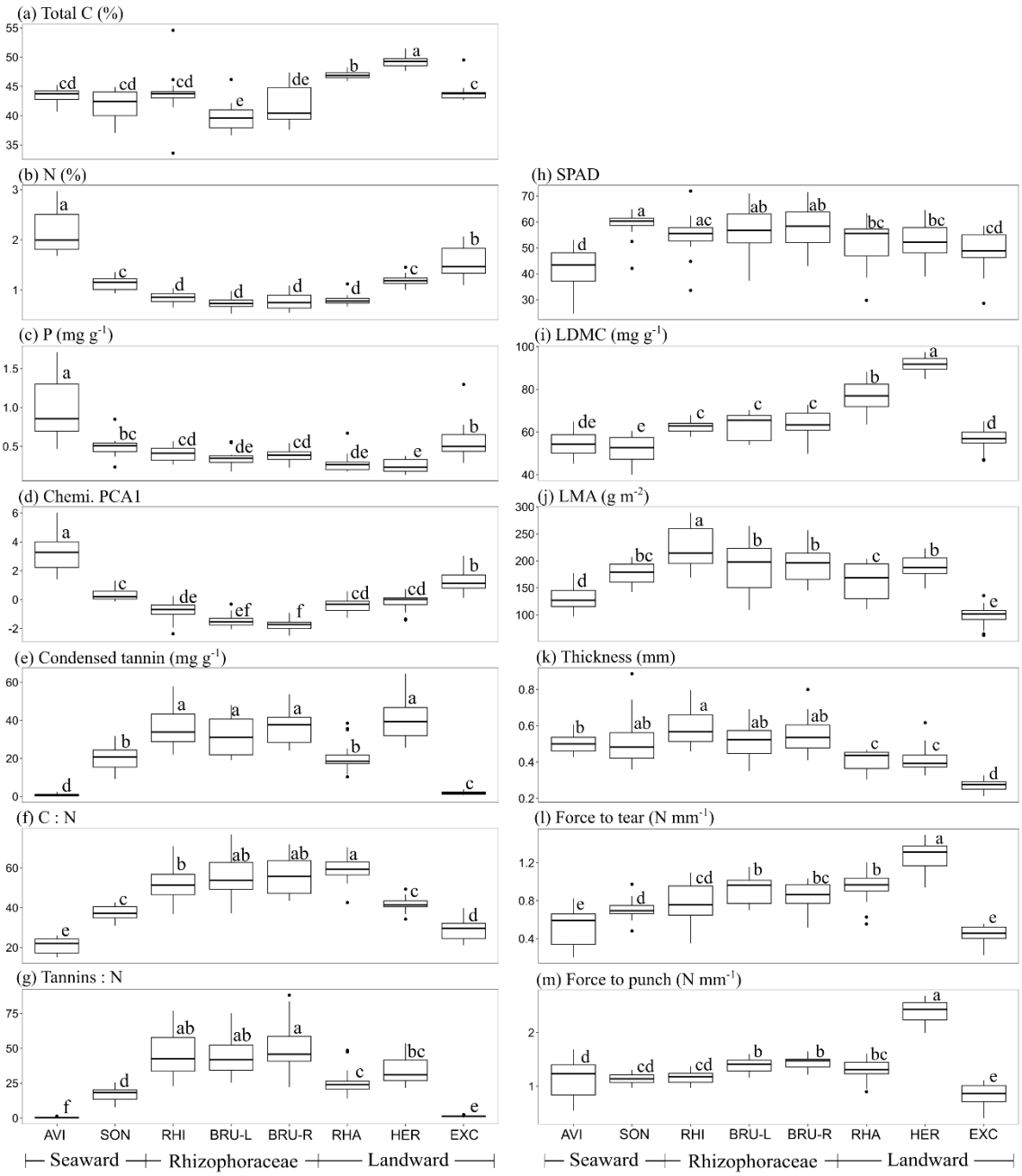


**Figure 2-4.** Leaf traits for seven mangrove tree species. Total C (a), N content (b), P content (c), the scores on axis 1 of PCA for the major leaf nutrient concentrations (Chemi. PCA1; d), condensed tannin content (e), C:N ratio (f), tannins:N ratio (g), SPAD (h), LDMC (i), LMA (j), thickness (k), force to tear (l), and force to punch (m). AVI = *Avicennia*, SON = *Sonneratia*, RHI = *Rhizophora*, BRU-L = *Bruguiera*-L, BRU-R = *Bruguiera*-R, RHA = *Rhaphiolepis*, HER = *Heritiera*, and EXC = *Excoecaria*. Different letters in the panels indicate significant differences among species according to Tukey's post hoc test ($P < 0.05$).

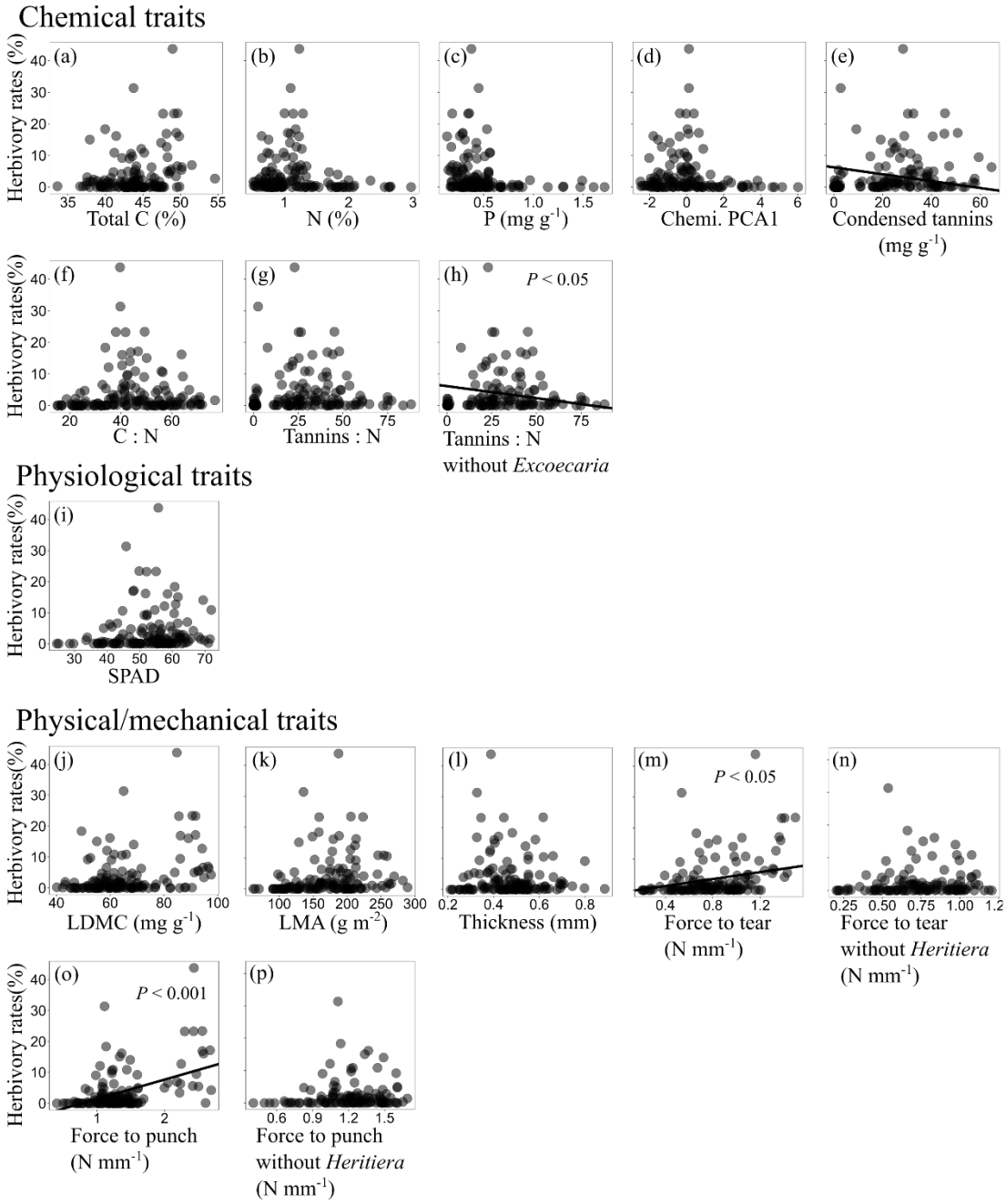


**Figure 2-5.** Relationships between herbivory rates and leaf traits: total C content (a), N content (b), P content (c), the scores on axis 1 of PCA for the major leaf nutrient concentrations (Chemi. PCA1; d), condensed tannins (e), C:N ratio (f), tannins:N ratio (g), tannins:N ratio without *Excoecaria* (h), SPAD (i), LDMC (j), LMA (k), thickness (l), force to tear (m), force to tear without *Heritiera* (n), force to punch (o), and force to punch without *Heritiera* (p). In each panel, each circle represents the value for each species, leaf phenotype, and leaf position. The solid line indicates the regression line of the linear mixed model.

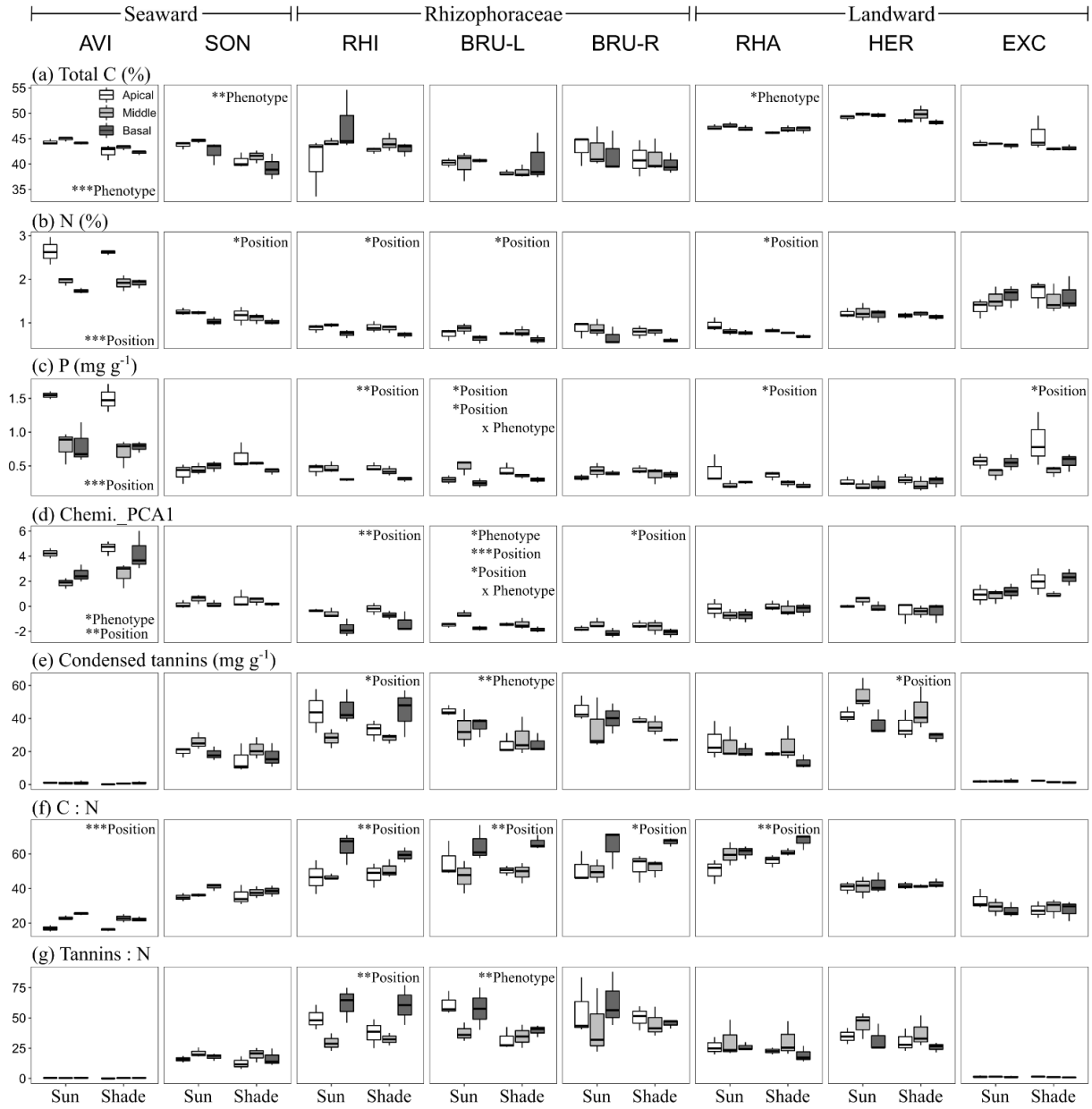


**Figure 2-S1.** Variations in leaf traits among leaf phenotypes and positions for seven mangrove tree species. Total C content (a), N content (b), P content (c), the scores on axis 1 of PCA for the major leaf nutrient concentrations (Chemi. PCA1; d), condensed tanniI(e), C:N ratio (f), tannins:N ratio (g), SPAD (h), LDMC (i), LMA (j), thickness (k), force to tear (l), and force to punch (m). AVI = *Avicennia*, SON = *Sonneratia*, RHI = *Rhizophora*, BRU-L = *Bruguiera*-L, BRU-R = *Bruguiera*-R, RHA = *Rhaphiolepis*, HER = *Heritiera*, and EXC = *Excoecaria*. Bars represent geometric means ± SE. Significant effects are briefly described in the figures, followed by two-way ANOVA model *P* values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

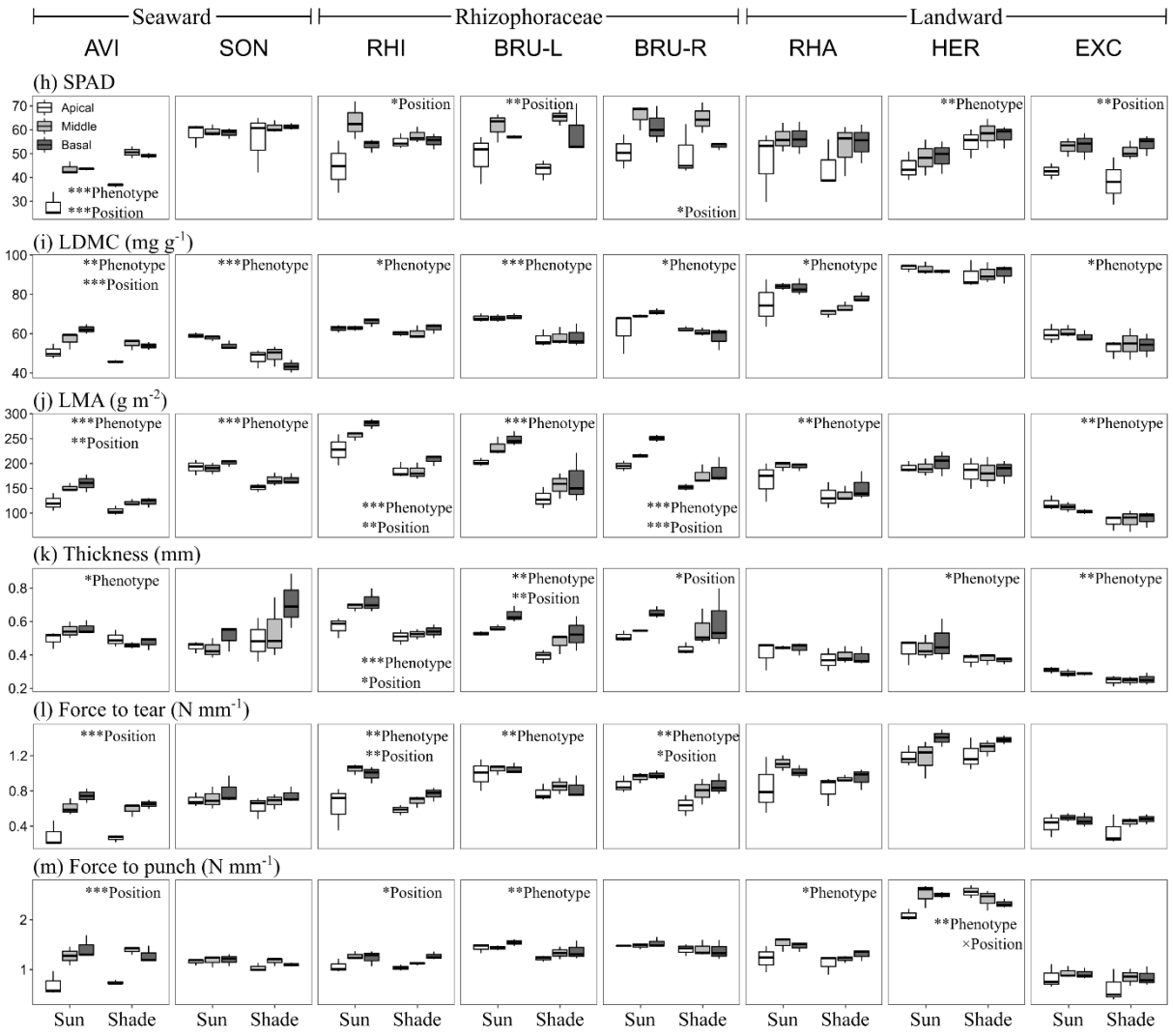


**Figure 2-S1.** Continuation.

## Chapter 3. Interspecific differences in the responses of foliar nutrients to nitrogen and phosphorus fertilization in Borneo

### 3.1. Introduction

In tropical soils, bioavailable P is often impoverished due to intense weathering (Walker and Syers 1976; Vitousek 1984; Vitousek and Sanford 1986; Crews et al. 1995). On such P-depleted soils, tropical trees show high P-use efficiency, thereby increasing carbon acquisition for a given amount of foliar P, which contribute to maintaining high productivity on P-poor soils (Vitousek 1984; Kitayama and Aiba 2002). Leaf nutrient resorption, which is a process by which plants degrade organic compounds and resorb their nutrient elements from senescing leaves (Stoddart and Thomas 1982), is one of the mechanisms to increase nutrient-use efficiency (Aerts and Chapin 2000).

Nutrients use strategies via such leaf nutrient resorption may differ among species with different successional status and mycorrhizal types (Zhang et al., 2018). Because there is a trade-off between growth rate and resource use efficiency, slow-growing climax species may have higher leaf nutrient resorption efficiency than fast-growing pioneer species (Reich 2014). In addition, ECM-associated trees had higher nutrient resorption efficiency in leaves than AM trees in boreal and temperate forests at a global scale (Zhang et al. 2018; Huang et al. 2023). This is because leaves of ECM trees often decay more slowly than leaves of AM trees (Cornelissen et al. 2001), nutrient cycling is slower in ECM-dominated ecosystems than in AM-tree-dominated ecosystems (Lin et al. 2017), and consequently ECM trees may have a more conservative strategy than AM trees (Reich et al. 2014). Furthermore, ECM trees may be less susceptible to N limitation because ECM-associated plants have a higher capacity to acquire N from the soil than AM-associated species (Lambers et al. 2008).

