

DISTRIBUTION AND CHARACTERISATION OF PHENOLIC COMPOUNDS IN *MACARANGA PRUINOSA* AND ASSOCIATED SOILS IN A TROPICAL PEAT SWAMP FOREST

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Phenolic compounds are abundant in tropical peat swamp forests and peat mostly comprises lignin and other phenolics. Water in these forests is darkened by tannins and humic acids and the flora exhibit high total phenolic contents (TPC). To further understand the functions of phenolics in peat swamp forests we investigated phenolic compounds (Folin Ciocalteu assays, HPLC) in a common tree, *Macaranga pruinosa* (Euphorbiaceae), over 18 months. We simultaneously studied peat phenolics with respect to composition, depth and season. TPC were highest in mature leaves compared with trunks, branches and roots, probably relating to their role in defence against herbivory and pathogens in low nutrient environments. Relative concentrations of low (phenolic acids, flavonoids) to high (tannins, derivatives) molecular weight phenolics decreased as leaves matured indicating conversion within leaves. TPC increased significantly at the peat surface and during the wet seasons. TPC of mature leaves were significantly higher during the wet season. This implies either plants synthesise phenolic compounds in response to flooding or phenolics are more readily available during the wet season due to increased detrital leaching and plants can absorb phenolics via their roots. Waterlogged conditions may not be stressful to adapted plants but may facilitate recycling of protective phenolic compounds.

Keywords: Tannins, flavonoids, lignin, quercetin, taxifolin, flooding, Malaysian peat swamp forest

INTRODUCTION

The tropical peat swamp forests of Indonesia and Malaysia contain one of the largest stores of terrestrial organic carbon in the world (Page et al. 2011). These forests support enormous biodiversity with many endangered species and trees up to 70 m high (Yule 2010). They owe their existence to phenolic compounds, particularly recalcitrant lignin and its derivatives, which form the substrate of peat (Andriess 1988), and tannins that inhibit decomposition and consequently promote peat accretion which is enhanced by the anaerobic conditions (Coq et al. 2010, Constabel et al. 2014). These waterlogged forests are seasonally flooded with water blackened by phenols such as tannins and humic acids which leach from the peat and fallen leaves. The extremely high levels of phenolics in the water and substrate are the key difference between peat swamp forests and freshwater swamp forests. Phenolic compounds are fundamental to the creation and maintenance of peat swamp forests. Accretion of peat occurs due to two factors. Firstly, the extreme conditions

of flooding with acidic, toxic, phenol-rich water impedes microbial decomposition of dead trees and roots (Evers et al. 2016). Secondly, the leaves are tough and rich in phenolic compounds to reduce herbivory and pathogen attack in the nutrient poor peat swamp forests environment, and these features make them resistant to microbial breakdown (Yule & Gomez 2009, Constabel et al. 2014).

When plants die or senescent leaves fall, they introduce phenolic compounds into the soil. Thousands of diverse phenolic compounds occur. They are found in all vascular plants where they function in structure (e.g. lignin), defense against herbivory and pathogens (e.g. flavonoids, tannins), colour (e.g. anthocyanins) and protection against ultraviolet (UV) light (e.g. phenylpropanoids) (Lattanzio et al. 2012, Constabel et al. 2014, Moctezuma et al. 2014). The composition and abundance of phenolic compounds within plants vary seasonally and spatially, depending on environmental conditions and different plant structures, for example they

are typically higher in mature than immature leaves (Lattanzio et al. 2012). Ferns, gingers and *Macaranga* (Euphorbiaceae) species (including *M. pruinosa*, *M. gigantea*, *M. hosei*, *M. hypoleuca*, *M. kingii* and *M. triloba*) growing in peat swamp forests had significantly higher levels of total phenolic compounds (TPC) and tannins than the same species growing on mineral soils (Lim et al. 2014; Yule et al. 2016). Concentrations of phenolic compounds in leaves of *M. pruinosa* and also in the peat substrate decrease along a gradient of increasing peatland degradation due to drainage and forest clearing (Yule et al. 2016).

