PLANT POLYPHENOLS IN THE CONTEXT OF BIODIVERSITY-ECOSYSTEM FUNCTIONING

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"Wohlfühlen ist [...] kein wissenschaftliches Kriterium. Wir können uns dabei warm und gemütlich fühlen, doch die historische Entwicklung der wissenschaftlichen Erkenntnis zeigt, dass 'warm' und 'gemütlich' von Zeit zu Zeit nur ein höflicher Ausdruck für 'falsch' ist."

Terry Pratchett, Ian Stewart & Jack Cohen (2006),
in: "Darwin und die Götter der Scheibenwelt"; Piper Fantasy

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Summary:

The present thesis had the aim to deepen the scientific understanding of the role of plant polyphenolic substances in the context of biodiversity-ecosystem functioning (BEF). The central focus was put on polyphenolic substances extracted from leaf material To give an introduction into this field of research and its rationale, the most important results from early BEF experiments are presented in the first chapter. As the present thesis was carried out in the framework of the BEF-China project, this project is described in chapter two. The third chapter gives a concise overview on the chemical properties as well as known effects of plant polyphenolics on a broad range of ecological aspects. Finally, a specification of the current research questions leads over to the central challenges and investigations carried out in this thesis.

The first achievement of this thesis was the establishment of a protocol that is optimized for the collection, sample storage and processing of samples that are collected for the quantification of polyphenolic substances in the context of biodiversity-ecosystem functioning. This protocol was designed to consider the multitude of peculiar conditions that are typical in BEF research and to ameliorate possible negative effects on the amount of extractable polyphenols from plant tissue resulting from these peculiarities. BEF-Experiments are frequently carried out in remote areas with only limited access to specialized laboratory equipment. In the case of BEF-China, which is located in the subtropical region of south-eastern China, high temperatures often occur during sample collection in the field. External factors such as high light intensities and elevated temperatures have been found to be detrimental to the amount of extractable polyphenols form leaf material in other studies. This also applies to the conditions of drying and long-term storage. Therefore, the novel sampling protocol was - inter alia- designed to keep temperatures low during sample collection. Drying the plant tissue by the means of lyophilization and storing the leaves in UV-tight vials are other important aspects of the optimized protocol. Another challenge was the high number of samples typically collected in BEF research. In many other research fields (e.g. chemical ecology or plant physiology) sample sizes are small, compared to BEF studies where often hundreds of samples are collected for a single study. Therefore, established methods to quantify plant polyphenols have been modified to allow for robust a quantification of leaf total phenolics and tannin concentrations, to increase the accuracy of quantification and to allow for a high sample throughput.

Moreover, this thesis addressed, in which way ecological conclusions were affected, when information on leaf polyphenolic concentrations that was gathered from investigations where the samples were collected according to established, non-optimized protocols. It is known that plant polyphenols are involved in important ecosystem processes such as nutrient cycling and thus may be relevant in studying ecological processes. Most established protocols for the sampling of leaf tissue in BEF contexts do not consider the sensitivity of polyphenolic substances to external influences during sample handling and storage. Thus, ecological conclusions drawn from investigations based such samples may be erroneous due to methodological shortcomings. To assess the impact of the sub-optimal sample handling on the amount of extractable polyphenolics, two sample sets collected from 20 tree species from the BEF-China species pool were compared for their concentrations of extractable polyphenols. The first set comprised samples collected according to the established protocol of Cornelissen *et al.* (2003), whereas the second set was collected according to the protocol established as a part of this thesis. Moreover, the samples were at different seasons of the year, a factor that has also been reported to affect leaf tissue polyphenol concentrations. Thus, the differences between the two sample set comprised the variations that typically encountered when collecting samples in the context of BEF experiments. Polyphenol concentrations were determined according to the procedures described in the optimized protocol in both sample sets. The variance brought about by suboptimal sample treatment was compared to the variance caused by the taxonomic range of species. To assess the significance of the taxonomic variation in this context, the extent of trait conservatism (phylogenetic conservation) of polyphenol concentrations in the investigated species was assessed. The study demonstrated that these concentrations are highly phylogenetically conserved, even to a greater extent than six other leaf traits incorporated in this study. This study moreover demonstrated that the variation in polyphenol concentrations brought about by non-optimal sample handling was widely overridden by the variation caused by the taxonomic extent. Successive reduction of the taxonomic extent considerably increased the impact of differences in sample handling on extractable polyphenol concentrations. However, total phenolics concentrations were found to be more robust to differences in sample treatment than tannin concentrations. Finally, using non-metric multidimensional scaling methods, it was shown that the interpretations derived from the two sample sets both led to similar ecological conclusions. Consequently, this study demonstrated that conclusions drawn from information on polyphenolic concentrations collected and processed under non-optimal conditions are reliable, given the taxonomic extent of the respective investigation is sufficiently wide.