Previous studies have reported that plants increase their P resorption efficiency (PRE) and P use efficiency to maintain their productivity as P limitation increases by studying the dominant species in tropical montane forests (Paoli et al. 2005; Hidaka and Kitayama 2011; Tsujii et al. 2017b). In addition, direct testing using field fertilization experiments suggests that P-use strategies in leaves differ among common tree species(Mayor et al. 2014; Yang 2018). However, most previous studies focused only a limited functional type (e.g. AM-associated or climax species only). Therefore, it is unclear whether nutrient-use strategies in leaves of tropical

tree species under P-depleted conditions differ among functional types.

Here, I present foliar nutrient concentrations, resorption proficiency and resorption efficiency in nine species with different mycorrhizal associations (ECM or AM) and successional status (climax or pioneer) using a 11-yr factorial N- and P- fertilized plots in lowland tropical rainforests in Sabah, Malaysia. I tested the following two hypotheses: (1) P fertilization decrease PRE of climax species, but not pioneer species, because climax species are more resource conservative and have higher leaf nutrient resorption efficiency than pioneer species. (2) AM species have greater PRE and decreased N resorption efficiency (NRE) in response to N addition, because N fertilization is more likely to alleviate N limitation and promote P limitation in AM-associated species reflecting the low N-acquisition capacity of AM fungi.

### 3.2. Materials and methods

*Study site and target species*

Study sites were located in Deramakot Forest Reserve and Tangkulap Forest Reserve in Sabah, Malaysian Borneo (5°14– 30′N, 117°11– 36′E) (Imai et al., 2009, 2010, 2012; Yokoyama et al., 2017). I selected nine evergreen broad-leaved species, representing a broad range of the taxa of the commonest woody species in my study sites (Table 1-S1). Details are described in Chapter 1, Section 1.5.

*Leaf sampling*

I selected a total of 115 trees of the nine target species within the experimental plots with diameters at breast height (dbh) ranging from 8.0 to 87.8 cm (Table 1-S1; green leaves: 9 species × 4 treatments × 3–6 individuals = 115 trees; fresh senescent leaves: 9 species × 4 treatments × 3–4 individuals = 111 trees). Fully expanded top canopy sun leaves were collected using a slingshot and a pole in September 2022 after annual nutrient applications had been applied 10 times (3 months after the last application). Fresh senescent leaves of the same trees were also sampled. Three to five 180 cm diameter litter traps of 0.25 mm mesh were set under the canopy of each target tree. Fresh litter was collected at 2-day intervals because relatively high potential solubility of P from fresh litter has been reported (Schreeg et al. 2013). To ensure that selected leaves were fresh, I visually examined if the abscission layers of petioles were still fresh.

*Chemical analyses*

All leaf samples were dried at 65 °C for 72 h and ground to powder for chemical analyses. The C and N concentrations of green and senescent leaves were determined by combustion coupled to gas chromatography using a N and C analyser (Sumigraph NC-220F, Sumika Chemical Analysis Service, Japan). To measure the P concentration, dried ground samples were digested with concentrated $HNO_3$ in an acid decomposition system in heat blocks (DigiPROBE, GL Science, Japan). Then, the P concentration of green and senescent leaves was determined by inductively coupled plasma optic emission spectrometry (ICPS-8100, Shimadzu Co., Japan).

Leaf N resorption efficiency (NRE) and leaf P resorption efficiency (PRE) were calculated as the proportion of N and P recovered from a senesced leaf (Killingbeck 1996; Tsujii et al. 2020). Mass loss during leaf senescence was corrected using a mass loss correction factor (MLCF; the ratio of senescent to green leaf mass, Vergutz et al. 2012). MLCF (=0.774) was calculated based on the data from tropical evergreen angiosperm in Vergutz et al. (2012).

$$NRE = \frac{N_{green} - (N_{senesced} \times MLCF)}{N_{green}} \times 100\ (\%),$$

$$PRE = \frac{P_{green} - (P_{senesced} \times MLCF)}{P_{green}} \times 100\ (\%),$$

where $N_{green}$ and $P_{green}$ were the N and P concentrations of green leaves, and $N_{senesced}$ and $P_{senesced}$ were the N and P concentration of senesced leaves.

*Statistical analysis*

The free statistical environment R 4.2.2 (R Core Team 2022) was used for all analyses. I used linear mixed models (LMMs) to assess the significant effects of treatment and functional types on the leaf N and P nutrient properties (leaf N and P concentrations, NRE, and PRE) using the lmer function in the lmerTest package (Kuznetsova et al. 2017). I treated a species as a random effect and N and P fertilizer treatments and functional types as fixed effects in the LMMs. The model equation was as follows: leaf N and P nutrient properties ~ treatment (control or P fertilization or N fertilization or N and P fertilization) + functional types (Climax-ECM or Climax-AM or Pioneer-AM) + interaction between treatment and functional types + random effect (species). I also used LMMs for each functional type to assess the significant effects of P and N fertilization on the leaf N and P nutrient properties. The model equation was as follows: leaf N and P nutrient properties ~ P fertilization (P fertilization or non- P fertilization) + N

fertilization (N fertilization or non- N fertilization) + interaction between P fertilization and N fertilization +random effect (species).

### 3.3. Results

*N and P concentrations in green leaves*

When the effects of fertilization and functional types were separately evaluated using the data for all species, the linear mixed models showed that green leaf N and P concentration (mg $g^{-1}$) differed among functional types and were higher in the pioneer species than in the climax species (Figure 3-1a–f, Table 3-S1). Moreover, Green leaf N/P in the pioneer species tend to be lower than in the climax species (Figure 3-1g–i, Table 3-S1).

Green leaf N concentration did not increase following N and unchanged following P fertilization across functional types (Table 3-S1), although the green leaf N concentration for the climax-ECM species marginally increased following N fertilization (Figure 3-1a). Green leaf P concentration for the climax-ECM species significantly increased following P fertilization, but not for climax- and pioneer- AM species (Figure 3-1d–f). Green leaf N/P ratios for climax- ECM and AM species decreased following P fertilization, but not for pioneer species (Figure 3-1g–i). It had no effects of N fertilization on green leaf P concentration and N/P ratios in all functional types (Figure 3-1d–i).

*N and P concentrations in senesced leaves*

When the effects of fertilization and functional types were separately evaluated using the data for all species, the linear mixed models showed that senesced leaf N and P concentration (mg $g^{-1}$) did not differ among functional types (Figure 3-2a–f, Table 3-S1). In the control plots, however, the median value of senesced leaf N concentration for the pioneer species was lower than the climax species (Figure 3-2a–c).

The N/P ratios of senesced leaves responded to P fertilization in a similar manner as green leaves, with parallel declines observed in all functional types. However, P concentration of senesced leaves increased with P fertilization not only in climax-ECM species, but also in climax-AM and Pioneer-AM species (Figure 3-2d–f). As a result, N/P ratios of senesced leaves decreased with P fertilization in all functional types (Figure 3-2g–i). In addition, for the climax-AM species, N fertilization decreased the P concentration and increased the N/P ratios in

senesced leaves (Figure 3-2e,h).

*NRE and PRE*

When the effects of fertilization and functional types were separately evaluated using the data for all species, the linear mixed models showed that NRE marginally differed among functional types, and that the median value of NRE of pioneer-AM species was more than 1.5 times higher than the climax species (Figure 3-3a–c, Table 3-S1). It had no significant effects of N or P fertilization (separately or in combination) on NRE for each functional type, or across functional types (Figure 3-3a–c, Table 3-S1).

When the effects of fertilization and functional types were separately evaluated using the data for all species, the linear mixed models showed that PRE in the control plots did not differ among functional types (Figure 3-3d–f, Table 3-S1). PRE decreased in the climax-ECM species following P fertilization (Figure 3-3d), while PRE marginally increased in the climax-AM species following N fertilization (Figure 3-3e). In contrast, it had no significant effects of N or P fertilization (separately or in combination) on PRE in the pioneer-AM species (Figure 3-3f).

**3.4. Discussion**

P fertilization increased P accumulation of canopy leaves for climax-ECM species and decreased foliar N/P ratios for climax- ECM and AM species (Figure 3-1d, g, h). Similarly, in response to the increase in P concentration of senesced leaves, N/P ratios of senesced leaves also decreased for the climax species (Figure 3-2d,e,g,h). An increase in leaf P and a decrease in leaf N:P ratio after P fertilization have also been reported at the species level (Mayor et al. 2014) and at the plot level (Wright et al. 2019; Ostertag 2010), suggesting that most climax species have leaves with low P concentration under the P-depleted condition. Moreover, in ECM-associated species, P fertilization decreased PRE (Figure3-3d). Previous studies have reported that tree species inhabiting P-poor environments increase PRE by improving the degradation of recalcitrant compounds (Hidaka and Kitayama 2011; Tsujii et al. 2017b). Similarly, this result suggests that ECM species maintain high PRE under P-depleted condition. In climax AM species, however, PRE did not change following P fertilization, and increased following N fertilization (Figure 3-3e). In general, nutrient resorption requires the decomposition of the organic P compounds in leaves via enzyme activities, and it is necessary to invest N in these enzymes. The resorption

processes of AM species may be limited by N due to the lower N-acquisition capacity of AM fungi than ECM fungi (Lambers et al. 2008).

By contrast, in pioneer species, P fertilization did not change the P concentration of the green leaves (Figure 3-1f,i). In addition, green leaf P concentrations of the pioneer species were higher than those of the climax species (Figure 3-1f,i, Table 3-S1). Moreover, PRE of the pioneer species did not change following N and/or P fertilization (Figure 3-3f). These results suggest that pioneer species, which have higher growth rates and luxurious resource-use strategies, may invest N and P in their leaves regardless of soil nutrient concentrations.

N fertilization did not increase green leaf N concentration regardless of functional types (Figure 3-1a–c). These results suggest that N concentrations in the green leaves of most tropical trees in my sites would already be at optimal levels because my site is a relatively N saturated condition (Yokoyama et al. 2017, Hirano et al. 2022). Moreover, N concentration of senesced leaves in the climax-ECM and pioneer species increased following N fertilization (Figure 3-2a,c). The climax-ECM and the pioneer species produce N-rich litter with extra-N by fertilization and have N-leaky strategies, while climax-AM species may not show such a response due to the low N availability via the AM fungi.

On the other hand, N resorption efficiency was higher in pioneer species than in climax species (Figure 3-3a-c). This result may reflect the high N demand of the pioneer species. Pioneer species, which have higher growth rates than climax species, have high wood productivity. On the other hand, the wood is composed by many structural proteins, and thus requires more N than P for wood growth. Therefore, pioneer species may invest N resorbed from senesced leaves in wood growth by increasing NRE in leaves. In addition, it is also possible that the large fraction of labile N fraction in the leaves of pioneer species allows them to maintain high NRE with relatively low cost.

My results suggest that ECM species, but not AM species, maintain high PRE in leaves under P-depleted conditions. The P resorption processes of climax-AM species may be limited by N due to the lower N acquisition capacity of AM fungi. My results also suggest that pioneer species have leaves with high NRE, reflect the high N demand of pioneer species. I suggest that successional status and mycorrhizal types regulate N and P use strategies of tropical trees, respectively. Understanding these mechanisms is important for comprehensive appreciation of nutrient cycling and ecosystem functions in lowland tropical rainforests where diverse species

coexist.

**Table 3-S1.** Statistical tests derived from linear mixed effect models (LMMs). Test statistics and *P* values were calculated by estimating the denominator degrees of freedom using Satterthwaite's method.

| Type of leaf trait | Variable | *F* value | *P* |
|---|---|---|---|
| Leaf N | Functional type | 7.75 | **< 0.05** |
| | Treatment | 1.58 | 0.2 |
| | Treatment: Functional type | 1.53 | 0.177 |
| Leaf P | Functional type | 6.38 | **<0.05** |
| | Treatment | 4.07 | **<0.05** |
| | Treatment: Functional type | 1.3 | 0.28 |
| Leaf N:P | Functional type | 5.18 | **<0.1** |
| | Treatment | 18.17 | **<0.001** |
| | Treatment: Functional type | 2.32 | **<0.05** |
| Leaf litter N | Functional type | 0.2 | 0.825 |
| | Treatment | 1.37 | 0.256 |
| | Treatment: Functional type | 2.78 | **<0.05** |
| Leaf litter P | Functional type | 0.04 | 0.962 |
| | Treatment | 15.79 | **<0.001** |
| | Treatment: Functional type | 2.23 | **<0.05** |
| Leaf litter N:P | Functional type | 1.52 | 0.293 |
| | Treatment | 13.81 | **<0.001** |
| | Treatment: Functional type | 2.34 | **<0.05** |
| NRE | Functional type | 4.34 | **<0.1** |
| | Treatment | 0.63 | 0.598 |
| | Treatment: Functional type | 0.42 | 0.415 |
| PRE | Functional type | 0.5 | 0.71 |
| | Treatment | 2.88 | **<0.05** |
| | Treatment: Functional type | 0.91 | 0.494 |

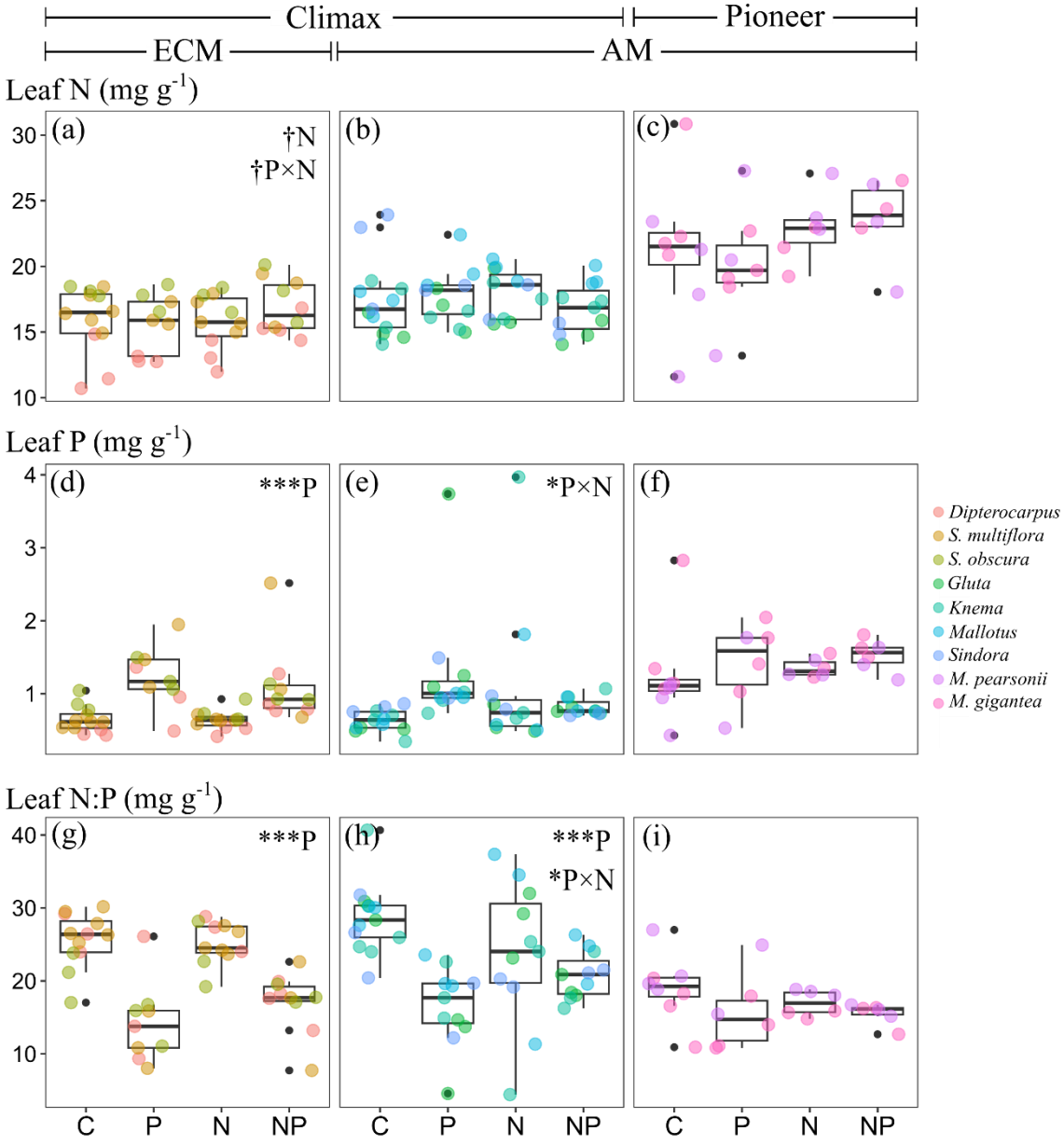


**Figure 3-1.** Canopy leaf N and P concentrations (a–f), and N/P ratios (g–i) in nine dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. ECM or AM indicate ectomycorrhizal or arbuscular mycorrhizal type, respectively. Letters (+N, +P, or N × P) indicate significant overall N and P effects or an N × P interaction effect, followed by linear mixed model *p* values. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

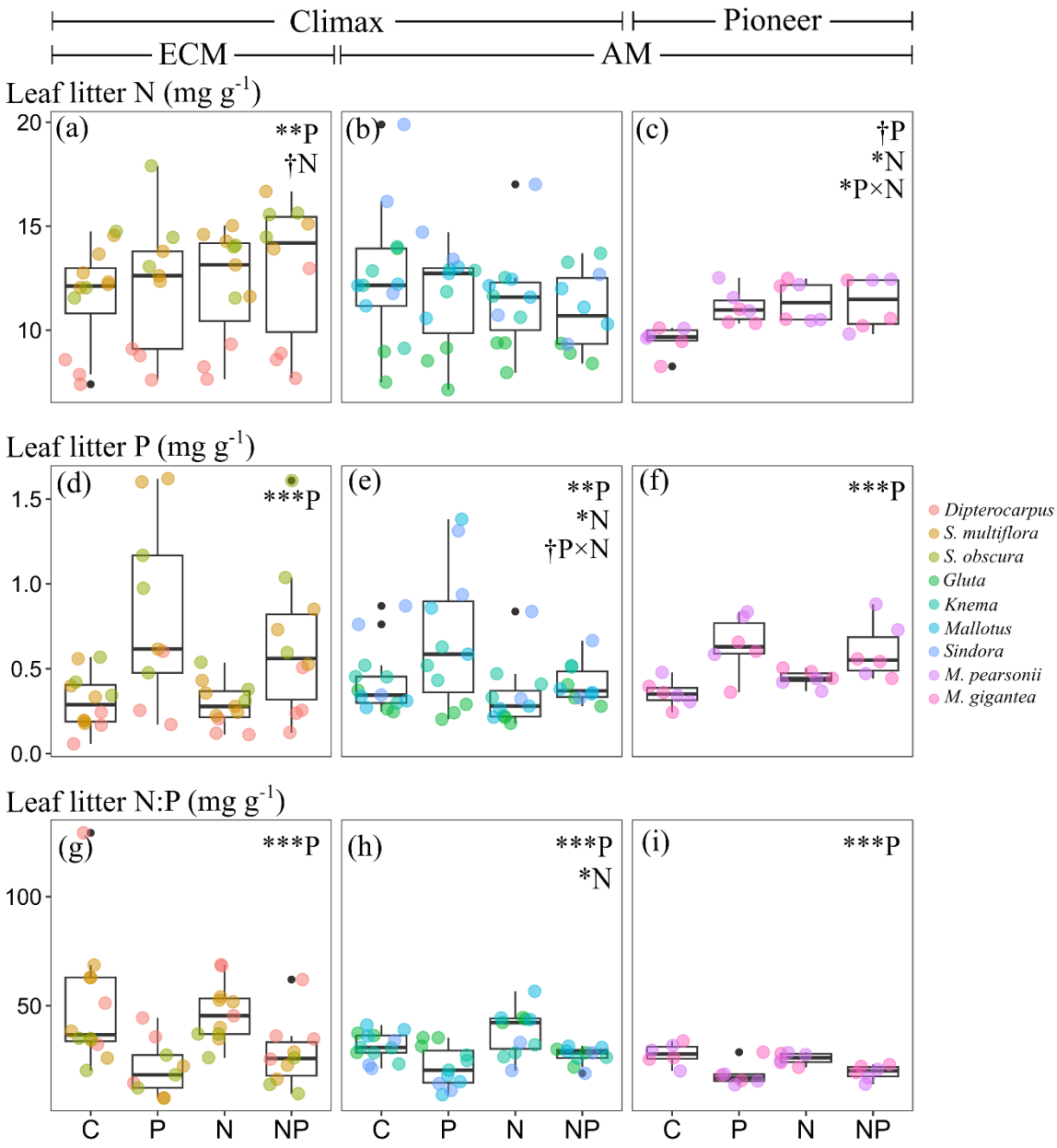


**Figure 3-2.** Senesced leaf litterfall N and P concentrations (i.e., resorption proficiency; a–f), and N/P ratios (g–i) in nine dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. The meanings of the abbreviations and letters are the same as in Figure 3-1.