Peat swamp forests of Indonesia and Malaysia originally covered about 25 million ha, but less than 15% presently remain (Hooijer et al. 2010) because they are being rapidly drained, cleared and burnt mostly for conversion to oil palm plantations (Koh et al. 2011). Dry peat is highly flammable due to the high percentage of lignin (~80%) (Andriess 1988). Seasonal fires to clear peatlands can burn underground for years and cause regional transboundary haze with huge economic and health impacts as well as globally detectable greenhouse gas emissions, i.e. up to 3.1% of current global CO₂ emissions from fossil fuel combustion (Page et al. 2002, Hooijer et al. 2010).

Most regional peat swamp research has focused on biodiversity, carbon storage and emissions and agricultural conversion, but little is known about the ecosystem functioning of peat swamp forests. To further understand the dynamics of phenolic compounds in peat swamp ecosystems this study investigated the following questions:

(1) What is the distribution and composition of phenolic compounds within *M. pruinosa*?

(2) Are concentrations of phenolic compounds in leaves of *M. pruinosa* affected by the difference in water levels between wet and dry seasons?

(3) What is the composition of phenolic compounds in the peat substrate and do they vary with depth and between wet and dry seasons?

MATERIALS AND METHODS

Study sites and sample collection

The study was conducted in the peat swamp forest in Sungai Karang Forest Reserve, North Selangor (3° 39' N, 101° 19' E), about 100 km north-east of Kuala Lumpur, Malaysia. It is a mixed swamp forest that was selectively logged 25 years previously and the canopy height is now approximately 30 m. Although a drain is regularly excavated to prevent flooding of the road along the northern edge of the forest, the forest floor still floods seasonally. Ten sites along the northern edge of the forest were chosen, and at each site, 25 *M. pruinosa* trees were selected and tagged (n = 250 trees). Each tagged tree was growing on peat and no more than 5 m high, so the leaves were accessible via a long-handled pruner. The study was conducted over 1.5 years covering a dry, then wet, then dry season. On days 0, 90, 180, 360 and 540 triplicate samples of leaves, twigs, branches, trunks and roots (Table 1) were collected for analyses from each of the 250 trees. Sampling of peat was carried out at the same time from five of the sites. Peat samples were collected using soil corer from the surface, 25 and 50 cm (n = 5 for each depth at days 0, 90, 180, 360 and 540).

Table 1 Plant structures collected for analyses

Plant structure	Condition	Description
Leaf	Young	Soft and light green in colour
	Mature	Leathery and dark green in colour
	Old	Leathery, dark green coloured leaves with presence of yellow spots
	Senescent	Freshly fallen, yellowish to brown leaves
Twig		Succulent stems connecting the petiole of the leaves to the branches
Branch		Woody stems connecting the twigs to the trunk of the plant
Trunk		Main woody stump of the plant
Root	Fine	Fine network of roots < 2 mm in diameter
	Thick	Root network connecting the buttress of the plant to the fine roots, > 2 mm in diameter

Sample preparation

Plant extracts were prepared and analysed as in Lim et al. (2014) as follows: 1 g of each of the fresh samples (in triplicate) was crushed mechanically into powder in liquid nitrogen. The crushed samples were extracted with 50 mL of 100% methanol by shaking the suspension on an orbital shaker for 1 hour and then the extracts were filtered under suction. The extracts were stored at -20 °C until further use.

Total phenolic contents

The Folin Ciocalteu assay (Kähkönen et al. 1999) was used to determine TPC. Samples (300 µL, in triplicate) were placed in test tubes followed by 1.5 mL of Folin Ciocalteu's reagent (10× dilution) and 1.2 mL of 7.5% sodium carbonate. Tubes were left to stand in the dark for 30 min prior to measurement of absorbance at 765 nm. Total phenolic content was expressed as gallic acid equivalent (GAE) in mg GAE/100 g material. The calibration equation was $y = 0.0111x - 0.0148$ ($r^2 = 0.9998$) where y = absorbance and x = concentration of gallic acid in mg L⁻¹.