Another main objective addressed in this thesis was the investigation of the role of leaf polyphenolic substances in community leaf litter decomposition during secondary forest succession. In the second study presented in this thesis, shifts in the community weighted means (CWM) of 14 functional leaf traits were assessed across 27 forest communities that represented a gradient of secondary succession. All the incorporated traits have been reported to relate to leaf litter decomposability in previous studies. *In-situ* leaf litter decomposition rates (k-rates) were calculated for each of the 27 communities. Firstly, the study investigated the direction and patterns of shifts in CWM along the successional gradient. The detected shifts in CWM leaf traits generally followed logarithmic patterns, whereas community k-rates did not significantly change along the successional gradient. The encountered shifts in CWM leaf traits indicated a shift in resource-use strategy from high nutrient acquisition in young forest stands to high nutrient retention in old forest stands. Secondly, in order to test whether the shifts in CWM traits related to the dynamics of community k-rates with ongoing succession, multiple regression analyses were carried out. It was demonstrated that the k-rates were related to shifts in CWM leaf traits along the successional gradient. CWM traits that are related to leaf nutritional quality (leaf phosphorous concentration) promoted community leaf litter decomposition. Simultaneously, traits related to leaf chemical (leaf polyphenols) and physical (leaf toughness) resistance decreased the k-rates. These results indicated that community decomposition rates were not affected by single leaf traits, but resulted from the interactions of different traits that jointly affect decomposition in opposing directions.

The third investigation presented as part of this thesis considered plant polyphenols (total phenolics and tannin) in relation to leaf toughness (LT) of 51 tree species. Both these traits may be interpreted as aspects of leaf defense, and both traits rely on carbon as a precursor. As carbon limitation may restrict plants to either invest into leaf structural (i.e. LT) or chemical defense (plant polyphenols), a trade-off between leaf toughness and plant polyphenolic substances due to constraints in carbon allocation was hypothesized in this investigation. Most previous studies on trade-offs between different aspects of plant defense are based on the use of species mean traits. This use of species trait means is, however, only valid under the assumption that the differences in the trait values between species are bigger than the differences within species. Thus, the relative importance of intraspecific trait variability (ITV) was investigated in the presented study. In addition, the effect of the incorporation of ITV when assessing the strength and direction of a putative trade-off was investigated. Species-specific analyses did not show a consistent pattern of trade-offs between leaf polyphenols and leaf

toughness for all 51 species. In some species, polyphenols were positively correlated to LT, in some species negative covariations were detected; in most species no significant trait interrelation-ships were encountered. However, when the analyses were carried out acorss all species using species trait means, the analyses pointed to an over-all a negative covariation (i.e. trade-off) between the two aspects of leaf defense. The incorporation of ITV in these analyses significantly lowered the strength but not the direction of the trade-offs. Moreover, based on the results of the first manuscript presented in this thesis, plant polyphenol concentrations as well as leaf toughness were hypothesized to be highly phylogenetically conserved. In order to test this, phylogenetic information on the 51 investigated species was included in the analyses. All investigated defense traits showed strong phylogenetic signals. However, the timing of the intensity of this conservatism as well as the degree of trait divergence differed between the two aspects of leaf defense during their evolution. When this phylogenetic non-independence was stochastically accounted for, strong trade-offs between physical and chemical defense were detected. The results of this study indicate that the encountered trade-offs between the two different aspects of leaf defense were dominated by evolutionary, rather than by resource allocation constraints.

In summary, this thesis provided evidence for the importance of plant polyphenolic substances on important ecosystem processes such as leaf litter decomposition rates. Moreover, the present thesis demonstrated that the evolution of plant polyphenols is coupled to the evolution of leaf toughness. The encountered trade-offs are not straightforward, but are rooted in the species' phylogeny. In consequence, polyphenols and leaf toughness may not be considered as independent defense traits but must be interpreted in combination when examining their effects on ecosystem processes. In this respect, the interplay of leaf polyphenols, leaf toughness and functional traits related to leaf nutritional value that was found to determine the litter decomposition rates along the gradient of secondary succession, provides a good example. The relevance of such interplays may, however, also apply to othe ecosystem processes such as herbivory. Conclusively, the most important finding of this thesis was, that plant polyphenols and their effects on ecosystem functioning should not be investigated isolated from other traits, but rather under the view of combined, occasionally opposing effects.

Zusammenfassung:

Die vorliegende Arbeit hatte zum Ziel, das wissenschaftliche Verständnis für die Rolle pflanzlicher Polyphenole im Kontext der Biodiversitäts-Ökosystemfunktionsforschung (*biodiverstiy-ecosystem functioning*, BEF) zu vertiefen. Der Fokus dieser Arbeit lag dabei auf den Polyphenolgehalten in Blättern. Um dem Leser einen Einstieg in dieses Forschungsfeld sowie dessen Grundannahmen zu vermitteln, wurden die wichtigsten Ergebnisse früher BEF Experimente im ersten Kapitel dieser Promotionsschrift zusammengefasst. Da die vorliegende Dissertation im Rahmen des Projektes BEF-China angefertigt wurde, wurde der Aufbau dieses Projektes im zweiten Kapitel umrissen. Im dritten Kapitel findet sich eine knappe Übersicht über die chemischen Eigenschaften pflanzlicher Polyphenole sowie über die bisher bekannten Effekte dieser Stoffgruppe auf verschiedene ökologische Aspekte. Schließlich leitet eine Zusammenfassung der spezifischen Fragestellungen die der vorliegenden Arbeit zugrundeliegen über zu den zentralen Untersuchungen, die im Rahmen dieser Dissertationsschrift durchgeführt wurden.