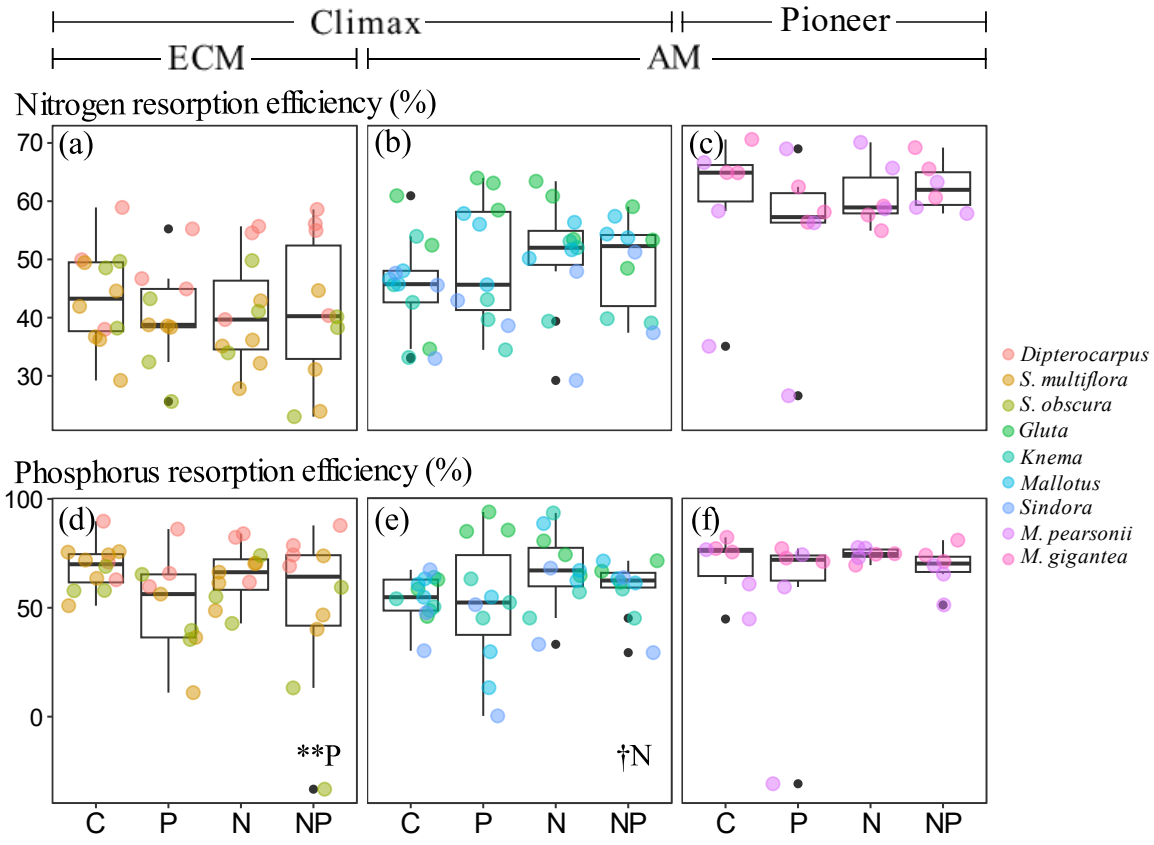


**Figure 3-3.** Nitrogen resorption efficiencies (a–c) and Phosphorus resorption efficiencies (d–f) in nine dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. The meanings of the abbreviations and letters are the same as in Figure 3-1.

## Chapter 4. Interspecific differences in the responses of root phosphatase activities to nitrogen and phosphorus fertilization in Borneo

### 4.1. Introduction

The niche theory predicts that species diversity advances through trade-offs that lead to the partitioning of limiting resources among species. This means that different species use unique strategies to acquire a resource in limited supply (Tilman 2004). It is well known that plant species may be specialized in their capacity to acquire different chemical forms of soil N at N-limited sites, such as in temperate and arctic ecosystems (McKane et al. 2002; Miller and Bowman 2002; Kahmen et al. 2006; Zhou et al. 2021). A similar phenomenon may occur with soil P. This is because P resources exist in various organic forms that require different types of enzymatic hydrolysis before they can be exploited by plants (Turner 2008; Liu et al. 2018).

Phosphorus, which is originally derived from bedrock, is thought to limit biological processes in lowland tropical rainforests because most soils are strongly weathered and the availability of soil P is impoverished (Vitousek 1984). Under such conditions, the form of available P may shift from inorganic P pools to hydrolyzed organic P pools (Imai et al. 2010; Kitayama 2013; Cabugao et al. 2021). There are various forms of organic P, which must be hydrolyzed to inorganic P by different classes of phosphatase enzymes before the P can be taken up by plants as a mixture of monoester P (including labile monoester P and phytate) and diester P. These organic P is the most abundant form in tropical soils (Turner and Engelbrecht 2011). Labile monoester P (e.g., glucose phosphate, mononucleotides) and phytate (salts of myo-inositol hexakisphosphate) are hydrolyzed by phosphomonoesterase (PME) and phytases (PhTs), respectively. Diester P (e.g., DNA and RNA) is hydrolyzed by both PME and phosphodiesterase (PDE), which is more costly to acquire than labile monoester P (Turner and Haygarth 2005). The acquisition of phytate probably incurs the highest metabolic cost among organic P compounds because phytate must be solubilized before its enzymatic hydrolysis, as it is robustly stabilized on mineral soil surfaces (Turner 2008). Plant species may increase their expression of different classes of phosphatases (PME, PDE, and PhT) to depend on these different forms of organic P under P-limited conditions. This may lead to resource partitioning for soil organic P among species (Turner 2008; Ahmad-Ramli et al. 2013; Yokoyama et al. 2017).

Acquisition strategies for soil organic P may differ among species of different

successional status and mycorrhizal types (Yokoyama et al. 2017). It is possible that climax species, which dominate primary forests, have a stronger ability than pioneer species to use organic P other than labile monoester P, reducing the competition for organic P (Huang et al. 2013). This is possibly because belowground competition for organic P by plants will be greater in the later stages of forest succession, with the accumulation of soil nutrients in wood biomass during this process and reduced cycling rates of limiting nutrients (Tang et al. 2011; Huang et al. 2013). Furthermore, trees that form associations with ECM fungi may depend more on soil organic P than AM-associated tree species (Phillips and Fahey 2006; Rosling et al. 2016). This is because ECM fungi have evolved from saprotrophs and are known to exploit not only inorganic P but also organic P via the exudation of phosphatase enzymes (Phillips & Fahey, 2006; Smith & Read, 2008), whereas AM fungi are thought to mainly increase the acquisition of inorganic P only (Dodd et al. 1987; Tarafdar and Marschner 1994; Joner et al. 2000). Turner (2008) specifically predicted that mycorrhizae are likely to play a central role in the partitioning of P by plants, and Liu et al. (2018) suggested that ECM-associated trees depend more on organic P sources, including recalcitrant phytate, than AM-associated trees. Phenomena such as P resource partitioning among plant species have been reported in temperate peatlands (Ahmad-Ramli et al. 2013), grasslands (Ceulemans et al., 2017; Phoenix et al., 2020), and tropical forests (Steidinger et al. 2015; Liu et al. 2018). However, most studies that have investigated P resource partitioning among species have used experiments with seedlings grown for very short periods. They have rarely examined phosphatase activities directly (e.g., Liu et al., 2018) or have focused on limited classes of phosphatases, and few have examined the effect of differences in successional status.

Yokoyama et al. (2017) measured three classes of fine-root phosphatase (PME, PhT, and PDE) using bulk soil/root samples from mixed species in the same factorial N and P-fertilized plots as I used in the present study. They found that P fertilization reduced the PME and PhT activities (but not PDE activity) at the stand level and suggested that most tree species do not depend greatly on diester P as a P source at the stand level in Bornean tropical rainforests. However, they did not examine interspecific differences in the response of root phosphatase activities to nutrient limitation. Therefore, the partitioning of soil organic P between species in lowland, species-rich, tropical rainforests remains unclear.

I measured the activities of three classes of phosphatase (PME, PDE, and PhT) of seven

mature tree species with different mycorrhizal associations (ECM or AM) and successional status (climax or pioneer) using factorial N and P-fertilized plots in Sabah, Malaysia. I tested the following three hypotheses: 1) P fertilization reduces the PDE and/or PhT activities in climax species but not in pioneer species, as climax species have a stronger capacity to use diester P and/or phytate to avoid competition for soil organic P in late successional stages; 2) P fertilization reduces the PhT activities of ECM-associated species but not AM-associated species because ECM-associated species depend more on recalcitrant organic P (phytate) than AM species (Liu et al. 2018); 3) N fertilization enhances the phosphatase activities in ECM species because these species may allocate excess N to the synthesis of extracellular phosphatases (proteins that have high N contents) to acquire P, which is reflected in the high phosphatase activity in ECM-associated roots (Treseder and Vitousek 2001; Phillips and Fahey 2006; Marklein and Houlton 2012).

## 4.2. Materials and methods

*Study site and target species*

Study sites were located in Deramakot Forest Reserve and Tangkulap Forest Reserve in Sabah, Malaysian Borneo (Imai et al. 2009a, 2010, 2012; Yokoyama et al. 2017; Hirano et al. 2022). I selected nine evergreen broad-leaved species, representing a broad range of the taxa of the commonest woody species in my study sites (Table 1-S1). Details are described in Chapter 1, Section 1.5.

*Fine-root sampling*

I selected a total of 86 trees of the seven target species within the experimental plots with diameters at breast height (dbh) ranging from 7.4 to 81.8 cm (Table S1). Fine root samples were collected from August to November 2019 after annual nutrient applications had been applied nine times (5 months after the last application). It is difficult to sample the fine roots of large adult trees in tropical rainforests because there are various root systems of many species in the soil (Aoki et al. 2012). Therefore, I sampled the fine roots of the target species from many saplings (0.3–1.5 m tall), prepared the root specimens (Figure 1-S2), and learned the colors, textures, sizes, wound exudates, and branching patterns of the roots and conducted several trials to sample the fine roots from large trees to understand the locations at which the fine roots attach

to the larger roots. Ultimately, I could precisely identify and sample the fine roots from large trees of the target species. I excavated the fine root systems (<2 mm diameter) of each tree using a manual shovel and then a trowel and skewer. The fine root systems were isolated carefully from the soil and organic matter to ensure that they were not damaged. The collected samples were immediately taken back to the laboratory and stored in a refrigerator at 4 °C until enzymatic analysis. All enzyme assays were conducted within 3 days of root sampling.

*Phosphatase analysis*

The root PME and PDE activities were measured using the method of Antibus, Sinsabaugh, and Linkins (1992), Treseder and Vitousek (2001), and McLachlan (1980), with some modifications. The measurement of root PhT activities was based on the modified method of George et al. (2008) and Yokoyama et al. (2017). I could verify the interspecific differences in phosphatase activities between ECM- and AM-associated species because this assay evaluates the activities of surface-bound and extracellular acid phosphatases associated directly with the plant roots and the fungal mantle (in ECM-associated plants, but not in AM-associated species) (Antibus et al. 1992; Steidinger et al. 2015).

To measure the root PME activities, the soil particles and leaf litter attached to the collected root samples were washed off briefly with tap water. Four to five root subsamples (40–100 mg fresh weight each, approximately first to fourth order roots), which contained root caps, were removed from the primary root sample and transferred into a 2 mL microtube (designated S1, S2, S3, [S4,] and P for each subsample, n = 3 or 4). I also prepared a 2 mL microtube without any root subsample as the control (designated C). Subsamples S1, S2, S3, P, and C received 1.75 mL of acetate buffer (pH 5.0) containing $NaN_3$. Subsamples S1, S2, S3, and C then received 0.25 mL of 40 mM *p*-nitrophenyl phosphate (pNPP) in acetate buffer as the substrate for the enzyme reactions, and subsample P received 0.25 mL of 5 mM *p*-nitrophenol (pNP). These subsamples were incubated in the dark at 25 °C for 30 min, and then 1 mL of the reaction solution was transferred to a 6 mL glass vial containing 0.5 mL of 0.5 M NaOH and 4.0 mL of pure water to stop the reaction and develop the color of the *p*-nitrophenol released as the product of the enzymatic reaction. The assay mixture was then shaken and the absorbance measured with a spectrophotometer at a wavelength of 410 nm (Tabatabai and Bremner 1969). The absorbance was compared with a standard curve of pNP and converted to the amount of pNP generated

during the incubation of the subsample.

The same procedure was used to measure the root PDE activity, but with a substrate solution of 40 mM bis-nitrophenyl phosphate (bis-NPP) in pure water.

To measure the root PhT activity, six root subsamples (40–100 mg each), which contained root caps, were removed from the primary root sample, and each was transferred into a 2 mL microtube (designated S1, S2, S3, C, P, and N, n = 3). I also prepared a 2 mL microtube with no root subsample (designated $B_1$ or $B_2$ for each subsample). All subsamples received 0.75 mL of acetate buffer (pH 5.0) containing $NaN_3$. Subsamples S1, S2, S3, and $B_1$ then received 0.25 mL of 8 mM phytic acid sodium salt in acetate buffer as the enzyme reaction substrate. Subsample C, N, and $B_2$ received 0.25 mL of acetate buffer. Subsample P received 0.25 mL of 10 mM $KH_2PO_4$. The subsamples were incubated in the dark at 25 °C for 14 h, and the enzymatic reaction was terminated by the addition of 0.06 mL of 100% trichloroacetic acid (TCA). Subsamples S1, S2, S3, P, N, and $B_1$ then received 0.94 mL of acetate buffer, whereas subsamples C and $B_2$ received 0.69 mL of acetate buffer and 0.25 mL of 8 mM phytic acid sodium salt in acetate buffer. Each assay mixture was then shaken, and 1 mL of the reaction solution was used to determine the concentration of orthophosphate using the molybdenum blue method. The absorbance at 712 nm was measured on a spectrophotometer (MP-1200, Erma Inc., Tokyo, Japan).

The activities of root PME and PDE were determined as:

$$\text{Activities} = \frac{S - C}{T \times P/Pb} \ (\mu\text{mol pNP } g^{-1}\ h^{-1})$$

The activities of root PhT were determined as:

$$\text{Activities} = \frac{S - C - B_1 + B_2}{T \times (P - N)/Pb} \ (\mu\text{mol } PO_4\ g^{-1}\ h^{-1})$$

S Mean amount of pNP or $PO_4$ in the solutions of subsamples S1, S2, and S3 after incubation per g dry weight of the subsample (μmol $g^{-1}$);

C Amount of pNP or $PO_4$ in the solution of subsample C after incubation per g dry weight of subsample (μmol $g^{-1}$);

$B_1$ Amount of $PO_4$ in the solution of subsample $B_1$ after incubation per g dry weight of subsample (μmol $g^{-1}$);

$B_2$ Amount of $PO_4$ in the solution of subsample $B_2$ after incubation per g dry weight of

subsample (μmol $g^{-1}$);

P Amount of pNP or $PO_4$ in subsample P after incubation per g dry weight of subsample (μmol $g^{-1}$);

N Amount of $PO_4$ in subsample N after incubation per g dry weight of subsample (μmol $g^{-1}$);

T Incubation time (h);

Pb Amount of pNP or $PO_4$ added to subsample P before incubation per g dry weight of subsample P (μmol $g^{-1}$).

*Statistical analysis*

The free statistical environment R was used (R Core Team 2021) for all analyses. I used linear mixed models (LMMs) to assess the significant effects of treatment and species on the classes of root phosphatase activities using the lmer function in the lmerTest package (Kuznetsova et al. 2017). I treated an individual tree as a random effect and N and P fertilizer treatments and species as fixed effects in the LMMs. The model equation was as follows: root phosphatase activity (PME, PDE, or PhT) ~ treatment (control or P fertilization or N fertilization or N and P fertilization) + species + interaction between treatment and species + random effect (individual tree). I also used LMMs for each species to assess the significant effects of P fertilization and N fertilization on the classes of root phosphatase activities. The model equation was: root phosphatase activity (PME, PDE, or PhT) ~ P fertilization (P fertilization or non-P fertilization) + N fertilization (N fertilization or non-N fertilization) + interaction between P fertilization and N fertilization + random effect (individual tree). Because I could not obtain adequate residual plots, PME activities were log-transformed before analysis. The interspecific differences in phosphatase activities in the control plots were also analyzed using LMMs. I treated individual trees as a random effect and species as a fixed effect. *Post hoc* tests were performed using the function glht in the multcomp package (Hothorn et al. 2008), using Tukey contrast for multiple comparisons to assess interspecific differences in the control plots.