Total flavonoid contents

Total flavonoid content was determined using the aluminium chloride colorimetric method described by Chang et al. (2002). An amount of 0.5 mL sample was diluted with 1.5 mL methanol after which 100 µL of 10% aluminium chloride, 0.1 mL of 1.0 M potassium acetate as well as 2.8 mL of distilled water was added into the extract solution. The final volume was 5.0 mL. This mixture was incubated for 30 min at room temperature. The absorbance of the extracts was measured at 435 nm against distilled water. For the blank, 0.1 mL of 10% AlCl₃ was substituted with distilled water. Total flavonoid content values were expressed as quercetin equivalent in mg per 100 g of material. The calibration equation used was $y = 0.0686x + 0.001$ ($r^2 = 0.9984$) where x = concentration in mg L⁻¹ and y = absorbance at 435 nm.

Total tannin contents

Determination of total tannin contents (TTC) was conducted using the similar method for the determination of TPC (Makkar et al. 1993).

Briefly, 0.3 mL of extract was mixed with 1.5 mL (1:10) Folin-Ciocalteu's reagent and 1.2 mL of 20% (w/v) sodium carbonate. The solution was allowed to stand for 30 min in the dark. Absorbance measured at 765 nm allowed the determination of TPC in terms of tannic acid equivalent (TAE). In a separate tube, 100 mg of polyvinylpyrrolidone (PVPP) was weighed and added to a mixture of distilled water and extract (1.0 mL each). The mixture was then vortexed at 4 °C for 15 min and centrifuged for 10 min at 3000 g. The supernatant, which contained only simple phenolics as tannins were bound to PVPP, was collected. The phenolic content of the supernatant was then determined using Folin-Ciocalteu's reagent. Tannin content of the sample was calculated as: total phenolics – non-tannin phenolics = tannins (mg TAE 100 g⁻¹). TTC was expressed as TAE in mg per 100 g of materials. The calibration equation was $y = 0.097x - 0.0012$ ($r^2 = 0.998$) where x = concentration in mg L⁻¹ and y = absorbance at 765 nm. The TTC:TPC ratio was determined by dividing total phenolic values with tannin values obtained previously. High molecular weight phenolics are represented by tannins and their derivatives (Pridham 1964).

High performance liquid chromatography analysis

Crude extracts were dissolved as much as possible in 1 mL of 30% methanol (sonicated). Hexane (1.0 mL) was added to each fraction, vortexed and allowed to stand for 1 min. Fractions were centrifuged for 30 s. The hexane layer (top layer) which contained fatty compounds was removed and the entire process was repeated twice. The aqueous layer was filtered through a membrane filter (pore size 0.45 µm) prior to injection into HPLC for chromatographic analysis. The HPLC consisted of a quaternary vacuum degasser pump and diode array detector. Samples were injected through a manual injector valve fitted with 20 µL sample loop. The column consisted of a phenyl-bound silica column (100 mm × 4.6 mm, 5 µm particle size).

Gradient mode was used in this analysis, involving two solvents, namely, 100% methanol and water, both acidified to approximately pH 2.5 with 0.1% trifluoroacetic acid. The elution profile was 40% mobile phase 1 to 60% mobile phase 2 in a linear gradient from 0 to 20 min.

The flow rate was set at 1 mL min⁻¹. The detection wavelengths used were 210, 245, 280 and 365 nm with reference wavelength set at 700 nm.

Identification of phenolic compounds

Specific phenolic compounds in the leaves of *M. pruinosa* and in the peat soil were identified based on retention time, spiking with known standard, and comparing the absorbance spectra of both spiked and existing compounds. The concentration of each compound was determined based on the construction of a standard curve for each of the phenolic compound identified. A total of four phenolic compounds were isolated from the leaves and four different standard curves were constructed. The content of each compound was expressed as mg 100 g⁻¹ of fresh leaves.

Analyses of peat samples

For chemical assays of TPC and TTC, approximately 10 g of freeze-dried peat was extracted with 70% methanol by shaking the suspension continuously for 2 hours. It was then filtered and the solution was stored at -20 °C. For reversed phase-HPLC analysis, 100 g of freeze-dried peat was extracted with 70% methanol overnight. It was then filtered and the

solution was stored at -20 °C. For nutrient and total organic carbon analysis, approximately 200 g of peat was air dried and kept at room temperature (25–27 °C) and the analyses (using the combustion method) were outsourced to the Land Management Department of Universiti Putra Malaysia, Serdang, Selangor.