Die erste Aufgabe, die in dieser Dissertationsschrift gelöst wurde, war die Etablierung eines Protokolls welches hinsichtlich der Entnahme von Pflanzenproben, deren Lagerung sowie deren Aufarbeitung zum Zwecke der Quantifizierung pflanzlicher Polyphenole im Rahmen von BEF Experimenten optimiert wurde. Dieses Protokoll wurde speziell angepasst um eine Vielzahl an Besonderheiten, welche typisch für Forschungen im Bereich von BEF sind, zu berücksichtigen. Des Weiteren beinhaltet dieses Protokoll Maßnahmen, die die möglicherweise negativen Auswirkungen dieser Besonderheiten auf die Menge an extrahierbaren Polyphenolen abschwächen. BEF Experimente werden häufig in abgelegenen Regionen durchgeführt, in denen der Zugang zu speziellen Laboreinrichtungen und -geräten beschränkt ist. Im Falle des BEF-China Projektes, welches in der subtropischen Klimazone des südöstlichen Chinas lokalisiert ist, herrschen häufig sehr hohe Temperaturen während der Probennahme. Es ist bekannt, dass sich äußere Faktoren wie hohe Temperaturen aber auch hohe Lichtintensitäten nachteilig auf die Menge der aus Pflanzenmaterial extrahierbaren Polyphenole auswirken. Diese Faktoren wirken ebenso während der Probentrocknung sowie deren Lagerung auf die untersuchten Blattsubstanzen ein. Daher umfasst das neue Probennahmeprotokoll unter anderem Vorkehrungen, um die Temperatur der Proben während der Entnahme durch Kühlung niedrig zu halten. Die Gefriertrocknung der gesammelten Blätter sowie eine Lagerung der Proben in UV-undurchlässigen Probengefäßen sind weitere wichtige Aspekte des optimierten Protokolls. Eine weitere Herausforderung stellte die hohe Anzahl an Proben dar, die

üblicherweise im Rahmen von BEF-Studien gesammelt werden. In vielen anderen Forschungsgebieten (z.B. der chemischen Ökologie oder der Pflanzenphysiologie) werden meist nur wenige Proben genommen. Für BEF-Studien hingegen werden häufig hunderte von Proben im Rahmen einer einzigen Studie gesammelt. Daher wurde bereits etablierte Methoden zur Quantifizierung des Totalphenol- sowie Tanningehaltes in pflanzlichen Materialien dahingehend modifiziert, einerseits robuste Ergebnisse zu liefern und deren Genauigkeit zu erhöhen, andererseits aber auch einen hohen Probendurchsatz zu ermöglichen.

Des Weiteren untersuchte die vorliegende Arbeit, inwieweit ökologische Schlussfolgerungen beeinträchtigt wurden, die sich aus der Interpretation von Informationen über pflanzliche Phenole ergaben, wobei die Proben auf der Basis von bereits etablierten Protokollen gesammelt wurden. Die meisten etablierten Protokolle zur Probennahme im Kontext von BEF Experimenten berücksichtigen die Sensitivität pflanzlicher Polyphenole hinsichtlich der Probenbehandlung und -aufbewahrung üblicherweise nicht. Es ist bekannt, dass pflanzliche Polyphenole Einfluss auf wichtige Ökosystemfunktionen, wie beispielsweise auf Nährstoffkreisläufe haben und daher von ökologischer Bedeutung sind. Daher können ökologische Schlussfolgerungen möglicherweise verfälscht werden, falls die suboptimale Behandlung der analysierten Proben nur unzuverlässige Aussagen über deren Polyphenolgehalt zulässt. Um die Auswirkungen einer solchen suboptimalen Probenbehandlung auf die Menge der extrahierbaren Polyphenole zu bewerten, wurden zwei Stichprobensätze von jeweils 20 Baumarten bezüglich der Konzentrationen an extrahierbaren Polyphenolen untersucht. Der erste der beiden Stichprobensätze wurde entsprechend dem etablierten Protokoll von Cornelissen *et al.* (2003) gesammelt, wohingegen der zweite Probensatz nach den Vorgaben des optimierten Protokolls gesammelt wurde. Darüber hinaus wurden beide Stichprobensätze zu unterschiedlichen Jahreszeiten gesammelt, ein Faktor der ebenfalls den Polyphenolgehalt in Pflanzenmaterial beeinflussen kann. Dementsprechend umfassten die Unterschiede zwischen den beiden Probensätzen alle Variationen auf, die für die Probennahme in BEF Experimenten typisch sind. Die Quantifizierung der Polyphenolgehalte erfolgte entsprechend den Methoden die im optimierten Protokoll beschrieben wurden. Die Variabilität in den Polyphenolgehalten, welche durch die suboptimale Behandlung der Proben hervorgerufen wurde, wurde mit der Variabilität verglichen, die sich durch die taxonomischen Unterschiede der einzelnen Pflanzenarten ergab. Um zu testen, ob diese taxonomischen Unterschiede in diesem Zusammenhang tatsächlich relevant sind, wurde das Grad der evolutionären Fixierung (phylogenetische Konserviertheit) dieser Blatteigenschaften untersucht. Es stellte sich heraus, dass sowohl die Totalphenol- als auch die Tanningehalte der unterschiedlichen Arten