## 4.3. Results

Root PME, PDE and PhT activities did not differ among the seven study species (Figure 4-1a–c). Phosphorus fertilization significantly reduced PME activity in all seven species (Figure 4-2a–

g). Similarly, fertilization tended to alter PhT activity, and the effect did not differ among species (Table 4-S1). P fertilization reduced the PhT activity more markedly in the four climax species (excluding *Knema*) than in the two pioneer species. Moreover, P fertilization reduced the PDE activity significantly only in *Knema* (Figure 4-2s), and the response of PDE activity to fertilization differed significantly among species (interaction between treatment and species for PDE: $P < 0.05$; Table 4-S1). Nitrogen fertilization had no significant effects on the PME or PhT (Figure 4-2a–n), but increased PDE activities in *Dipterocarpus* (an ECM-associated tree species) and *Knema* (an AM-associated tree species) (Figure 4-2p, s).

### 4.4. Discussion

*Responses of phosphatase activities to P fertilization*

Only a few studies have reported the root phosphatase activities of tropical trees. PME values in the control plots (50–210 μmol pNP $g^{-1}$ $h^{-1}$) were close to those of tropical tree species on Mount Kinabalu, Borneo, which ranged from 90 to 180 pNP $g^{-1}$ $h^{-1}$ (Kitayama 2013; Ushio et al. 2015). Most tropical tree species are essentially dependent on labile organic P, but their dependence on more-recalcitrant organic P differs according to their successional status. Fertilization with P significantly reduced the PME activity in all seven species (Figure 4-2a–g; Table 4-S1), indicating that a labile monoester P is important for most tropical tree species as a P source, as shown in previous P fertilization experiments (Treseder and Vitousek 2001; Zheng et al. 2015; Yokoyama et al. 2017). Phytase activity declined with P addition in four of five climax species (*Shorea*, *Dipterocarpus*, *Sindora*, and *Gluta*), but not in the two pioneer species (Figure 4-2h–n), although the response of PhT activity to fertilization did not statistically differ among species (Table 4-1). P fertilization also reduced the PDE activity in *Knema*, one of the five climax species (Figure 4-2s), but not in the two pioneer species (Figure 4-2m, n, t, u). Based on these results for PhT and PDE activities, recalcitrant organic P is more important for climax species as P sources rather than for pioneer species. Huang et al. (2013) reported higher PME activities in species that dominated in the later stages of forest succession rather than in those that dominated the early stages, and suggested that competition among plants for P is greater in later successional stages. Thus, in such P limited conditions, climax species may have a potential to avoid competition for P among species through depending on recalcitrant organic P other than labile monoester P (i.e., phytate and/or diester P), the acquisition of which incurs more metabolically high cost.

I detected no difference in the PhT activities of climax and pioneer species in the control plots (Figure 4-1b), and the climax species tended to depend more on phytate than did the pioneer species (Figure 4-2h–n). In contrast, Yokoyama et al. (2017) reported that the fine roots in secondary forests had higher PhT activity than those in primary forests, suggesting that the pioneer trees in secondary forests depend more on phytate than the climax trees in primary forests. These differences between the studies probably arise from the fact that even within pioneer species, different types of acquisition strategies for organic P could be used to acquire phytate. Typical pioneer species (including the two *Macaranga* species studied; Aoyagi, Imai, & Kitayama, 2013) would not mainly use phytate, whereas some pioneer species may have a strong capacity to acquire phytate via PhT activity. Future research should examine the variation in the P acquisition strategies of pioneer species in a larger number of species.

Fertilization with P reduced PhT activities not only in ECM-associated species (*Shorea* and *Dipterocarpus*) but also AM-associated species (*Sindora* and *Gluta*) (Figure 4-2h–k). This indicates that recalcitrant phytate is important for both ECM and some AM species as a P source. Previous studies have shown inconsistent findings on the dependence of AM tree species on recalcitrant organic P. For example, in an experiment in which seedlings were fertilized using different chemical forms of P, Liu et al. (2018) showed that ECM tree species exploited more-recalcitrant organic P (phytate) than AM tree species, indicating soil P partitioning between ECM and AM tree species. By contrast, Steidinger et al. (2015) reported that ECM and AM species exploit similar forms of organic P, and Moyersoen et al. (2001) suggested that AM tree species are actually distributed under conditions of high soil organic nutrients, as are ECM species. I have not directly compared the extracellular phosphatase activities of mycelia and could not evaluate the hyphal networks of AM fungi in this study. However, my study shows that AM species depend on recalcitrant organic P, like ECM species, which suggests that even AM species utilize organic P via phosphatases exudated directly by plant roots. This study covers only a few ECM and AM tree species out of the megadiverse tropical tree community. I nevertheless speculate that resource partitioning for soil organic P might not occur between ECM and AM trees in this target species studied in Malaysian Borneo.

*Responses of phosphatase activities to N fertilization*

Nitrogen fertilization had no significant effect on the PME or PhT activity in any of the seven

species (Figure 4-2a–n). This suggests that these species do not allocate excess N to the synthesis of PME or PhT. Because phosphatase proteins have relatively high N contents (8%–32%), an increase in phosphatase activity after N fertilization has been reported (Treseder and Vitousek 2001; Marklein and Houlton 2012; Zheng et al. 2015). However, some studies have shown that N fertilization does not increase phosphatase activity, even in tropical lowland forests on P-impoverished soils (Turner and Wright 2014; Yokoyama et al. 2017; Lugli et al. 2021). Specifically, Yokoyama et al. (2017) reported that N fertilization did not increase root PME, PhT, or PDE activities and concluded that these Bornean lowland forests are saturated with N. My results on species-specific root phosphatase activity also demonstrate that most tree species in the lowland tropical forest invest sufficient amounts of N in the synthesis of PME and/or PhT.

On the other hand, N fertilization increased PDE activity in *Dipterocarpus* (an ECM species) and *Knema* (an AM species; Figure 4-2p, s), suggesting that the two species allocate excess N to the synthesis of PDE to acquire soil P. In *Knema*, fertilization with P significantly reduced PDE activity, but not PhT activity (Figure 4-2s), indicating that *Knema* depends more strongly on diester P than the other species examined. To avoid competition with coexisting species, *Dipterocarpus* and *Knema* may invest more N in the synthesis of PDE (but not in PME or PhT) to acquire diester P, upon which the other tree species do not mainly use. In tropical forests, where diverse species coexist, several tree species may acquire different P resources to avoid competition among species (Turner 2008; Steidinger et al. 2015; Nasto et al. 2017; Liu et al. 2018).

*Knema* and *Dipterocarpus*, whose PDE activities increased following N fertilization, may have different P acquisition strategies from those of the other species. *Knema* in the control plots showed the highest root diameter and the lowest SRL (Chaper 5: Figure 5-4d). Furthermore, root morphological traits of *Knema* did not change after N and P fertilization (Chapter 5: Figure 5-4d,k,r). Therefore, *Knema* may invest N to acquire P by secreting phosphatases (PDE), rather than by extending its roots to scavenge soil P. P fertilization reduced the PME and PhT activities of *Dipterocarpus* (Figure 4-2b,i). Therefore, *Dipterocarpus*, an ECM species, may have the capacity to exploit all major soil organic P by secreting PME, PhT, and PDE. This may be reflected in the higher phosphatase enzyme activities in ECM-associated roots than in AM-associated roots (Phillips & Fahey, 2006). These mechanisms would explain why *Dipterocarpus* is one of the canopy-dominant species at this site. I suggest that these differences in organic P

acquisition strategies may also reflect the resource partitioning of soil organic P among tree species, and that organic P acquisition strategies are species-specific, but do not necessarily differ between ECM and AM species.

*Conclusions*

My results indicate that recalcitrant organic P (phytate and/or diester-P) is more important for climax species as P sources rather than for pioneer species in tropical rainforests. Moreover, some climax species acquire diester P by allocating excess N to the synthesis of PDE, independently of the mycorrhizal type. I conclude that resource partitioning for soil organic P, as in the conceptual model of Turner (2008), occurs in tropical rainforests and reduces the competition among coexisting tree species. This process may play a potentially important ecological role in promoting the coexistence of tree species in Bornean lowland, species-rich, tropical rainforests.

**Table 4-S1.** Statistical tests derived from linear mixed effect models (LMMs). Test statistics and *P* values were calculated by estimating the denominator degrees of freedom using Satterthwaite's method. "L" indicates that the data were log-transformed before analysis.

| Class of phosphatase | Variable | *F* value | *P* |
|---|---|---|---|
| $PME^{L}$ | treatment | 30.477 | **<0.001** |
| | species | 4.936 | **<0.001** |
| | treatment: species | 1.297 | 0.224 |
| PhT | treatment | 5.232 | **<0.01** |
| | species | 0.618 | 0.715 |
| | treatment: species | 0.651 | 0.843 |
| PDE | treatment | 3.585 | **<0.05** |
| | species | 0.17 | 0.984 |
| | treatment: species | 1.844 | **<0.05** |

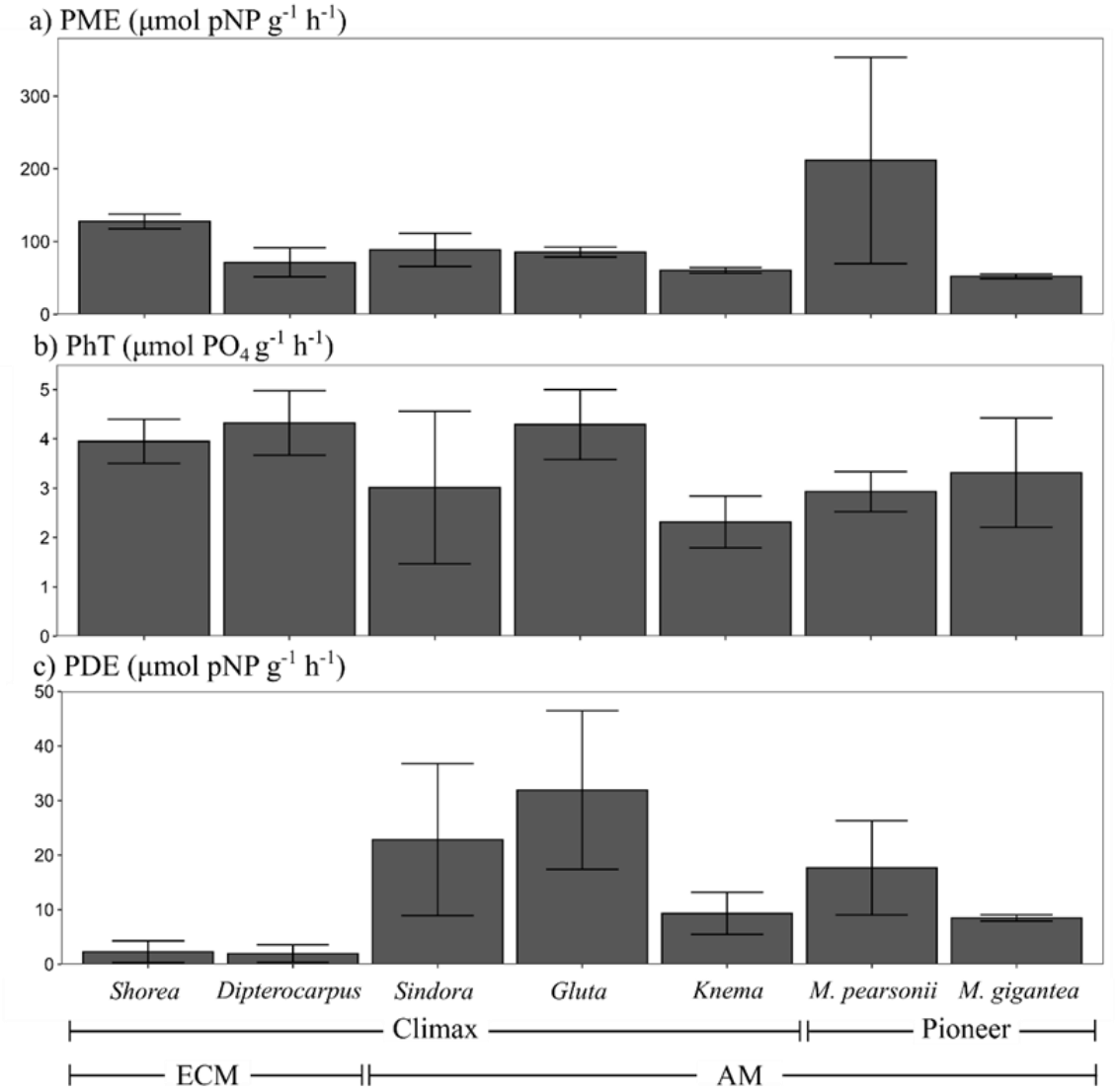


**Figure 4-1.** PME (a), PhT (b), and PDE activities (c) of the seven dominant tree species in the control plots in lowland tropical rainforest in Borneo. The definitions of the bars are the same as in Figure 4-1, and the meanings of the abbreviations and letters are the same as in Figure 3-1. There are no significant differences among species according to a post hoc Tukey test ($P < 0.05$).

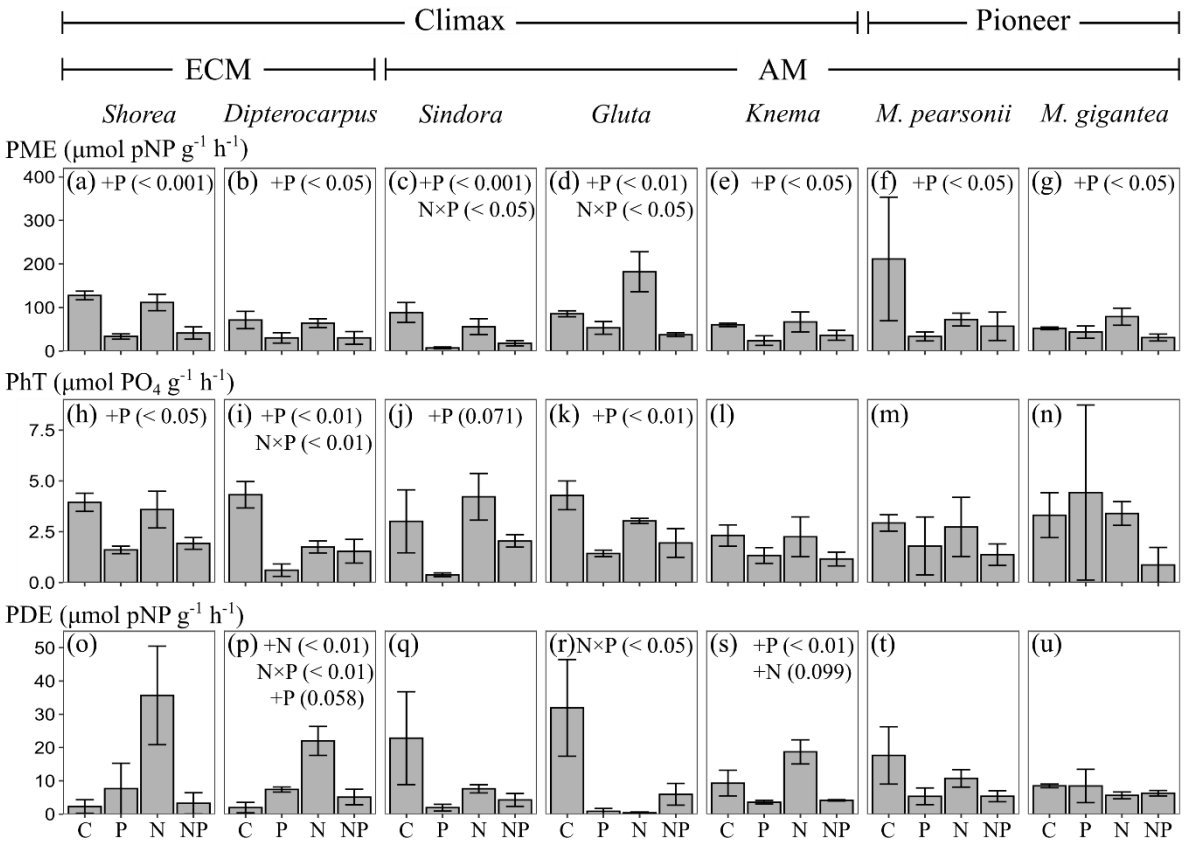


**Figure 4-2.** PME activity (a–g), PhT activity (h–n), and PDE activity (o–u) in seven dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. Bars represent geometric means ± SE. The meanings of the abbreviations and letters are the same as in Figure 3-1.

## Chapter 5. Interspecific differences in the responses of root exudation to nitrogen and phosphorus fertilization in Borneo

### 5.1. Introduction

The availability of soil P, which is largely derived from parent rock, reduces with long-term bedrock weathering and leaching progress in humid climates (Walker and Syers 1976). Especially in tropical rainforests, P is stabilized by incorporation into organic matter or adsorption onto aluminum (Al) and iron (Fe) oxides due to intense weathering (Tiessen and Moir 1993; Turner and others 2007). These processes may eventually limit NPP in tropical rainforests (Sollins 1998; Kitayama et al. 2000; Wardle et al. 2004). In Borneo, however, lowland tropical rainforests can maintain a huge amount of aboveground biomass and a high NPP even under P-depleted conditions (Kitayama et al. 2000; Kitayama 2005). In previous studies, several adaptive mechanisms to P-depleted conditions have been reported, including higher P use efficiencies in leaves (Kitayama et al. 2004; Mayor et al. 2014), acquiring P through mycorrhizal fungi (Lambers et al. 2008), fine root systems and root phosphatases degrading Po (Treseder and Vitousek 2001; Hirano et al. 2022). However, the importance of root exudation including organic acid exudation in P-limited tropical rainforests has not been verified (but see Aoki et al. 2012).