Statistical analyses

For comparison of mean TPC values in different plant parts and in different seasons (for both leaves and peat), one way analysis of variance was used and significant differences were identified using Tukey's HSD (honestly significant difference) test. Statistical analyses were conducted using SPSS version 16.0 and differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Phenolic compounds in *Macaranga pruinosa*

Throughout the 1.5 years study, leaves (particularly mature leaves) of *M. pruinosa* consistently had the highest concentration of phenolic compounds, followed by roots (in which fine roots had higher TPC than thick roots) and lastly woody parts (Figure 1). The presence of

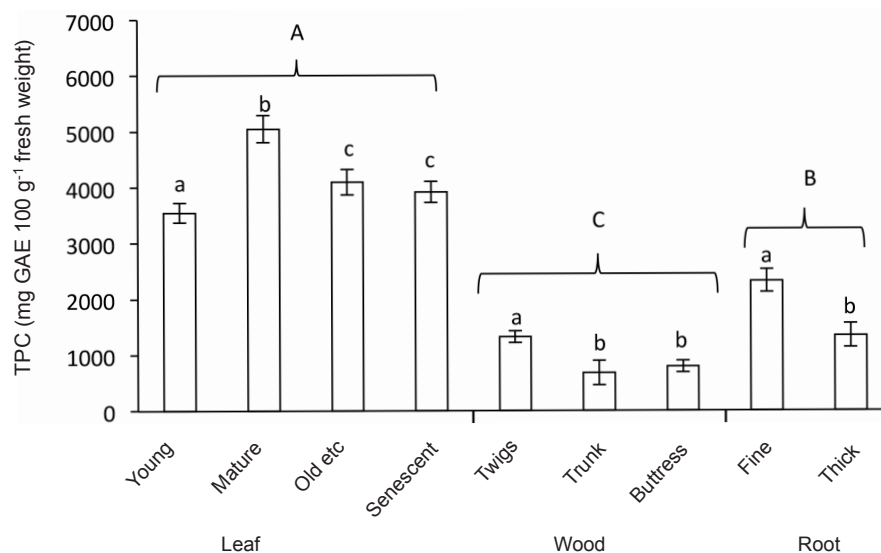


Figure 1 Mean total phenolics contents (TPC) (± 1 standard deviation (SD), $n = 250$ trees) of various *Macaranga pruinosa* parts over 1.5 years; for comparison within plant parts (leaves, wood and roots), bars with the same letter (a, b, c) above them are not significantly different at $p < 0.05$, for the comparison between leaves, wood and roots, bars with different letters (A, B, C) above the bars are significantly different at $p < 0.05$ (Tukey HSD test); GAE = gallic acid equivalent

higher concentrations of phenolics in the leaves probably relates to their role in defence against herbivory, microbial pathogens and UV light, whereby the leaves require protection due to their vital function in photosynthesis and lack of protective lignin (Lattanzio et al. 2012, Constabel et al. 2014, Moctezuma et al. 2014). The reason why fine roots have higher TPC than coarse roots may be because they are not as tough and so they require greater protection from microbial pathogens. Furthermore, they function in water and nutrient uptake, and absorb low molecular weight phenolic compounds from the peat water (Lim 2012).

Relative concentrations of high molecular weight phenolics (tannins and their derivatives) to low molecular weight phenolics (phenolic acids and flavonoids) increased as the leaves mature (Table 2). This suggests that young leaves

are capable of producing low molecular weight simple phenolic compounds (phenolic acids and flavonoids) and/or the plants absorb these from the peat substrate (Lim 2012). They confer initial chemical protection for the young leaves which are softer and so have less physical protection against herbivory, UV light and other factors. As the leaves mature, these simple low molecular weight phenolics are converted to more complex phenolics following the biosynthetic pathway (Niesh 1960, Schijlen et al. 2004):