hochkonserviert waren. Zudem zeigte sich, dass deren Fixierung im Vergleich zu sechs anderen untersuchten Blatteigenschaften wesentlich stärker war. Die Studie zeigte des Weiteren, dass die Variabilität, die durch die Unterschiede in der Probenbehandlung hervorgerufen wurde weitgehend durch die Variabilität der Polyphenolgehalte zwischen den Arten überlagert wurde. Eine sukzessive Reduktion des Artumfangs in der Analyse zeigte, dass der relative Einfluss der suboptimalen Probenbehandlung mit abnehmendem taxonomischem Umfang deutlich zunahm. Insgesamt zeigte sich dass der Totalphenolgehalt gegenüber suboptimalen Bedingungen während der Probennahme und deren Lagerung robuster waren als die Tanningehalte der untersuchten Proben. Abschließend wurden auf Grund nicht-metrischer multidimensionaler Skalierungen gezeigt, dass die Interpretationen, die auf Grund der beiden Datensätze gezogen wurden, zu vergleichbaren ökologischen Schlussfolgerungen führte. Zusammenfassen konnte gezeigt werden, dass ökologische Zusammenhänge, die auf der Basis von Informationen über den Gehalt an pflanzlichen Polyphenolen auch dann noch als zuverlässig angenommen werden können, wenn die analysierten Proben unter suboptimalen Bedingungen gesammelt wurden,. Dies trifft jedoch nur in solchen Fällen zu, in denen eine Vielzahl an Arten in die entsprechenden Untersuchungen mit einbezogen wurden.

Ein weiteres Hauptziel der vorliegenden Dissertationsschrift war es, den Einfluss von Polyphenolen in Blättern auf die Zersetzungsraten der Laubstreu von Pflanzengesellschaften entlang eines Gradienten sekundärer Sukzession zu untersuchen. In der zweiten Untersuchung, die Bestandteil der vorliegenden Arbeit ist, wurden die Änderungen von 14 Blatteigenschaften entlang von 27 Waldgemeinschaften untersucht, welche einen Bestandteil des BEF-China Projektes bilden. Hierbei wurden die untersuchten Blattmerkmale auf die Häufigkeit der untersuchten Arten in den entsprechenden Gesellschaften gewichtet. Die so erhaltenen Mittelwerte werden als *community weighted mean traits* (CWMs) bezeichnet. Die einbezogenen Blatteigenschaften wurden in vorangehenden Studien als relevant für die Zersetzbarkeit der Laubstreu eingestuft. Zusätzlich wurden die *in-situ* Raten der Laubstreuzersetzung (k-Raten) für jede der 27 Gemeinschaften berechnet. Zunächst wurden die Muster untersucht, welche der Verschiebung der CWMs entlang des Sukzessionsgradienten zugrunde liegen. Die gefundenen Verschiebungen folgten generell einem logarithmischen Muster, wohingegen die k-Raten der Waldgesellschaften keine signifikante Änderung entlang des Sukzessionsgradienten aufzeigten. Die gefundenen Änderungen in den CWMs der Blatteigenschaften deuteten auf eine Änderung in der Ressourcennutzungsstrategie hin: in jungen Waldgemeinschaften herrschte eine Strategie der hohen Nährstoffaufnahme vor, wohingegen in alten Waldgemeinschaften eine Strategie der starken Nährstoffkonservierung

vorherrschte. Weiterhin wurde untersucht, ob die beobachteten Änderungen der Blatteigenschaften in den Waldgesellschaften in Verbindung mit den Labstreuzersetzungsraten standen. Hierzu wurden multiple Regressionsanalysen durchgeführt. Es konnte gezeigt werden, dass die k-Raten der untersuchten Waldgesellschaften in Zusammenhang mit den untersuchten Blatteigenschaften standen. Blatteigenschaften die mit der Nährstoffqualität der Blattmischungen in Verbindung stehen, so z.B. der Phosphorgehalt, beschleunigten die Laubstreuzersetzung. Gleichzeitig erniedrigten Blattcharakteristika, welche die Widerstandskraft der Blätter in chemischer (pflanzliche Polyphenole) bzw. physikalischer (Blatthärte) Hinsicht erhöhen, die Zersetzungsraten der Laubstreu. Dies deutete darauf hin, dass die k-Raten von Waldgesellschaften nicht nur von einzelnen Blatteigenschaften abhängen, sondern dass sich die Zersetzungsraten aus dem Zusammenspiel verschiedener Blattcharakteristika ergeben, welche sich teilweise in entgegengesetzter Richtung auf die Zersetzungsraten auswirken.