Root exudation contains soluble organic compounds and provides labile C to soil from root (Walker et al. 2003). These exudates contain amino acids, organic acids, sugars, phenolics and other secondary metabolites (Jones et al. 2009). For herbaceous plants, there have been extensive investigations on the amount and chemical composition of root exudates. By exudating a wide variety of compounds, plant roots adjust the soil microbial community in their immediate vicinity, accelerate beneficial symbioses, change the soil chemical and physical properties (Nardi et al. 2000). In natural forest ecosystems, tree roots may also regulate microbial activity and nutrient availability by exudating root exudates (Phillips et al. 2009, 2012). This rhizosphere priming effect potentially can regulate soil C storage, nutrient mineralization, and nutrient availability of plants (Kuzyakov et al. 2000; Cheng et al. 2014). Therefore, it is important to evaluate root exudation rates among sites with different soil nutrient concentrations to better understand the overall role of root exudates in nutrient acquisition.

Root exudation rates may differ among functional types with different mycorrhizal types and successional status. ECM mycelia have the ability to solubilize nutrients via organic

acids, whereas AM mycelia do not. Thus, ECM trees may have roots with higher root exudation rates than AM trees (Phillips and Fahey 2006). Nutrient acquisition strategies via root exudate rates may also differ between climax and pioneer species because root exudation rates may differ among species with different growth strategies (Jiang et al. 2023). However, while many studies have examined in situ root exudation rates in temperate regions (Phillips et al. 2009; Xiong et al. 2020; Ataka et al. 2020; Jiang et al. 2021), few studies have examined root exudates in tropical regions. Furthermore, although some studies have examined root exudation rates of tropical trees along natural soil P gradients (Aoki et al. 2012; Jiang et al. 2022), no studies so far examined responses of root exudation to P and N fertilization in lowland tropical rainforests on P-impoverished soils. Therefore, the importance of nutrient acquisition mechanisms via root exudates in P-deficient soils and the differences in root exudation rates among functional types remain unclear.

In addition to the physiological traits of roots, such as their root exudation rates, many studies have investigated the adaptive mechanisms of root morphological traits. In general, plant species that have fine roots with a high specific root length (SRL; root length per root weight) have a strong capacity to acquire nutrients (Aerts and Chapin 2000; Ostonen et al. 2007). Therefore, SRL or the specific root surface area (root surface area per root weight, an index that correlates positively with SRL) may increase as the availability of soil P decreases along a natural soil P gradient (Powers et al. 2005; Ushio et al. 2015; Cabugao et al. 2021). It is also suggested that root exudation rates often positively correlates with SRL or the specific root surface area, allowing the plant to take up soil nutrients efficiently, and that both SRL and root exudation rates increase tropical tree species under low-P conditions (Aoki et al. 2012; Ushio et al. 2015; Lugli et al. 2020). Although two fertilization studies have investigated the responses of root morphology to P fertilization at the plot scale (Wurzburger and Wright 2015; Lugli et al. 2021), no study has yet examined the interspecific differences in morphological root adaptations in response to nutrient fertilization or the relationships between morphological traits and root exudation rates in lowland tropical rainforests (but see Aoki et al. 2012).

Therefore, in this study, I investigated the response of root exudation rates and root chemical and morphological properties to N and P fertilization in seven tree species with different mycorrhizal types and successional status using factorial NP- fertilized plots in Sabah, Malaysia. The aim of this study are: (1) to test the significance of nutrient acquisition strategies

via root exudation of tropical trees; and (2) to identify the differences in root exudation rates between species with different mycorrhizal types and successional status in tropical lowland forests.

## 5.2. Materials and methods

*Study site and target species*

Study sites were located in Deramakot Forest Reserve and Tangkulap Forest Reserve in Sabah, Malaysian Borneo (Imai et al., 2009, 2010, 2012; Yokoyama et al., 2017). I selected seven evergreen broad-leaved species, representing a broad range of the taxa of the commonest woody species in my study sites (Table 1-S1). Details are described in Chapter 1, Section 1.5.

*Fine root sampling*

I selected a total of 82 trees of the seven target species within the experimental plots with diameters at breast height (dbh) ranging from 7.4 to 81.8 cm (Table 1-S1). Fine root and exudation samples were collected from October 2022 to 2022 after annual nutrient applications had been applied 11 times (4 months after the last application). All root systems were exposed under the canopy of target trees at a depth of 10 cm from the top of the soil including the organic layer. Details of species identification and sampling methods are described in Chapter 4, Section 4.2.

*Root exudation sampling*

I followed the protocol using filter paper exudation traps to sample root exudates in situ (Akatsuki and Makita 2020). I conducted measurements on daytime (10:00–15:00 h) when the rate of photosynthesis was presumed to be maximum. For each target species, one–four (mostly two) intact larger root system was gently exposed from the soil and remained attached to the mother root during the collection process. The root systems were then washed with pure water. The fine roots, which were 1-4 order root systems, were then sandwiched between two 25 mm diameter glass-fiber filters (GA-100, Advantec). To approximate the environment in the filters, 200 µl of a carbon-free nutrient solution (containing 0.5 mM $NH_4NO_3$, 0.1 mM $KH_2PO_4$, 0.2 mM $K_2SO_4$, 0.2 mM $MgSO_4$, and 0.3 mM $CaCl_2$; Phillips et al. 2008) was soaked into the filters

using a micropipette. The glass-fiber filters and aluminum foil used in this study were previously heated in a muffle furnace (FUL230FA, Advantec) at 450°C for 3 hours to remove organic matter. In addition, to prevent carbon contamination, the entire filter installation process was performed with tweezers and nitrile gloves. One control sample was prepared per individual tree. The entire root system was covered with an aluminum foil and a window shade sheet to prevent drying and incubated for 2 hours. Then, the absorbed filters were removed from the root and kept in zipper storage bags (UNI-PACK A-4, Seisannipponsya Ltd, Japan). The part of the root sample, which was sandwiched by filters, was excised for morphological and chemical analyses after exudation collection. Both root and filter samples were placed in an icebox and transported to the laboratory. A total of 530 filters and root systems (7 tree species × 4 treatments × 3 individuals × 7-3 (mostly 6) samples) were sampled. In addition, one control sample per individual was prepared. In the laboratory, the filter was freeze-dried for 72 h, cut into small pieces and wrapped in a capsule of tin foil. Then the total C amount from absorbed filters was analyzed by an NC analyzer (Delta V Advantage, Thermo Fisher J-Science Lab Co., Ltd, Japan).

*Root morphology and chemical analyses*

After the root exudation were measured, the root subsamples in which they were measured were washed briefly with tap water and placed in a laboratory dish. The laboratory dish was placed on a double-lamp bed scanner (GT-X970, Epson, Japan), and a digital image of the root subsample was taken. The image was used to calculate the mean root diameter (mm), total root length (cm), total surface area ($cm^2$), and total volume ($cm^3$) using ImageJ (http://rsbweb.nih.gov/ij/) and the macro program IJ_Rhizo (www.plant-image-analysis.org/ software/IJ_Rhizo), an image analysis system designed specifically for root measurement (Pierret et al. 2013). In this process, because the debris removal function of IJ_Rhizo was not working properly, I removed debris other than root systems of the target trees using the paint software Clip studio paint (https://www.clipstudio.net/ja/), and then performed the root morphological analyses using IJ_Rhizo. I used “Root_Dia_C” for mean root diameter, “Kimura_length” for root length, and “Eq_Vol” for root volume in the IJ_Rhizo system. These root diameter, total root length, and total root volume were estimated by assuming that the roots were cylindrical in structure. The root samples were then dried in an oven at 40 °C for 48 h, and the dry root weight was measured. SRL ($m\ g^{-1}$) and the root tissue density ($g\ cm^{-3}$) were calculated from the total length, root

volume, and dry mass of the samples. The dried root samples were then ground to a fine powder and analyzed for tissue N content (%) and C/N using the NC analyzer (Delta V Simple, Thermo Fisher J-Science Lab Co., Ltd, Japan). In total, the root morphology of 530 root samples (6–7 root subsamples × 2–5 individuals × 4 treatments × 7 species) and the root CN of 250 root samples (3-6 root subsamples × 2–5 individuals × 4 treatments × 7 species) were analyzed. Three root samples for CN analysis were selected for each species and fertilization zone, with three samples from each individual showing typical morphological values. The average values were taken as the value for an individual plant and used for downstream statistical analyses.

*Data analyses*

The free statistical environment R was used (R Core Team 2023) for all analyses. We used ANOVA models to assess the significant effects of treatment and species on the root exudation rates or the root chemistry or root morphology. We treated N and P fertilizer treatments and species as explanation variables in the ANOVA models. The model equation was as follows: root exudation or root chemical properties (N, P, and N/P ratio) or root morphological properties (root diameter, root tissue density, or SRL) ~ treatment (control or P fertilization or N fertilization or NP fertilization) + species + interaction between treatment and species. I also used linear mixed models (LMMs) for each species to assess the significant effects of P fertilization and N fertilization on the root exudation rates, the root chemistry, and the root morphology using the lmer function in the lmerTest package (Kuznetsova et al. 2017). The model equation used was: root exudation or root chemical properties (N) or root morphological properties (root diameter, root tissue density, or SRL) ~ P fertilization (P fertilization or non-P fertilization) + N fertilization (N fertilization or non-N fertilization) + interaction between P fertilization and N fertilization + random effect (individual tree). However, for root P content and N/P, linear models were used because the data structure did not allow to treat individuals as a random effect. The interspecific differences in root exudation rates in the control plots were also analyzed using LMMs. For this model, I treated individual trees as a random effect and species as a fixed effect. *Post hoc* tests were performed using the function glht in the multcomp package (Hothorn et al. 2008), using Tukey contrast for multiple comparisons to assess interspecific differences in the control plots. Moreover, the relationships between root exudation rates and root chemical/morphological properties were also analyzed using LMMs. For these models, I treated fertilization treatments

and species as a random effect and root chemical and morphological properties as a fixed effect.

Finally, I constructed a LMM and conducted a model selection analysis to identify the factors that influence the root exudation rates. In the LMM, I treated the root exudation rates as a response variable, fertilization treatments, root chemical properties, and root morphological properties as explanatory variables, and species as a random effect. To address multi-collinearity, I developed a correlation matrix for all coefficient estimates and eliminated any combinations of variables that correlated strongly (absolute value of correlation coefficients > 0.7; Dormann et al. 2013), leaving a total of seven explanatory variables (including fertilization treatments). I performed the model selection based on the corrected Akaike's information criterion (AICc) using the MuMIn package (Barton 2022) and assessed the significance by the *p*-values of the explanatory variables for the best model.

In general, it is known that root exudation rates are often strongly influenced by plant phylogeny (Sun et al. 2020; Williams et al. 2022). Phylogenetic trees were constructed for all species studied using v.phylomaker2 (Jin and Qian 2022), a package designed to generate phylogenies for vascular plants in R software (v.3.3.1; R Core Team, 2023). V.phylomaker2 uses the mega-tree derived primarily from GBOTB (GenBank taxa with backbone provided by Open Tree of Life v.9.1) for seed plants (Smith and Brown 2018) and Zanne et al.'s (2014) phylogeny for pteridophytes(Jin and Qian 2019). The phylogenetic tree across all species was then visualized with the annotation of functional type using the ggtree package (Yu et al. 2017) in R. Then, I calculated Blomberg's parametric K to evaluate the phylogenetic conservatism for each root trait using the picante R package (Blomberg et al. 2003; Münkemüller et al. 2012). A significant phylogenetic signal indicates that the trait is constrained by phylogeny, and a higher value of Blomberg's K indicates higher phylogenetic conservatism (Blomberg et al., 2003). However, because the target species group was within a relatively narrow phylogenetic distance, I conclude that it is difficult to discuss from this study whether the root exudation rates of a wide range of species groups are influenced by phylogeny in the lowland tropical rainforest (Text 5-S1, Table 5-S2, Figure 5-S2, S3).

In all statistical analyses, the variables were ln (x + 1)-transformed when necessary to meet the assumption of normality.

## 5.3. Results

*Root exudation rates*

In control plots, root exudation rates were high for *Gluta*, intermediate for ECM species (*Shorea* and *Dipterocarpus*), and low for the other AM species (Figure 5-1, 5-2a–c). P fertilization did not decrease the root exudation rates in most species (Figure 5-2). In one of the climax-AM species (*Gluta*), however, P or N fertilization decreased the root exudation rates (Figure 5-2c). Surprisingly, P fertilization increased the root exudation rates in AM species including one pioneer species (*Sindora* and *M. gigantea*) (Figure 5-2e,g). Similarly, N fertilization increased the root exudation rates in one pioneer-AM species (*M. pearsonii*) (Figure 5-2f).

*Root chemistry*

All chemical traits (root tissue N content, P content, and N/P) differed significantly among the species (Table 5-S1). Overall, root tissue N concentration did not respond to N and/or P fertilization (Figure 5-3a–g). In contrast, P fertilization increased root tissue P concentration in six species (Figure 5-3h–n) and decreased root tissue N/P ratios in all seven species (Figure 5-3h–u).

*Root morphology*

All morphological traits (SRL, root diameter, and root tissue density) differed significantly among the species (Table 5-S1). The responses of SRL to fertilization also differed significantly among the species (Table 5-S1).

P or N fertilization increased SRL significantly in the two pioneer species (*M. pearsonii* and *M. gigantea*; Figure 5-4f,g). In contrast, no significant effects of N or P fertilization (separately or in combination) were detected in the five climax species (Figure 5-4a–e). In general, the increase in SRL is caused by a reduction in root diameter and/or root tissue density (Ostonen et al., 2007). The root diameter decreased significantly in the two pioneer species, but the root tissue density was unchanged after N and/or P fertilization (Figure 5-4m,n).

*Relationship between root exudation rates and root chemical/morphological properties*

When all data including root chemical and morphological properties and fertilization effects were analysed together, the LMMs showed that only SRL significantly affected the variation in

root exudation rates across target species (Table 5-1 and 5-2). In the LMM for each root trait, root exudation rates significantly increased with increasing SRL and decreased with decreasing root diameter (Figure 5-5d,e). In contrast, the root chemical traits (tissue N, P concentration, or N/P ratios) did not correlate the root exudation rates across target species (Figure 5-5a–c).

### 5.4. Discussion

I used the glass–fiber filter method to investigate potential root exudation rates of intact fresh root segments of seven dominant species in lowland tropical rainforests, followed by Akatsuki and Makita (2020). Mass-based root exudation rates in control plots in my study ranged from 0.00 to 4.80 mg C $g^{-1}$ $h^{-1}$, compared with rates of 0.00 to 3.76 mg C $g^{-1}$ $h^{-1}$ in a temperate forest where Akatsuki and Makita (2020) studied using the same method. Thus, the exudation values in my study were comparable to the values in a cool temperate forest ecosystem (Akatsuki and Makita 2020). However, in studies using the hydroponic exudation trap approach with moist glass beads (Phillips et al. 2008), mass-based root exudation rates ranged from 0.006 to 0.01 mg C $g^{-1}$ $h^{-1}$ in tropical forests (Aoki et al. 2012) and from 0.007 to 0.3 mg C $g^{-1}$ $h^{-1}$ in temperate forest (Phillips et al. 2011; Brzostek et al. 2013; Yin et al. 2014; Abramoff and Finzi 2016; Sun et al. 2017; Tückmantel et al. 2017; Gougherty et al. 2018). The exudation values obtained in my study were similar to values in these studies using the hydroponic exudation trap approach in forest ecosystems, but my highest values were higher than those in other studies. This difference in root exudation rates may be due to the size of root samples measured (Akatsuki and Makita 2020). A large segment of the terminal root (1 or 2 mm average diameter with laterals) was measured in several studies (e.g., Phillips et al. 2011; Aoki et al. 2012; Yin et al. 2013, 2014; Sun et al. 2017), because it is technically difficult to evaluate the root exudation rates for extremely small-sized roots using an hydroponic exudation trap approach (Phillips et al. 2008). Thus, the estimation of exudation rate per unit dry mass using a large segment may be underestimated in the previous studies using the hydroponic exudation trap approach, because roots with smaller diameters and higher root exudation rates were not measured separately from roots with larger diameters and masses and lower root exudation rates. In contrast, I observed a large range of exudation rates of narrow-diameter roots at a small scale by 25-mm diameter glass filter. My approach allows the use of a small root segment of even the terminal root (<0.5 mm) (mean dry mass 0.01 g) for each measurement, is relatively easy to set up, and minimizes heterogeneity

among roots with different diameters. Consequently, I suggest that the exudation values in my measurements are reflected the high exudation rates from the smaller-diameter roots with first- and second-order branching. Further, the short collection period in this technique contributed to less microbial decomposition and sorption to the soil matrix. This technique can consider the effects of the size and diameter distribution of root samples and reduce the effect of incubation period on the exudation rate estimates.

Root exudation rates for *Gluta* and ECM species (*Shorea* and *Dipterocarpus*) were higher than the other AM species (Figure 5-2, 5-1). These three genera dominate in my study sites (Imai et al. in preparation). Aoki et al. (2012) have reported that root exudation rates of dominant species are higher at the P-poor site than the P-rich site in tropical montane forests. Thus, these results suggest that species groups with particularly high dominance in lowland tropical rainforests may acquire P and/or other nutrients more efficiently by having roots with higher root exudation rates than the other species. Especially in *Gluta*, one of the climax-AM species, N and P fertilization reduced root exudation rates (Figure 5-2b), suggesting that some climax species often maintain high root exudation rates for N and/or P acquisition.

Unexpectedly, P fertilization increased root exudation rates for a pioneer species (*M. gigantea*) and a climax-AM species (*Sindora*) (Figure 5-2e,g). These results suggest that the root exudation rate increased with the increased N demand in trees following P fertilization. In fact, P fertilization also decreased root tissue N/P (Figure 5-3), suggesting that P fertilization may promote N deficient of most tropical tree species. Moreover, P fertilization would increases the concentration of inorganic P in the soil at this site by fertilizing triple superphosphate (Lugli et al. 2021; Mori et al. 2023). Thus, it is possible that N deficiency is more pronounced in AM species than in ECM species due to the high uptake capacity of inorganic P through AM fungi. Furthermore, in addition to P availability, N availability is also often low in tropical regions, especially in secondary forests due to the removals of plant and/or wood resources out of the ecosystems (Davidson et al. 2007). Taken together, some AM species, including the pioneer species dominant in secondary forests, would have increased root exudate rates, reflecting increased N requirements following P fertilization and the relatively low N availability of secondary forests.

Similarly, N fertilization increased root exudation rates in one of the pioneer species (Figure 5-2f). This result suggest that the increased N availability may increase the activity of

some pioneer species, which results in increasing absolute C partitioning to the underground biomass (Ataka et al. 2020; Jiang et al. 2021). In contrast, no increase with fertilization was observed for other climax species (Figures 5-1a-d). This may be related to the higher capacity of ECM species to acquire organic P compounds via fungi and the slow growth rate and resource conservative strategy of the climax species. My results suggest that the combination of mycorrhizal types and successional status regulate nutrient acquisition via root exudates.