Effect of season on TPC of *M. pruinosa* leaves

Mean TPC values of mature leaves were significantly higher during the wet season than the dry seasons (Figure 2). TPC significantly increased at the peat surface and 25 cm depth during the wet season (Figure 3). This suggests increased leaching of phenolic compounds from newly submerged leaf litter, leading to two possibilities: (1) the plants synthesise TPC in response to waterlogging stress due to higher water table during the wet season, or (2) there is increased availability of simple phenolics during the wet season for absorption via the root system due to increased leaching into the water from submerged leaf litter. During the dry season, the water table is below the level of the surface root mat. However, the higher water table during the

Table 2 Ratio of total tannin contents (TTC) to total phenolic contents (TPC) in leaves and roots of *Macaranga pruinosa*

Plant structure	Age (visual observation)	TTC:TPC
Leaf	Young	0.64
	Mature	0.69
	Old	0.96
	Senescent	1.00
Root	Thick	0.42
	Fine	0.30

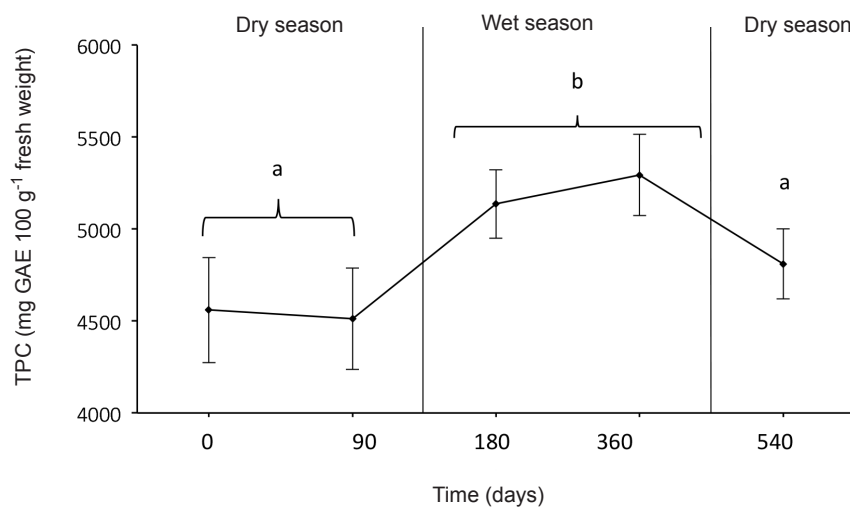


Figure 2 Effect of season on mean total phenolics contents (TPC) (± 1 SD, n = 250 trees) of mature *Macaranga pruinosa* leaves; values followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test)

wet season can make phenolics available to the roots resulting in higher uptake to the leaves. Water levels may be as low as 30 cm or more below the peat surface during the dry season, but the forest floor may be flooded by over 50 cm during the wet season (Pahang Forestry Department 2005).

Although TPC varied significantly with changing seasons, the concentrations of ferulic

acid and p-coumaric acid (major components of lignin) and three flavonoids (kaempferol, quercetin and taxifolin) in mature leaves of *M. pruinosa* showed no significant variation with time over 1.5 years indicating that their production and occurrence were fairly consistent throughout the year (Figures 4 and 5). Small increases in ferulic acid, quercetin and taxifolin could partly