Die dritte Untersuchung die im Rahmen der Vorliegenden Dissertationsschrift durchgeführt wurde, untersuchte pflanzliche Polyphenole im Zusammenhang mit der Blatthärte (leaf toughness, LT). Beide Blatteigenschaften können als Aspekte der der Blattverteidigung interpretiert werden; beide Blattcharakteristika werden aus dem Grundbaustein Kohlenstoff synthetisiert. Es ist denkbar dass Pflanzen, bedingt durch die beschränkte Verfügbarkeit an Kohlenstoff, diesen entweder in physikalische (LT) oder in chemische (hier: Polyphenole) Widerstandseigenschaften investieren können. Daher wurde für die hier beschriebene Untersuchung ein Trade-Off zwischen der Konzentration an pflanzlichen Polyphenolen und der Blatthärte postuliert. Ein Großteil der vorangegangenen Untersuchungen zu diesem Thema wurde auf der Grundlage von Artmittelwerten durchgeführt. Ein solches Vorgehen ist jedoch nur unter der Voraussetzung gerechtfertigt, dass die Unterschiede bezüglich der untersuchten Blattmerkmale *zwischen* den Arten größer sind als die entsprechenden Unterschiede *innerhalb* der einzelnen Arten. Daher wurde in der hier präsentierten Arbeit der relative Anteil der intraspezifischen Variabilität (ITV) der untersuchten Blattmerkmale bestimmt. Des Weiteren wurde der Effekt der Einbeziehung der ITV in die Analysen auf die Richtung und Stärke des vermuteten Trade-Offs untersucht. Ausgehend von den Erkenntnissen der ersten Untersuchung die Teil der vorliegenden Arbeit ist, wurde zusätzlich postuliert, dass sowohl die Polyphenolgehalte als auch die Blatthärte phylogenetisch hochkonserviert seien. Um diese Hypothese zu testen wurden Informationen zur Phylogenie der 51 untersuchten Arten mit einbezogen.

Regressionsanalysen auf der Ebene der einzelnen Spezies zeigten keine einheitlichen Muster, die auf einen generellen Trade-off zwischen dem Polyphenolgehalt und der Blatthärte schließen ließen. Während bei einigen Spezies der Polyphenolgehalt negativ mit LT korrelierte, zeigte sich für andere Baumarten ein positiver Zusammenhang zwischen diesen beiden Blattcharakteristika. Die meisten der untersuchten Arten zeigten keinen signifikanten Zusammenhang zwischen den beiden Aspekten der Blattverteidigung. Wurden die Analysen auf Grundlage von artspezifischen Mittelwerten über alle Arten hinweg untersucht, so zeigte sich, dass beide Blattmerkmale im Mittel negativ miteinander covariierten (d.h. ein Trade-Off wurde gefunden). Die Einbeziehung der ITV in diese Analysen schwächte den gefundenen Trade-off signifikant ab, hatte jedoch keinen Einfluss auf die Richtung des Zusammenhangs. Eine phylogenetische Analyse der drei untersuchten Blattcharakteristika zeigte ein starkes phylogenetisches Signal in allen untersuchten Blatteigenschaften (Totalphenol- und Tanningehalte sowie LT). Jedoch unterschied sich die Blatthärte bezüglich der Intensität und des Grades der Divergenz des phylogenetischen Signals deutlich von den Signalen der Polyphenolgehalte entlang der phylogenetischen Entwicklung der untersuchten Arten. Wurde diese phylogenetische Abhängigkeit bei den Untersuchungen zum Trade-Off zwischen den beiden Aspekten der Widerstandskraft durch stochastische Verfahren berücksichtigt, so zeigten sich über alle Arten hinweg starke negative Korrelationen zwischen der Blatthärte und den Polyphenolgehalten. Die Ergebnisse dieser Studie deuten darauf hin, dass die gefundenen Trade-Offs zwischen den Konzentrationen an Tanninen bzw. Phenolen und der Blatthärte weitgehend von evolutionären Zusammenhängen bestimmt wurden und nicht, wie ursprünglich angenommen, durch Beschränkungen in der Kohlenstoffverfügbarkeit.

Zusammenfassend machte die vorliegende Dissertation die Wichtigkeit pflanzlicher Polyphenole in Bezug auf wichtige Ökosystemprozesse wie Beispielsweise die Zersetzungsraten von Laubstreu deutlich. Darüber hinaus zeigte die Arbeit auch, dass pflanzliche Polyphenole evolutionär mit der Blatthärte zusammenhängen. Der beschriebene Trade-Off ist jedoch nicht direkt erkennbar, sondern findet seine Ursache tief in der Evolution der untersuchten Arten. Daher dürfen diese beiden Aspekte nicht unabhängig voneinander betrachtet werden, wenn es darum geht, um die funktionelle Bedeutung der Blattpolyphenole auf Ökosystemprozesse geht. Solche Untersuchungen sollten immer auf Basis der Kombination der beiden Blattcharakteristika durchgeführt werden. In dieser Hinsicht stellt das in dieser Arbeit beschriebene Zusammenspiel zwischen pflanzlichen Polyphenolen, der Blatthärte und anderen Blattcharakteristika die im Zusammenhang mit dem Nährwert der Blätter stehen, ein gutes Beispiel dar.