P fertilization increased root tissue P for most species (Figure 5-3h–n) and decreased root tissue N/P for all target species studied (Figure 5-3o-u). These results would reflect the higher nutrient availability in the soil solution after fertilization and a change in P source from primarily organic to inorganic P (Lugli et al. 2021). On the other hand, root tissue N did not change with fertilization for most species studied (Table 5-S1, Figure 5-3a–n). Similar results have been reported in a field fertilization experiment in the tropical Amazon (Lugli et al. 2021). These results suggest that the extra N added to these already N-rich soils was not taken up by plants and/or that N concentrations in the root could already be at their optimal levels, with N being retranslocated to other plant tissues (Wurzburger and Wright 2015; Lugli et al. 2021). Therefore, plants growing in this Bornean tropical rainforest strongly responded to the alleviation of rock-derived nutrient limitation, regardless of functional types.

The fertilization responses of root morphological traits differed among species with different successional status. Previous studies have reported that the fine root length/biomass and the specific root surface area (an index that correlates positively with SRL) increase as the availability of soil P decreases along a natural soil P gradient (Powers et al. 2005; Ushio et al. 2015). Therefore, it is possible that P fertilization reduces SRL in all the target species because plant species with high SRL may be better able to acquire nutrients (Aerts and Chapin 2000; Ostonen et al. 2007). Surprisingly, N and P fertilization increased SRL in the two pioneer species, but not in the climax species (Figure 5-4a–g). Moreover, the root diameter decreased in the pioneer species following N and/or P fertilization (Figure 5-4m,n), and the root tissue density of these species unchanged following fertilization (Figure 5-4t,u). Similarly, Wurzburger and Wright (2015) also demonstrated that the fertilization of N, P, and K together in tropical forests increased SRL at the stand level. Roots with high SRL and low root diameter are short lived and suited for rapid resource acquisition, but they incur the high costs associated with root tissue construction and maintenance (Aerts and Chapin 2000; Gill et al. 2002). Given the P and/or N

limitation in the early successional stages of tropical forests (Davidson et al. 2007) and resource-luxurious strategies of pioneer species (Reich 2014), the alleviation of nutrient limitation may cause the roots of pioneer species (but not those of climax species) to shift to the expression of functional traits that produce short-lived roots. Actually, it is known that the production of fine roots is faster in more fertile areas than in infertile areas (Doughty et al. 2014).

Root morphology may be a factor that affects root exudation rates rather than root chemistry in tropical rainforests. Aoki et al. (2012), who measured root exudation rates along a natural soil P gradient in a tropical montane forests, have also reported that root surface area and root exudation rates are positively correlated. Similarly, root exudation rates increased with increasing SRL in this study (Table 5-1, 5-2, Figure 5-5d), while decreased with decreasing root diameter (Figure 5-4e). Thinner roots would have higher physiological activities, leading to higher root exudation rates. However, there is no relationship between root exudation rates and root tissue N or P, or N/P (Table 5-1, 5-2, Figure 5-5a–c). Several studies reported that root tissue N is often positively correlated with root exudation rates (Sun et al. 2017; Akatsuki and Makita 2020). Further, Sun et al. (2017) reported a positive correlation of root exudation with root tissue N, as a result of enhanced fine root uptake of N mobilized by root exudation (Phillips et al. 2011; Yin et al. 2014). In the tropics, however, root tissue N concentrations may already be optimal as mentioned earlier, and thus may not correlate with root exudation rates. Moreover, because roots is a non-photosynthetic organ with the higher proportion of dead cells and the relative lower importance of P compared to leaves or other highly active organs (Aoyagi and Kitayama 2016), root tissue P would not correlate with root exudation rates.

My results indicate that dominant species maintain higher root exudation rates under P- and/or nutrient- depleted condition to acquire nutrients than the other species. Specifically, some climax-AM species may have roots with higher root exudation rates to acquire N and/or P. My results also suggest that a combination of different mycorrhizal types and successional status regulate the responses of root exudation rates to changes in nutrient availability. These results would be important for understanding belowground C allocation and adaptative mechanisms to P–limitation in tropical rain forests.

**Text 5-S1.**

A phylogenetic tree was built using GGTREE packages in R and V.PHYLOMAKER2, a package designed to generate phylogenies for vascular plants in R software (v4.3.1; R Core Team, 2023). Then, I calculated Blomberg's parametric K to evaluate the phylogenetic conservatism for each root trait using the picante R package (Blomberg et al., 2003; Münkemüller et al., 2012).

The phylogenetic tree for the target species studied was shown in Figure 5-S2, suggesting that the variations of root diameter, SRL, and root exudation rates exhibited a strong phylogenetic signal (Table 5-S2). However, when a conifer *Dacrydium gracilis* (Podocarpaceae, AM-associated tree), which commonly distributed in Malaysian Borneo, was included in the phylogenetic tree with the target species studied, I found that the phylogenetic distance between the target species and the conifers was far apart (Figure 5-S3), and that the above results on the phylogenetic signal were obtained within a relatively narrow phylogenetic distance. Therefore, I conclude that it is difficult to discuss from this study whether the root exudation rates of a wide range of species groups are influenced by phylogeny in the lowland tropical rainforest.

**Table 5-1.** Model selection for root exudation rates in lowland tropical rainforest in Borneo; only the top five models (1 to 5) are shown.

*Note*. Models were sorted by increasing values of second-order Akaike's information criterion (AICc, AIC corrected for small sample sizes) and Delta AIC, which represents the difference in AICc between the current and the most appropriate model.

| Model No. | Fixed term | AICc | Delta AICc |
|---|---|---|---|
| 1 | SRL | 21.0 | 0.00 |
| 2 | SRL, root tissue density | 22.9 | 1.88 |
| 3 | SRL, root tissue P | 25.2 | 4.17 |
| 4 | SRL, N fertilization | 26.8 | 5.79 |
| 5 | SRL, P fertilization | 26.9 | 5.90 |

**Table 5-2.** Statistical tests derived from the corrected Akaike's information criterion (AICc)-best model (model 1 in Table 5-1).

| Parameter | Estimate | SE | *t* value | *P* |
|---|---|---|---|---|
| (Intercept) | 0.238 | 0.061 | 3.92 | < **0.01** |
| SRL | 0.012 | 0.002 | 5.38 | < **0.001** |

**Table 5-S1.** Statistical tests derived from ANOVA models.

| Root traits | Variable | *F* value | *P* |
|---|---|---|---|
| Root exudation | treatment | 0.894 | 0.45 |
| | species | 6.061 | **<0.001** |
| | treatment: species | 2.749 | **<0.01** |
| N content | treatment | 0.345 | 0.793 |
| | species | 15.975 | **<0.001** |
| | treatment: species | 3.097 | **<0.001** |
| P content | treatment | 58.71 | **<0.001** |
| | species | 16.778 | **<0.001** |
| | treatment: species | 2.241 | **<0.05** |
| N/P | treatment | 58.958 | **<0.001** |
| | species | 6.458 | **<0.001** |
| | treatment: species | 0.986 | 0.489 |
| SRL | treatment | 4.966 | **<0.01** |
| | species | 171.214 | **<0.001** |
| | treatment: species | 2.274 | **<0.05** |
| Root diameter | treatment | 2.923 | **<0.05** |
| | species | 109.217 | **<0.001** |
| | treatment: species | 1.088 | 0.389 |
| Root tissue density | treatment | 0.633 | 0.597 |
| | species | 67.111 | **<0.001** |
| | treatment: species | 1.175 | 0.314 |

**Table 5-S2.** Blomberg's *K* values for eight root traits of seven dominant tree species in the control plots in lowland tropical rainforest in Borneo.

*Note*. The significance level was set at $P < 0.05$. Bold value denotes that the trait exhibited a significant phylogenetic signal.

| Root traits (abbreviations) | Blomberg's *K* | *P* |
|---|---|---|
| Diameter | 1.296 | **0.006** |
| Specific root length (SRL) | 0.777 | **0.02** |
| Specific root area (SRA) | 0.265 | 0.13 |
| Root tissue density (RTD) | 0.472 | 0.10 |
| Exudation | 1.021 | **0.02** |
| N concentration | 0.021 | 0.78 |
| P concentration | 0.105 | 0.31 |
| N/ P ratio | 0.040 | 0.67 |

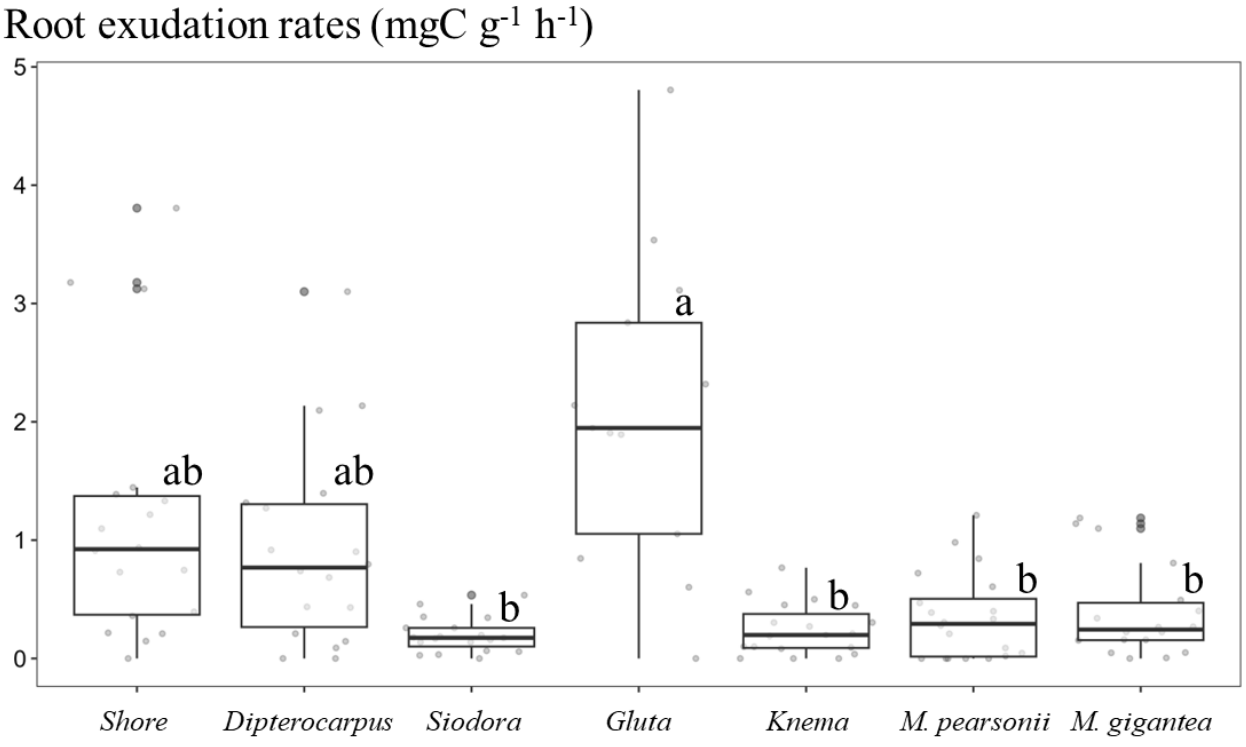


**Figure 5-1.** Root exudation rates of the seven dominant tree species in the control plots in lowland tropical rainforest in Borneo. Different letters in the panels indicate significant differences among species according to a post hoc Tukey test ($P < 0.05$).

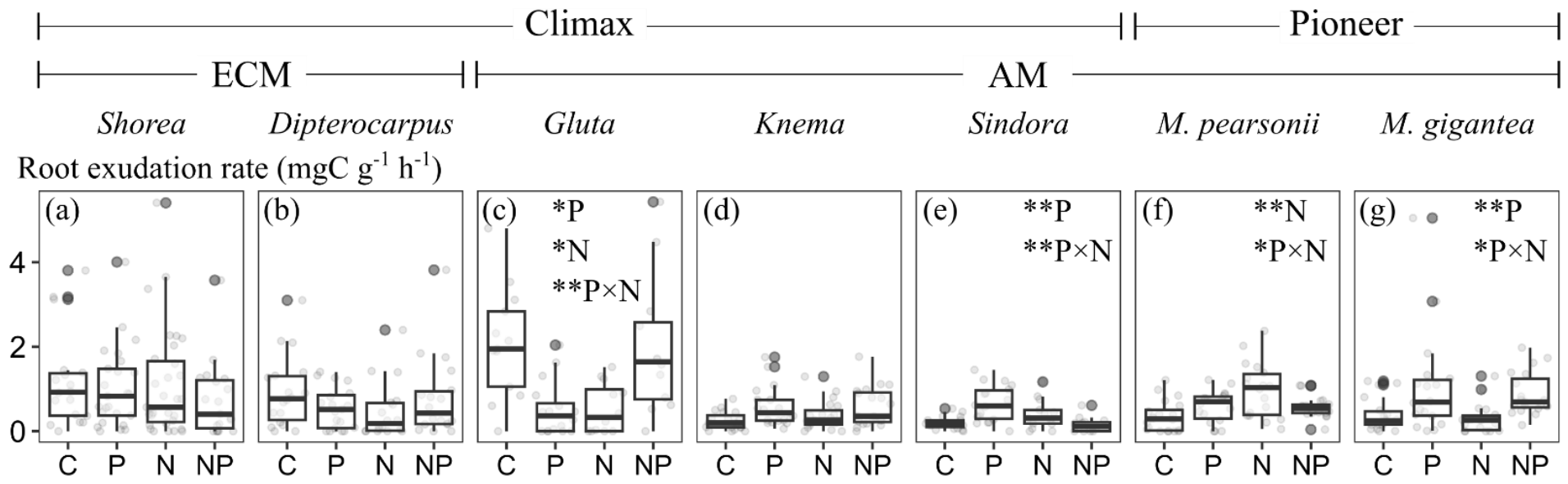


**Figure 5-2.** Root exudation rate (a–g) in seven dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. The meaning of abbreviations and letters are the same as in Figure 3-1

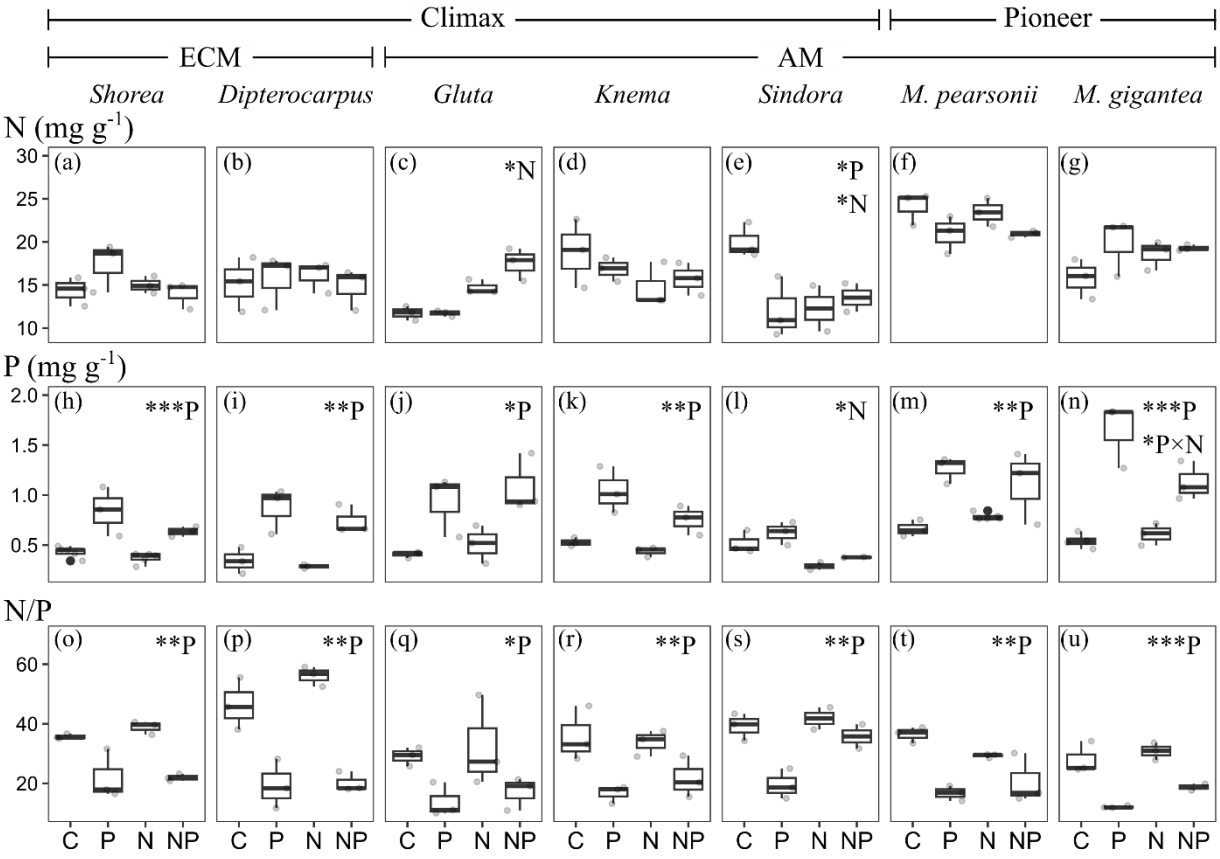


**Figure 5-3.** Root N content (a–g), P content (h–n), and N/P ratio (o–u) in seven dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. The meaning of abbreviations and letters are the same as in Figure 3-1.