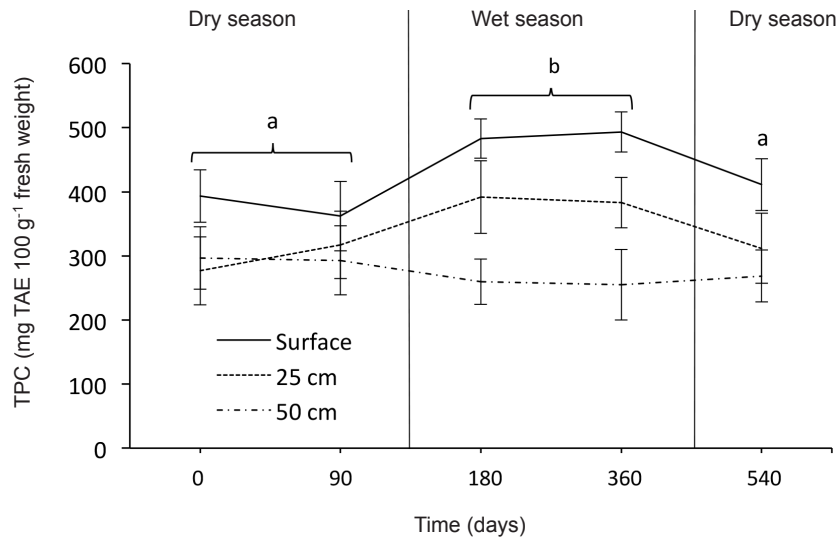


Figure 3 Effect of season and depth on mean total phenolics contents (TPC) (± 1 SD, $n = 10$) of peat collected at 0, 25 and 50 cm depth; values followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test) for surface peat; TAE = tannic acid equivalent

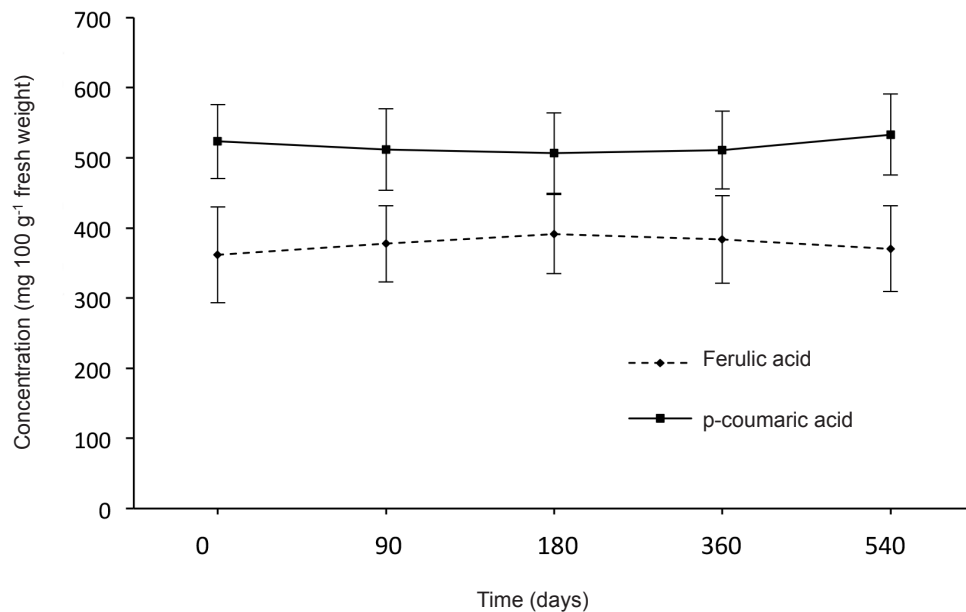


Figure 4 Mean concentration of phenolic acids over time in mature *Macaranga pruinosa* leaves (± 1 SD, $n = 250$)