Schlussendlich ist die wichtigste Erkenntnis die im Laufe der vorliegenden Dissertation gewonnen wurde die, dass der Effekt pflanzlicher Polyphenole auf die Funktion von Ökosystemen nicht unter Ausschluss der Einflüsse anderer funktioneller Blattmerkmale durchgeführt werden sollte. Vielmehr sollten deren Auswirkungen in Kombination mit anderen Blattcharakteristika interpretiert werden, deren Einfluss teilweise entgegengesetzt zu dem der Polyphenole wirken kann.

1. Introducing the framework of Biodiversity-Ecosystem Functioning

1.1 Biodiversity and Ecosystem functions

One of the key achievements of the United Nations Conference on Environment and Development which took place in 1992 in Rio de Janeiro was the formulation of the Convention on Biodiversity (CBD). Within the CBD biodiversity has been identified as a key feature for the global stock of 'goods and services' that can be provided by ecosystems. The CBD defined biodiversity as 'the variability among living organisms from all sources, including terrestrial, marine and other aquatic ecosystems as well as the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems' (CBD, 2001). In addition, this definition also includes the different functions that the aforementioned components of biodiversity can have in ecosystems. However, in the last decades biodiversity was found to decrease at increasing rates (e.g. Millennium Ecosystem Assessment, 2005). The goods that are provided by ecosystems encompass vital aspects such as the provision of food and fibre, shelter and building materials. Important examples for regulating services provided by ecosystems are the sequestration of atmospheric carbon dioxide as well as the stabilization and moderation of the earth's climate. The basis on which ecosystems provide these services is the maintenance of numerous fluxes such as oxygen genesis, carbon uptake as well as nutrient cycling within these systems. The biological, geochemical and physical processes that take place within ecosystems are defined as ecosystem functions (Maynard *et al.* 2010). These functions determine the productivity, decomposition and nutrient retention capacities of an ecosystem.

Biodiversity itself is not only dependent on these functions, but also exerts influence on ecosystem properties (Loreau *et al.* 2002). During growth, plants take up nutrients from the soil and shed woody detritus and leaves which are ultimately mineralized by destruents (e.g. bacteria and fungi). By this, the plants feed back to the soil nutrient status. Soil fertility, in turn, is not only dependent on plants but also on the complex interplay between plants, soil fauna and microorganisms. These organisms create rich top soils by cycling nutrients from both decaying organic matter and mineral-rich bedrocks (Blume *et al.* 2010). With their ability to produce biomass through photosynthesis, plants form the major proportion of primary producers. They provide the basis for the majority of goods and services in most

ecosystems, and thus a great proportion of biodiversity research is carried out on plants (Balvanera *et al.* 2006). The present thesis addressed one particular aspect of BEF relationships in forest ecosystems: the effects of leaf traits with emphasis on leaf polyphenolic substances on leaf litter decomposition in forest communities (see Chapter 4.3).

1.2. Linking biodiversity and ecosystem functions: The biodiversity ecosystem experiments

Recognizing the importance of the complex interplays between biodiversity and ecosystem functions, ecologists met at a conference held in Bayreuth, Germany, in 1991 which was initiated by E.D. Schulze and H.A. Mooney (Schulze and Mooney 1993). They invited community ecologists (which initially were interested in the effect of extrinsic factors such as site fertility, disturbance or climate on biodiversity) and ecosystem biologists (mainly focusing on the rates and stability of fluxes in ecosystems) to join their efforts in synthesizing studies that considered both, biodiversity and ecosystem functioning. Since then, the interface of biodiversity and ecosystem functioning became a major focus of contemporary ecology (Naeem *et al.* 2002).

The central hypothesis in biodiversity and ecosystem functioning theory states, that a decline in biodiversity will result in reduced ecosystem-level processes (the so-called biodiversity-complementarity hypothesis, see Naeem *et al.*, 2002). This hypothesis predicts higher over-all means in ecosystem functions (e.g. total biomass production) in plant communities comprising species mixtures than expected from monocultures. During the following decades, numerous studies on this topic have been carried out, leading to the establishment of many experiments trying to link biodiversity and ecosystem functioning (hereafter BEF).