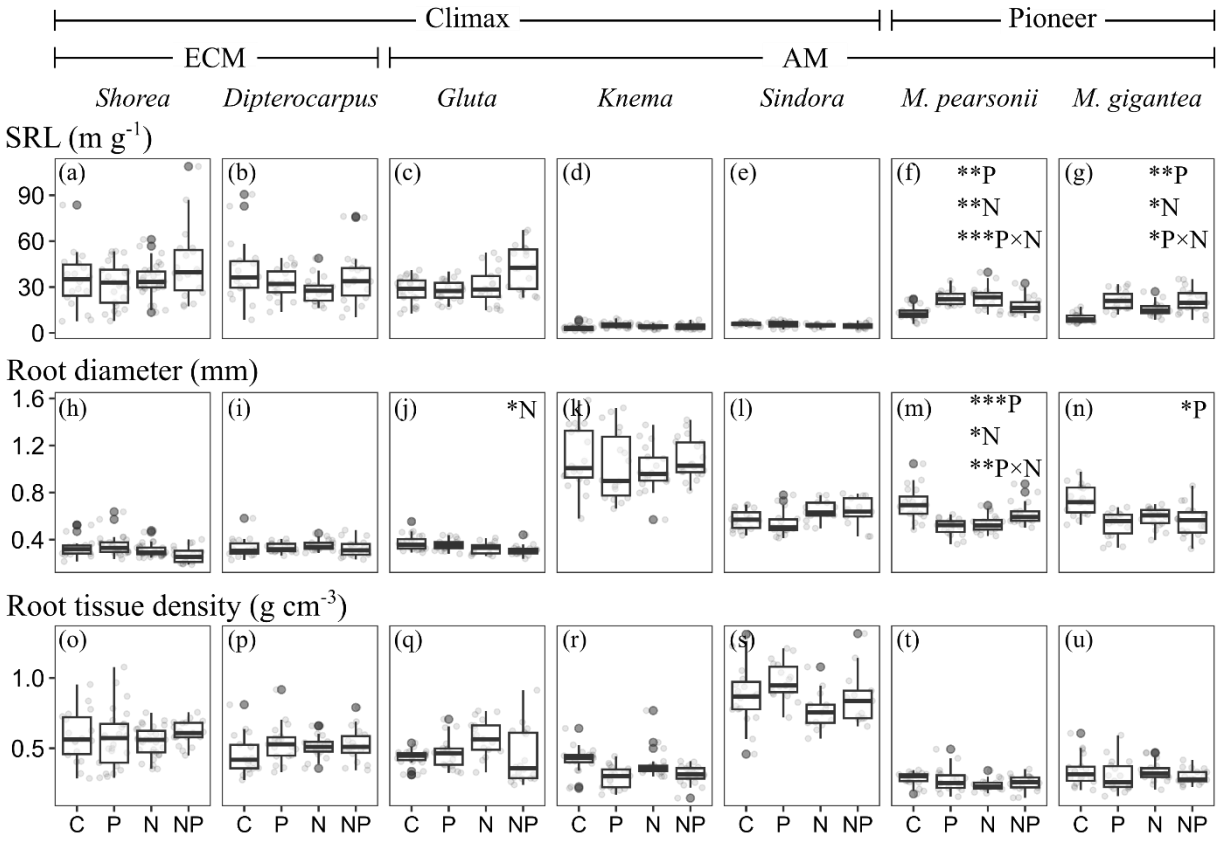


**Figure 5-4.** Specific root length (SRL) (a–g), root diameter (h–n), and root tissue density (o–u) in seven dominant tree species in the control, N, P, and N and P fertilization plots (C, N, P, and NP, respectively) in lowland tropical rainforest in Borneo. The meaning of abbreviations and letters are the same as in Figure 3-1.

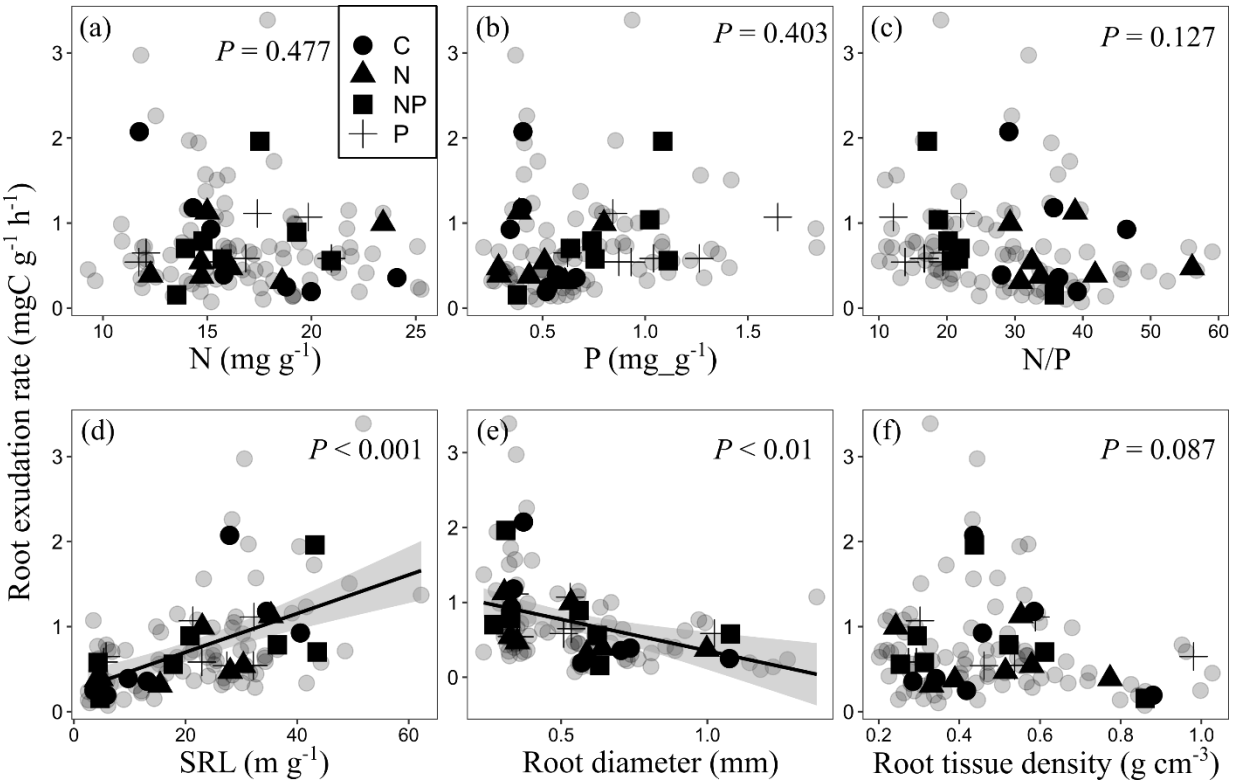


**Figure 5-5.** Relationships between root exudation rates and root chemical (N content; P content; and N/P, a–c) and morphological properties (specific root length (SRL); root diameter; and root tissue density, d–f) at the levels of individual trees and species. The meanings of gray circles and solid line are the same as in Figure 4-3. *P* values are results for root chemical or morphological properties in the linear mixed models.

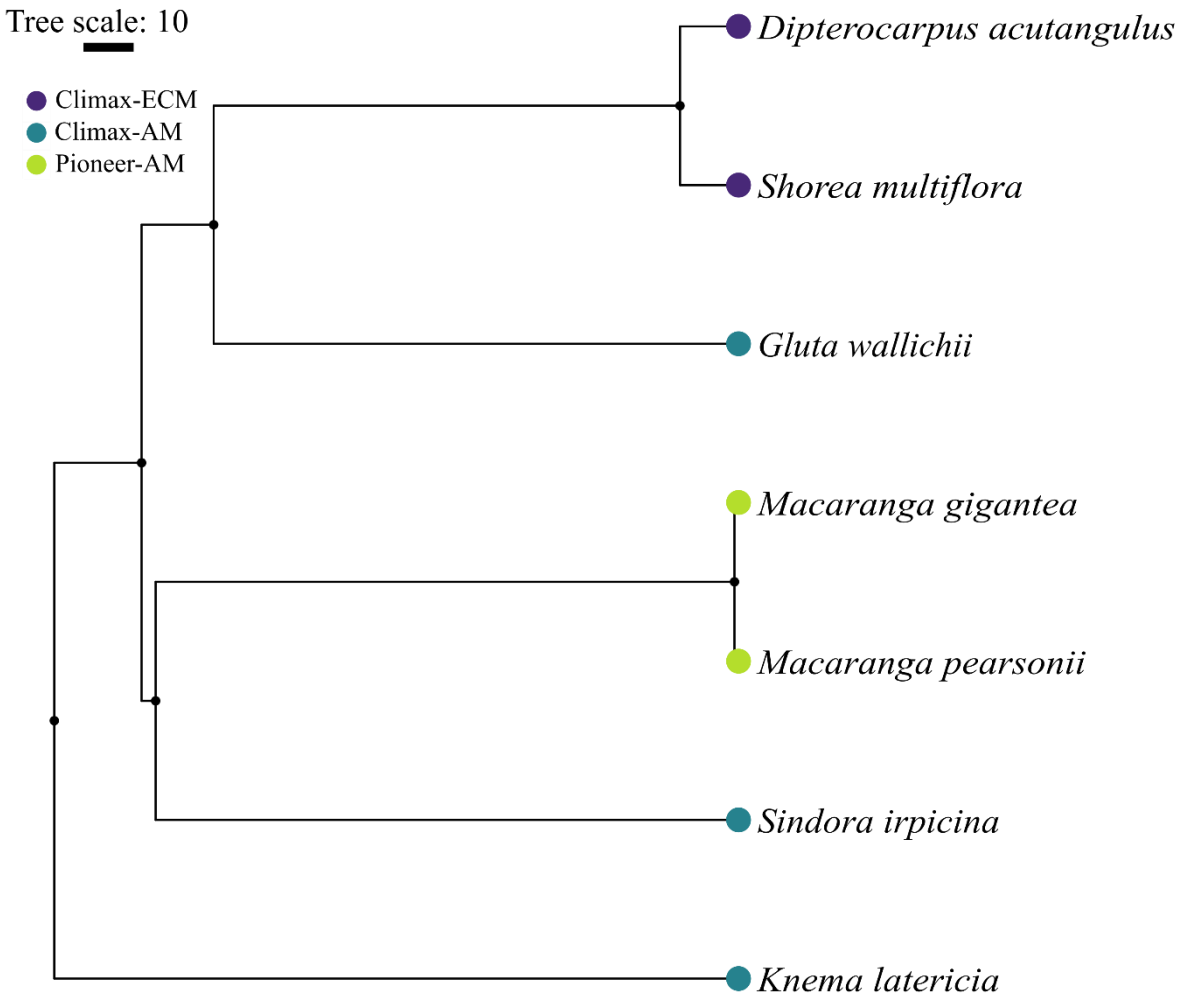


**Figure 5-S2.** The phylogenetic tree of seven target species in this study. The phylogenetic tree was built using GGTREE package in R and V.PHYLOMAKER2, a package designed to generate phylogenies for vascular plants in R software (v4.3.1; R Core Team, 2023). Circles at the tip of the phylogenetic branch denote functional type coloured in purple for climax-ECM species, in blue for climax-AM species, or in light green for pioneer-AM species.

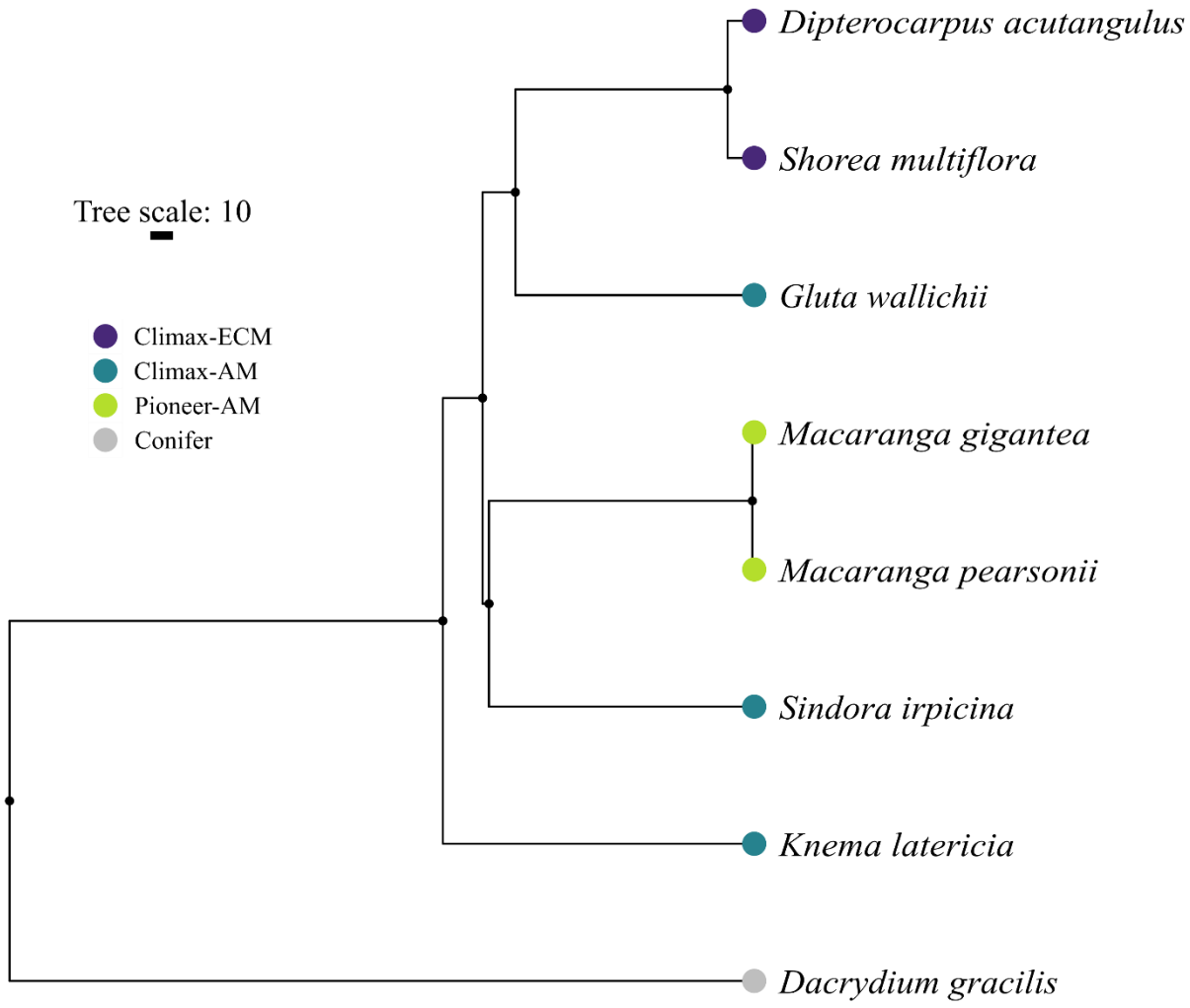


**Figure 5-S3.** The phylogenetic tree of seven target species in this study and a conifer *Dacrydium gracilis* (Podocarpaceae, AM-associated tree). The phylogenetic tree was built the same way as in Figure 5-S2. The meaning of circles are also the same as in Figure 5-S2.

## Chapter6. General discussion

In this study, I have discussed the differences in P-use and -acquisition strategies among functional types and/or species. First, I showed that climax-ECM species use P efficiently by increasing PRE in leaves under P-depleted condition, but climax- and pioneer-AM species do not (Chapter 3). In addition, pioneer species would have higher NRE in leaves than climax species probably because of the high N demand reflecting high growth rates of pioneers (Chapter 3). In P-depleted conditions, climax species increase PhT and/or PDE to acquire phytate and diester-P, but pioneer species do not (Chapter 4). Furthermore, highly dominant-climax species including climax ECM species may acquire N, P, and other nutrients via root exudates, while other species may not (Chapter 5).

On the other hand, there was no relationship between P -use trait (leaf PRE) and -acquisition traits (root phosphatase activity or root exudation rates) (Figure 6-1a–d). Ushio et al. (2015) reported a negative correlation between leaf P concentration and root phosphatase activity in tropical montane forests, suggesting that species with lower PRE in leaves have higher root phosphatase activities. However, as mentioned above, in the lowland tropical rainforests, each species and functional type has a variety of strategies, which may result in a lack of correlation between P-use and -acquisition traits across functional types.

The overall nutrients -use and -acquisition strategies also differed among functional types. To examine the overall P fertilization response of P-use and -acquisition traits, I subjected a total of 6 functional traits, including NRE, PRE, PME, PDE, PhT, and root exudation rates, to PCA analysis and showed the results in Figure 6-2. First, in climax-ECM species, overall traits changed along the positive direction of the PCA2 axis following P fertilization (Figure 6-2b). In addition, the PCA2 axis of climax-ECM species negatively correlated with PRE (Figure 6-2a). These results suggest that climax-ECM species prominently increase PRE under P-depleted conditions. Second, in climax-AM species, overall traits changed along the negative direction of the PCA2 axis following P fertilization (Figure 6-2d). In addition, the PCA2 axis of climax-AM species negatively correlated with PhT and PDE (Figure 6-2c). These results suggest that climax-AM species prominently increase PhT and/or PDE under P-depleted conditions. Finally, in pioneer-AM species, overall traits changed along the positive direction of the PCA1 axis

following P fertilization (Figure 6-2f). However, no traits correlated with the PCA1 axis of pioneer-AM species (Figure 6-2e), suggesting that there are no prominent changes of overall traits of pioneer-AM species under P-depleted conditions.

The results of the PCA analysis and the previous chapters suggest that nutrient-use and -acquisition strategies differ among functional types. The overall results of this study are shown in Figure 6-3. Climax-ECM species would have an efficient P-use and -acquisition strategy, primarily with high PRE, but also with P and nutrient acquisition via root phosphatase and exudation (Figure 6-3a). Climax-AM species would have an efficient P-acquisition strategy, primarily with high activities of phosphatases (i.e., PDE, PhT), but also with the high root exudation rates in some dominant species (Figure 6-3b). Pioneer-AM species would have an effective N-use strategies, primarily with high NRE in the leaves, to maintain their high wood growth rates (Figure 6-3c). Such different nutrient-use and -acquisition strategies among functional groups would promote the niche partitioning among species and/or functional types. Understanding these mechanisms is critical for a comprehensive appreciation of the mechanisms of P-limitation, multispecies coexistence, and biomass maintenance in tropical rainforests.