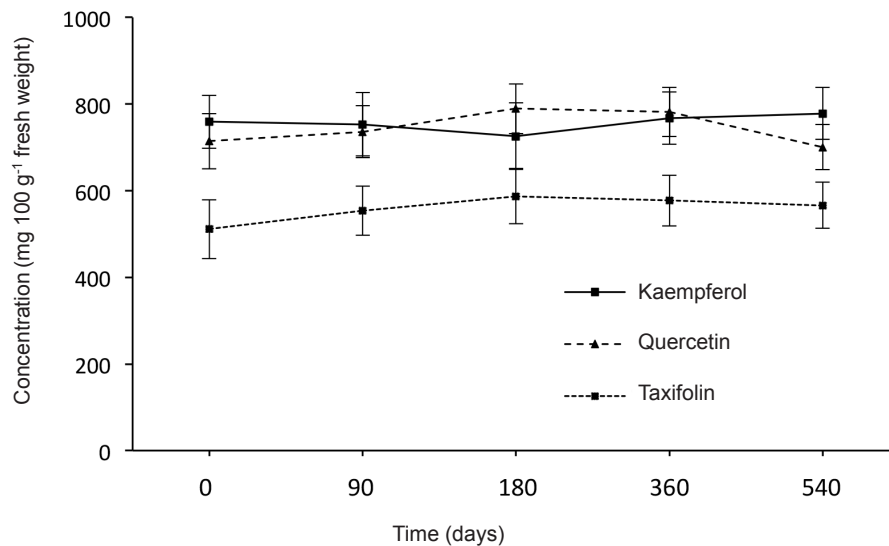


Figure 5 Mean concentration of flavonoids over time in mature *Macaranga pruinosa* leaves (± 1 SD, $n = 250$)

explain the higher TPC values in the wet season but clearly there must also be variations in other phenolic compounds in the leaves.

Phenolic compounds in peat

Levels of TPC were highest at the peat surface and generally decreased with increasing depth (Figure 3). TPC of surface peat and up to 25 cm deep increased significantly during the wet season (Figure 3). This is probably due to increased leaching from newly submerged leaf litter with rising water table and also increased rainfall. Levels of tannins were consistently higher at the surface (Figure 6). In another study of the same peat swamp forest (i.e. Sungai Karang Forest Reserve) it was observed that leaf litter, twigs and branches of two common emergent tree species (*Koompassia malaccensis* and *Shorea uliginosa*) fell throughout the year, although there were several months with significantly higher leaf fall (Ong et al. 2015). This means that the forest floor perpetually receives freshly fallen leaves and other dead plant materials. The higher water table and rainfall of the wet season would result in increased leaching of phenolic compounds from these materials compared with the dry season. Thus, during the wet season the surface peat contains higher concentrations of phenolic acids (simple phenolics) and flavonoids which are usually polar by nature and remain dissolved in water. Conversely, TTC initially

dropped at the surface with the start of the wet season (Figure 6), possibly due to dilution of peat water by rainfall. Levels of TTC at 25 cm and 50 cm remained stable throughout 1.5 years.

Identity of phenolic compounds in surface peat

The most abundant phenolic compounds identified in surface peat were two flavonoids—quercetin ($1671 \mu\text{g } 100 \text{ g}^{-1}$) which was found throughout the peat profile, followed by taxifolin ($716 \mu\text{g } 100 \text{ g}^{-1}$ fresh weight) (Table 3). Given the high levels of these and other phenolic compounds in the leaves, roots and woody biomass of the peat swamp forest plants (Tables 1 and 2, Figure 1), it is clear that these must be the major sources of the TPC we measured in peat. The presence of ferulic acid and p-coumaric acid (constituents of lignin) were also reported in peat sampled from cleared and agricultural sites in Malaysia by Katase (1993) who suggested that these compounds could be the cause for poor growth of rice crops on newly converted peat swamp forests soil. The author reported levels of total carbon and phenolic acids less than 50% of those determined in this study—a probable consequence of logging (removing the source of phenolic compounds) and drainage (removing fluvial phenolics and resulting in peat oxidation) at the sites.

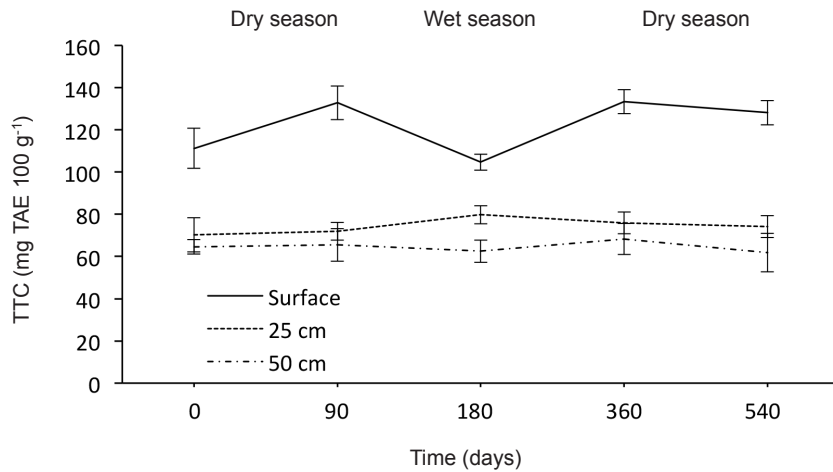


Figure 6 Mean total tannin content (TTC) (± 1 SD, $n = 10$) of peat collected at 0, 25 and 50 cm; TAE = tannic acid equivalent