Reducing natural complexity in the framework of experimental set-ups has always been a promising way to address relevant questions in complex systems. Early experiments were conducted by keeping the majority of putative influential factors (e.g. light, temperature) as constant as possible while only varying the levels of biodiversity (i.e. number of species in the experiment). To this end, Naeem and colleagues (e.g. Naeem *et al.* 1994; Naeem *et al.* 1995) established the first biodiversity experiment, carried out in the 'Ecotron' in the early to mid-nineties. This facility provided full control over environmental factors such as air temperature, air flow, relative humidity, water supply to the plants and initial soil conditions (Lawton *et al.* 1993). Across three hierarchical levels of biodiversity, where the less diverse ecosystem was

designed as a subset of the species included in the next higher diversity level (thus simulating species loss), the authors could demonstrate the importance of biodiversity on important ecosystem functions, e.g. CO_2-fluxes and primary productivity. The highest diversity levels were found to perform best (Naeem *et al.* 1994). However, despite the impressive groundwork provided by the Ecotron experiment, the design of this experiment was unapt to answer many issues that emerged out of the complexity of biodiversity and ecosystem functioning. Some of the main weaknesses of the Ecotron design were the absence of external, non-constant influential factors such as climate and the fact that species loss in the hierarchy was random, which is unlikely in natural ecosystems (e.g. Grime 2002). Moreover, this design lacked the ability to identify and quantify the so-called sampling effect.

The sampling effect describes the fact that, the higher the number of species in a community, the higher the probability to include a species with well defined effect on the ecosystem function/process under consideration. Ignoring this sampling effect inevitably leads to the impossibility to disentangle other biodiversity effects such as species complementarity (i.e. non-additive effects of species on the respective function) from idiosyncratic effects evoked by adding a particular species.

This critique led to improved experimental designs which included either a subset of environmental variables (e.g. mesocosms with controlled soil fertility placed outside to ensure natural in-situ climatic variation like temperature and rainfall) or by deliberately establishing the experiments in natural ecosystems. Mesocosms have often been criticized to only comprise synthetic, unnatural communities or only a subset of the natural biodiversity. Moreover, the inclusion of only a subset of environmental factors has been criticized, as this renders it difficult to infer results from such experiments to real communities or ecosystems (Srivastava and Vellend 2005).

Establishment of experiments in natural ecosystems, however, has been criticized for the fact that there is no experimental control possible. The results are only of correlative nature and thus may be interpreted differently under different aspects. Moreover, due to the complexity of natural ecosystems, such experiments do not allow for straightforward replication. This is because confounding factors such as soil conditions or rain events cannot be controlled in natural ecosystems. Attempts have been made to circumvent the problem of non-replicability of in-field experiments such as choosing sites with similar abiotic attributes as well as by recording *in-situ* environmental factors. For example, in the BEF-China project study plots were established in an evergreen broad-leaved subtropical rainforest. For each of these plots,

environmental factors such as soil nutrient state, light inception and microclimatic conditions have been determined *in-situ* (see Chapter 2.1 as well as Bruelheide *et al.* 2011).

Important conclusions from previous BEF experiments were that, with increasing biodiversity, key ecosystem functions such as primary productivity or ecosystem resilience to severe disturbance events (e.g. drought) increased (Tilman and Downing, 1994). However, different ecosystems as well as ecosystem processes responded differently to changes in levels of biodiversity (Naeem *et al.* 2002), highlighting the importance of idiosyncrasy within ecosystems (Scherer-Lorenzen 2005).

In these initial experiments, the number of species included in a study has been often used as proxy for functional entities, and thus species richness was used as a surrogate for functional diversity. However, it turned out that the diversity of functional groups (e.g. herbs, forbs, legumes, perennials, evergreens) generally had more pronounced effects than the mere number of species (see Scherer-Lorenzen 2005 for a summary).

The insight that species, although different in their identity (e.g. species name, family affiliation), may be similar in terms of their characteristics triggered a different approach in BEF research: the use of functional traits.

1.3. The dawn of trait-based ecology: The concept of functional traits

In the so-called 'trait-based approach', species are characterized according to certain traits (e.g. morphological or physiological attributes) rather than being considered as taxonomic entities. After a thorough definition of the concept of 'functional traits' (i.e. 'a measurable and quantifiable morpho-physio-phenologic character that- directly or indirectly- impact fitness via their effects on growth, reproduction and survival'; Violle *et al.* 2007), scientists began to resolve species into more abstract, multidimensional entities based on the quality as well as quantity of multiple features. Depending on the amount of work required to obtain these traits, Hodgson *et al.* (1999) defined the terms of 'soft' and 'hard' traits. While the former can be assessed with comparatively low laboratory and time effort (e.g. fresh weight, dry weight, leaf area), the latter are collected with more expenditure (e.g. leaf anatomy, chemical composition).