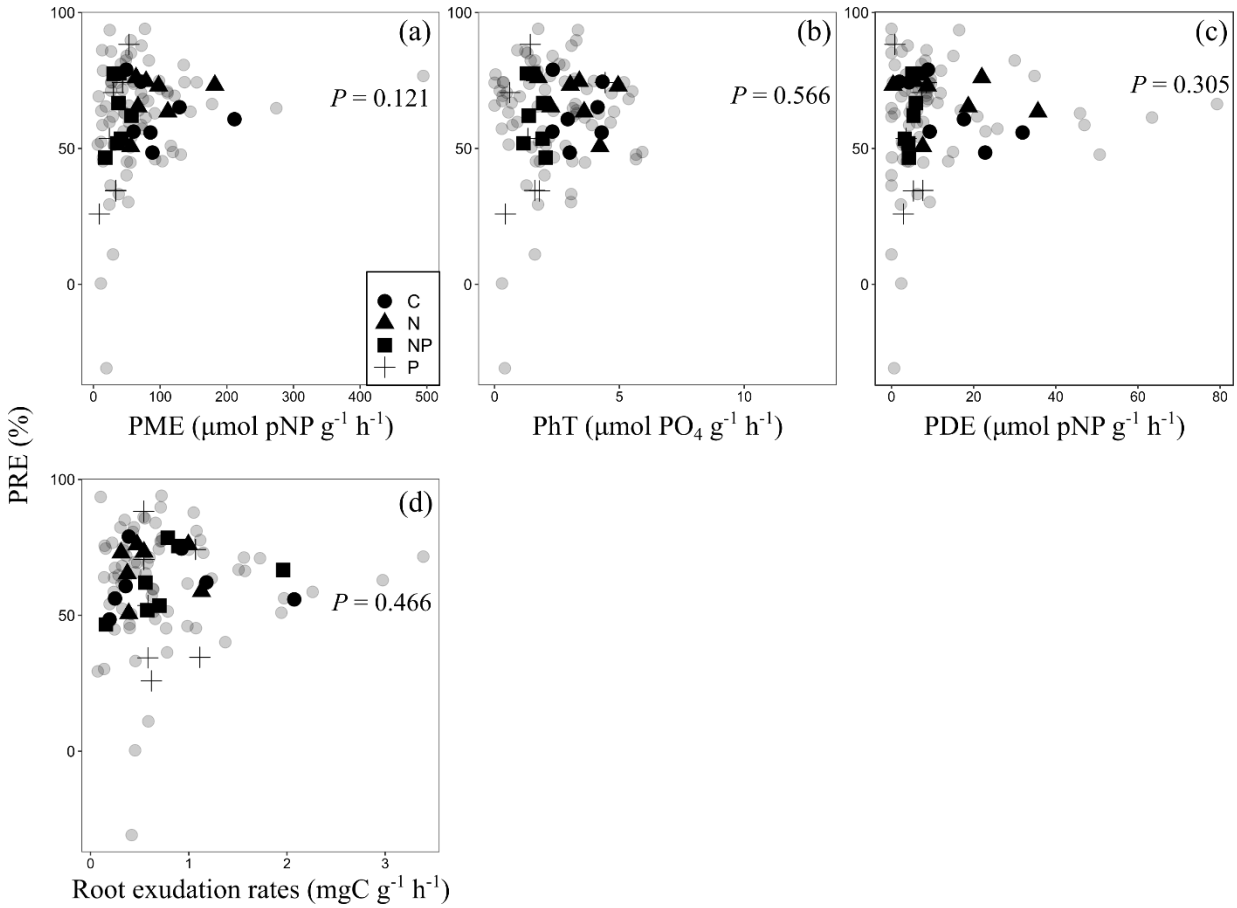


**Figure 6-1.** Relationships between phosphorus resorption efficiency and root physiological properties (PME activities, PDE activities, PhT activities, and root exudation rates) at the levels of individual trees and species. Gray circles indicate values at the level of individuals. Solid line indicates the regression line estimated using linear model analysis. *P* values are results in the linear mixed models treated species and treatments as random effects.

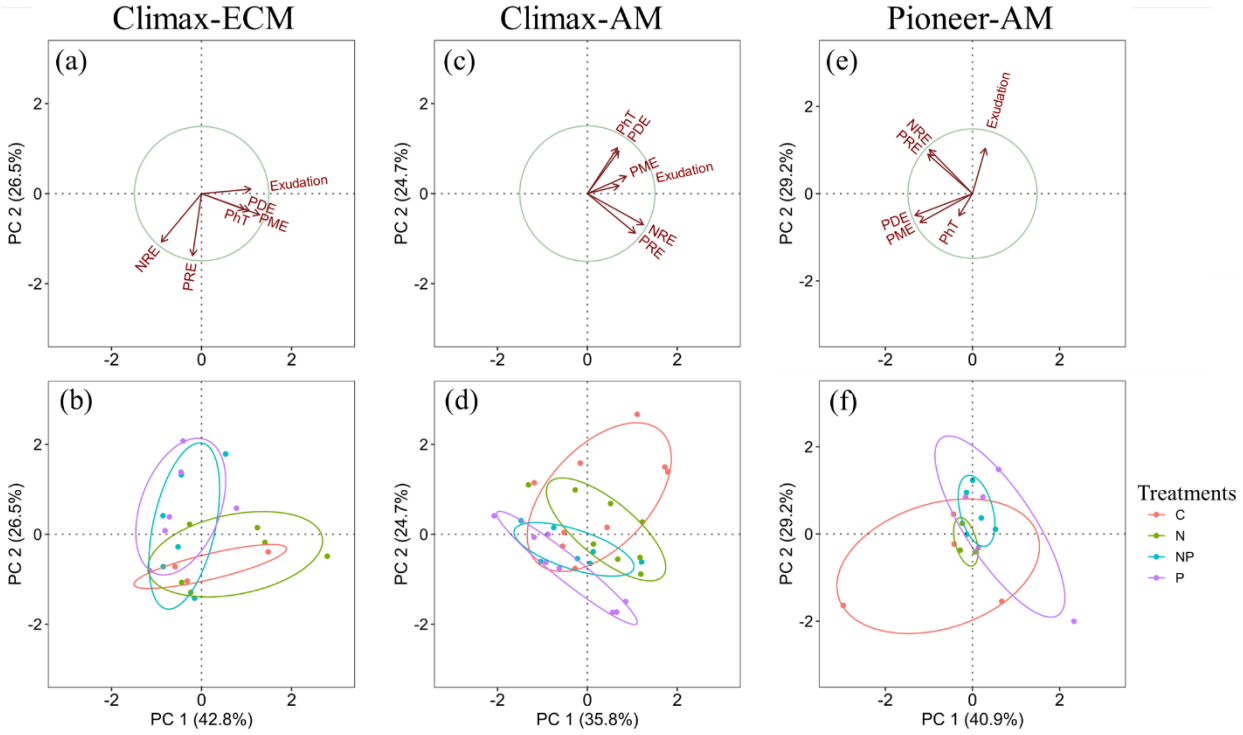


**Figure 6-2.** Principal components analysis (PCA) of nutrient-use and -acquisition traits (leaf nutrient use efficiency (PRE and NRE), phosphatase activities (PME, PhT, and PDE activities), root exudation rates) of trees for each functional types on an individual tree level. (a, c, e) Load of functional traits on two axes. (b, d, e) Individual distribution on the first two dimensions. Ellipses represent the 95% confidence interval of four treatment groups (control, N, P, and N and P fertilization plots) on the two dimensions.

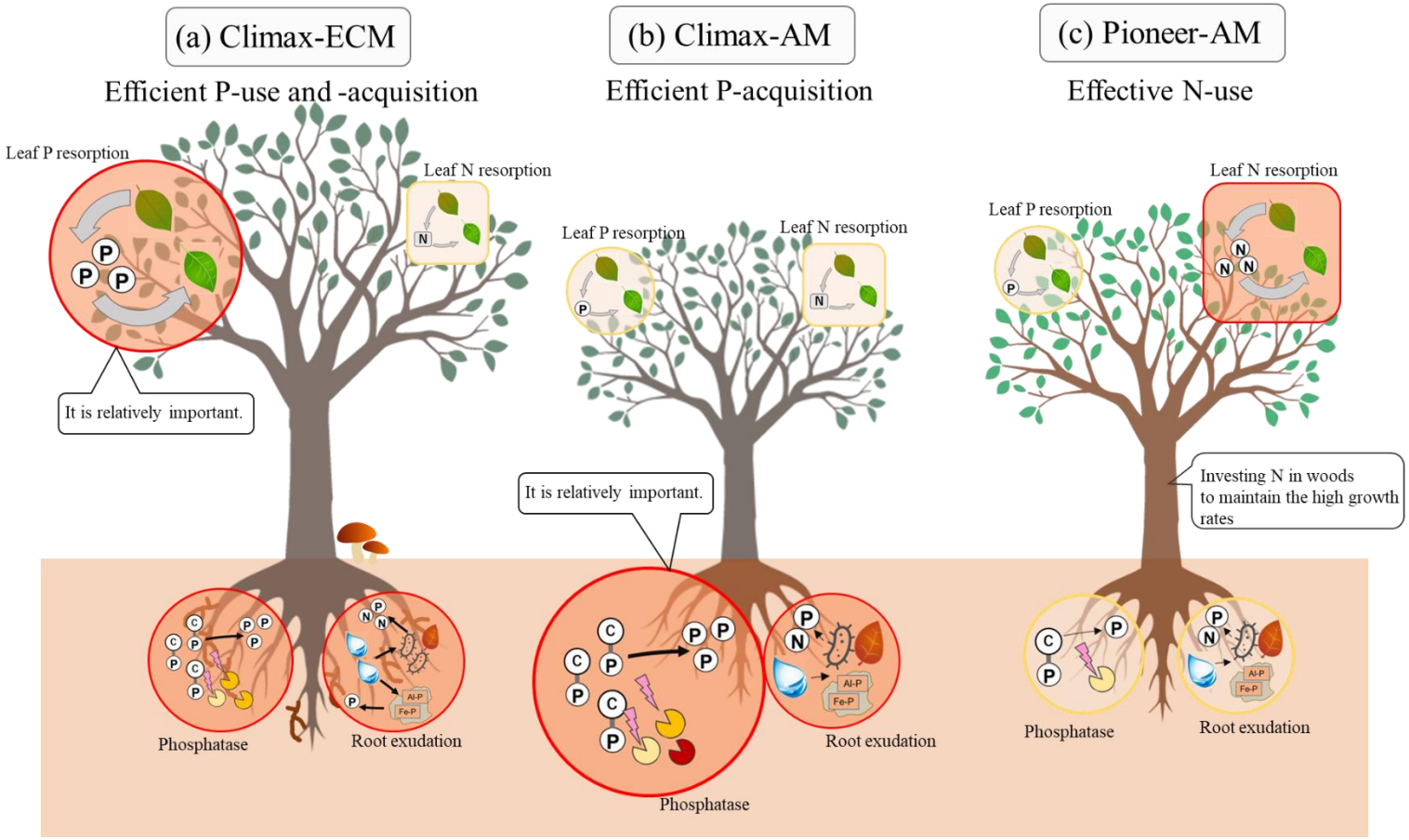


**Figure 6-3.** Illustration of the nutrient-use and -acquisition strategies of trees for each functional types (a: Climax-ECM; b: Climax-AM; and c: Pioneer-AM) in this study. Leaf P or N resorption is an indicator for plant P or N use; P and N are remobilized from senescing leaves before they fall and is reused in sink organs. Phosphatases (yellow or brown) are enzymes that degrading Po. Root exudation (blue waterdrop), including low-molecular-weight organic acids, can increase the availability of major soil nutrients (e.g., N and P) via enhancing soil microbial activity and mobilizing nutrients.

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**Summary**

In tropical rainforests, soil total phosphorus (P) is impoverished due to intense weathering. Despite this, tropical lowland rainforests maintain huge biomass, reaching 50-60 m in height. Tropical trees may maintain the productivity by increasing leaf P resorption efficiency and P acquisition capacity via root exudates and phosphatases. On the other hand, such nutrient-use and -acquisition strategies may differ among functional groups with different mycorrhizal types (arbuscular mycorrhizal (AM) or ectomycorrhizal (ECM) species) and successional status (climax or pioneer species).

In Chapter 1, I summarized previous knowledges on P-use and -acquisition strategies and reviewed studies using field fertilization experiments in tropical rainforests. I found that field fertilization experiment sites are unevenly distributed in the Neotropics, where AM species dominate, and that few studies have examined root traits in tropical forests at the species level. For this reason, there remains a complete lack of studies examining P-use and -acquisition strategies among functional types with different mycorrhizal types and successional status. Therefore, I decided to examine P-use and -acquisition strategies among functional types by examining the responses of P-use and -acquisition traits to N and P fertilization of three functional types, climax-ECM, climax-AM, and pioneer-AM species, in field fertilization experimental plots in Malaysian Borneo.

In Chapter 2, I examined the relationship between leaf traits, including leaf nutrient concentration, and leaf herbivory. Leaf herbivory is important for understanding carbon and nutrient cycling in forest ecosystems and diverts resources from the detrital pathway to the grazing pathway, but its determinants remain unclear. Therefore, I investigated the relationship between leaf traits and leaf herbivory rates in a mangrove forest, which is suitable for studying ecological characteristics at the species level along an environmental gradient. I suggest that the concentration of condensed tannins in the leaves moderately drive the leaf herbivory rates and are one of the factors driving the nutrient cycling in this mangrove forest ecosystem.

In Chapter 3, I examined the response of N and P resorption efficiency (NRE and PRE, respectively) in leaves to N and P fertilization in Bornean tropical rainforests. NRE and PRE were calculated by examining nutrient concentrations in sun leaves and senescent leaves of mature trees. The results indicate that climax-ECM species enhance leaf PRE under P deficiency to maintain their productivity, while climax-AM species and pioneer-AM species do not.

Moreover, pioneer species may have higher NRE in leaves than climax species, probably due to their high wood growth rates and high N demand.

In Chapter 4, I examined the response of fine root phosphatase activities to N and P fertilization. Strategies of the acquisition for soil organic P resources with different degradation characteristics (monoester P, diester P, and phytate) were examined by measuring three classes of root phosphatase activities (phosphomonoesterase, PME; phosphodiesterase, PDE; and phytase, PhT) in each functional type. The results indicate that, regardless of the mycorrhizal type, the climax species were more dependent on more recalcitrant organic P (phytate and/or diester P) than the pioneer species.

In Chapter 5, I examined the response of root exudation rates to N and P fertilization. Root exudation rates were measured by attaching the glass filter traps to the root systems of target trees and incubating them. My results suggest that, regardless of functional group, the dominant species (two primary-ECM and one primary-AM species) in my old-growth forests show higher root exudation rates than other AM species. In particular, some climax-AM species may often acquire N and P via root exudates.

In Chapter 6, I discussed the differences in P-use and -acquisition strategies among functional types. I performed the PCA analysis including all P-use (NRE and PRE) and -acquisition (three classes of phosphatase and root exudates) traits for each functional type, to examine the overall response of P-use and -acquisition traits to P fertilization. Considering the results of the PCA analysis and the previous chapters, I suggested that climax-ECM species would have an efficient P-use and -acquisition strategy, primarily with high PRE, that climax-AM species would have an efficient P-acquisition strategy, primarily with high activities of phosphatases (i.e., PDE, PhT), and that pioneer-AM species would have an effective N-use strategies, primarily with high NRE in the leaves, to maintain their high wood growth rates. Such different nutrient-use and -acquisition strategies among functional groups would promote the niche partitioning among species and/or functional types. Understanding these mechanisms is critical for a comprehensive appreciation of the mechanisms of P-limitation, multispecies coexistence, and biomass maintenance in tropical rainforests.

## 要旨

熱帯林では激しい土壌風化により土壌中のリン（P）が少ないため、P が欠乏状態にあると言われている。しかし、それにもかかわらず、熱帯低地林では樹高 50-60 m に達する巨大なバイオマスが維持されている。P 欠乏下の熱帯樹木は、葉の窒素（N）及び P 再吸収効率を高めたり、有機酸などの根滲出物や土壌有機態（土壌 Po）分解酵素（ホスファターゼ）を介した P 獲得能を高めたりすることで、その生産性を維持している可能性が考えられる。一方、このような栄養塩利用・獲得特性は遷移段階や菌根菌タイプが異なる機能群間で異なることも予想される。

そこで第 1 章では、P 利用・獲得戦略に関するこれまでの知見をまとめるとともに、熱帯林における野外施肥実験をレビューした。その結果、既存の野外施肥実験サイトがＡM種優占の新熱帯域に偏って分布していること、熱帯樹木の根形質を種レベルで検証した研究がほとんどないことにより、遷移段階や菌根菌タイプが異なる機能群間におけるＰ利用・獲得戦略の差異について検証できていないことが分かった。そこで、マレーシア・ボルネオ島の野外施肥実験区（対照、P 施肥、N施肥、NP 施肥）において、極相―ECM 種、極相―AM 種、パイオニア―AM 種それぞれ 2-3 種ずつを対象に NP 施肥に対する葉・根形質の応答を調べることで、P 利用・獲得戦略の機能群間の差異を検証した。

第 2 章では、葉の栄養塩濃度などを含めた葉形質と葉の被食率との関係性を検証した。葉の被食は、森林生態系における物質循環、腐食連鎖系から生食連鎖系への資源量変化を理解するうえで重要であるが、その規定要因はよく分かっていない。そこで、環境勾配に沿った生態特性の検証に適したマングローブ林において、葉の形質と被食率の関係を調べた。その結果、葉の縮合型タンニンは変動する葉の被食率を緩やかに規定し、生食連鎖系を駆動する要因の 1 つとなっていると考えられた。

第 3 章では、ボルネオ熱帯低地林において、NP 施肥に対する熱帯樹木の葉におけるＮ・Ｐ再吸収効率の応答を検証した。その結果、ECM 種は、極相・AM 種とは異なりＰ施肥によりＰ再吸収効率が低下したことから、P 欠乏下で葉の P 再吸収率を高く維持していることが示唆された。また、パイオニア種は、極相種よりもＮ再吸収効率が高く、Ｎを効率的に利用している可能性が考えられる。

第 4 章では、NP 施肥に対する熱帯樹木の細根ホスファターゼ活性の応答を検証した。機能群ごとに 3 種類の細根ホスファターゼ活性（PME; PDE; PhT）を測定することで、分解特性の異なる土壌 Po 資源（モノエステル態、ジエステル態、フィチン酸など）の獲得戦略を検証した。その結果、極相種はパイオニア種と異なり、Ｐ施肥に

より PhT・PDE 活性も減少した。極相種はホスファターゼを介し、より難分解な土壌 Po（フィチン酸やジエステル態Ｐ）を獲得していると考えられる。

第５章では、NP 施肥に対する熱帯樹木の根滲出物速度の応答を検証した。対象樹木の根系にガラスフィルタートラップを設置し培養することで、根滲出物速度を定量した。その結果、機能群に関わらず、原生林の優占種は他種に比べて根滲出物速度が高いことが示唆された。特に極相・AM 種の一部の種は、根滲出物速度を高めることでＮ・Ｐ獲得を行っている可能性が示唆された。

第６章では、機能群特異的なＰ利用・獲得戦略について議論した。Ｐ利用・獲得特性の全体的なＰ施肥応答を検証するため、機能群ごとにＰ利用特性（Ｎ・Ｐ再吸収効率）および獲得特性（ホスファターゼ、根滲出物）を全て含めてＰＣＡ解析を行った。このＰＣＡ解析と各章の結果から、極相―ECM 種は主に葉における Ｐ 再吸収効率を、極相―ＡM種は主に難分解な土壌 Po 獲得能を、それぞれ高めることで、その生産性を維持していると考えられる。加えて、原生林で特に優占する極相種は、菌根菌タイプに関わらず高い根滲出物速度を維持していると考えられる。また、パイオニア－ＡM種は、材成長に必要なＮを葉のＮ利用効率を高めて節約的に利用することで、その高い生産性を維持していると考えられる。このような栄養塩の利用・獲得戦略の機能群間の違いは、熱帯林における Ｐ 制限メカニズムや多種共存・バイオマス維持機構の理解を促進するものである。