Table 3 Concentration of phenolic compounds in surface peat

Compound	Concentration ($\mu\text{g } 100 \text{ g}^{-1}$ fresh weight \pm SD)
Ferulic acid	217 \pm 23
p-Coumaric acid	319 \pm 45
Kaempferol	553 \pm 87
Quercetin	1671 \pm 119
Taxifolin	716 \pm 101

The peat was composed of 55–67% organic carbon (Table 4) which is typical of peat soil elsewhere in both temperate and tropical regions (Andriess 1988). Levels of nutrients were higher in surface peat and decreased with depth and they were higher in the dry season than the wet season (Table 4). The major source of nutrients in peat swamp forests is leaf litter (due to the lack of inflowing rivers bringing nutrients). Thus, litter decomposition dynamics have a major influence on nutrient cycling. Consequently, most of the nutrients are on the surface where the senescent leaves fall and where nutrients are taken up by trees, leaving only small amounts to be sequestered in the accreting peat layers (Ong et al. 2015). Leaf litter in north Selangor peat swamp forests decomposed faster under dryer conditions (on hummocks) than submerged (in pools) which would suggest greater release of nutrients in the dry season (Ong et al. 2015). Furthermore, high water content of peat in the

wet season could dilute the nutrients (Table 4). Higher pH and lower percentage of total organic carbon of the wet season (Table 4) are further examples of the diluting effects of the higher water table. Waterlogging and lower nutrients could cause stress to the plants during the wet season but are possibly partly offset by the slightly higher pH.

CONCLUSIONS

Phenolic compounds in north Selangor peat swamp forests varied spatially and temporally. Mature leaves of *M. pruinosa* and surface layers of peat had the highest TPC compared with other plant parts and deeper peat layers and the concentrations increased significantly in the wet season. Fine roots had higher TPC than coarse roots which supported the proposition that they are active in absorption of phenolic compounds from the peat water and enable recycling of phenols. The results suggest that the extreme, waterlogged, phenol-rich peat swamp forests may offer the opportunity for adapted plants to recycle phenolic compounds back into the plants via the roots. Phenolic compounds are then concentrated in mature leaves to protect the leaves from herbivory and attack by microbial pathogens.

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Table 4 Available nutrients, water content and pH in peat according to season and depth (mean \pm 1 SD, n = 5)

	Surface		25 cm		50 cm		
	Dry	Wet	Dry	Wet	Dry	Wet	
TOC (%)	62.19 \pm 1.96	58.42 \pm 0.82	67.22 \pm 1.25	55.19 \pm 2.11	66.27 \pm 1.04	55.33 \pm 0.74	
Nutrient	N (%)	1.72 \pm 0.01	1.31 \pm 0.02	1.05 \pm 0.02	0.47 \pm 0.02	0.57 \pm 0.04	0.42 \pm 0.05
	P (ppm)	53.73 \pm 4.32	39.12 \pm 5.66	53.72 \pm 4.55	32.16 \pm 3.21	24.09 \pm 1.66	15.34 \pm 2.12
	K (ppm)	291.28 \pm 23.01	114.32 \pm 12.11	155.23 \pm 9.35	87.45 \pm 5.34	148.29 \pm 9.11	101.32 \pm 15.23
Water content (%)	80.21 \pm 10.69	90.45 \pm 15.47	76.54 \pm 9.01	88.52 \pm 12.22	71.97 \pm 18.14	86.35 \pm 9.68	
pH	3.29 \pm 0.02	3.45 \pm 0.01	3.31 \pm 0.01	3.42 \pm 0.02	3.31 \pm 0.05	3.42 \pm 0.04	

TOC = total organic carbon, N = nitrogen, P = phosphorus and K = potassium

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