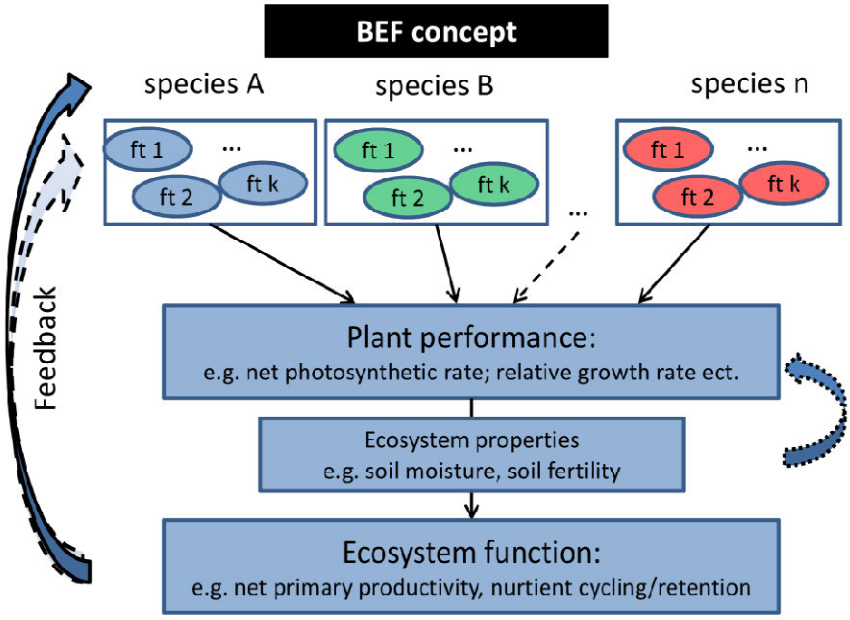


Figure 1-1: The integrative concept of functional traits in the BEF approach. Left hand side: BEF concept, integrating functional traits into ecosystem functioning; Species A to n indicate different species under consideration in the respective study; ft 1 to k: functional trait measured for individuals of certain species. Functional traits affect species' performances; species performances affect ecosystem properties (which may in turn affect the performance of certain species; dotted blue arrow); Ecosystem properties determine ecosystem functions. These, in turn feed back to either species composition (i.e. presence or absence of certain species; solid blue arrow) or affect individual trait values (i.e. phenotypic plasticity).

Basically, irrespective of traits being classified as 'soft' or 'hard', the morpho-physio-phenological characteristics defined as 'functional traits' influence performance of a certain individual (e.g. maximum relative growth rates, maximum photosynthesis rates). These functional traits, in turn, were found to affect ecosystem properties (Figure 1-1).

This trait-based approach also allows for generalization, as species are not characterized as distinct entities in trait-space but are resolved into a more general, multidimensional continuum of traits. Most importantly, this concept of functional traits allows to infer the effects of changes in certain traits on ecosystem functions from measurements based on the individual level to whole ecosystems (Figure 1-1).

As shown in Figure 1-1, while on the one hand, biodiversity (i.e. species and their connected functional traits) may affect ecosystem functions, these may feedback to biodiversity. This can be achieved via either changing the composition of species in the ecosystem, or by affecting the magnitude of trait expression of existing species (i.e. the so called 'phenotypic plasticity') or both (see e.g. Violle *et al.* 2007).

Albeit the fact that the magnitude of a trait may be affected by environmental conditions (Albert *et al.* 2012; Auger and Shipley 2013), the majority of contemporary BEF studies have been carried out using species mean values. As an inevitable consequence, these studies ignore intraspecific variability, i.e. the trait variability within species (but see e.g. Albert *et al.* 2010; Albert *et al.* 2011; Albert *et al.* 2012). Omitting intraspecific variability, however, is only valid under the assumption that the magnitude of intraspecific trait variability is negligibly small when compared to the variability between species (interspecific variability). The studies by Albert and colleagues have highlighted that this is not always the case. However, Auger and Shipley (2013) could show that intraspecific variability is most important when investigations are carried out at short environmental gradients (e.g. soil moisture) but decrease in importance when species mean values compiled from studies along more pronounced gradients (e.g. on landscape scale or global studies). One of the manuscripts presented in this thesis is devoted to this problem of intraspecific variability in investigations on trade-offs between leaf defense traits (see Chapter 4.4).

1.4. BEF-Experiments: early experiments and present state

In the last decades, experimental approaches have been successfully applied to in-field experiments such as grassland communities (e.g. Tilman and Downing 1996; Tilman *et al.* 1997; Hector *et al.* 1999; Weigelt *et al.* 2010) as well as in aquatic systems (e.g. Emmerson *et al.* 2001; Monaghan *et al.* 2001; Mehner *et al.* 2002). Due to the short-term character of such experimental set-ups (usage of organisms with short generation times, fast growing, often annual plant species), these systems were appealing for the aim to establish and test first hypotheses in BEF research.

Analyzing the draught resistance of experimental plots in the Cedar Creek Natural History Area, Minnesota USA, Tilman and Downing (1996) reported a higher resistance in communities with high species richness than in communities with lower numbers of species. They also found that species-rich plots recovered faster to pre-drought total biomass production and therefore exhibited a higher resilience than species-poor plots. This study has, however, bee criticised by many other scientists because of the sampling effect: species mixtures with higher diversity had a higher probability to contain drought-resistan species.

In a synthesis covering seven years of the Jena grassland Experiment, Proulx *et al.* (2010) found that plant species richness led to a decrease in the variability of community biomass production, resistance to non-resident plant species as well to a stabilization and an increasing

complexity of food webs across trophic levels. Thus, higher levels of biodiversity resulted in a higher stability of important ecosystem processes such as nutrient turnover. In the BIODEPTH experiment, covering experimental grassland plots across seven countries, Hector *et al.* (1999) demonstrated that species-richness as well as functional richness were both positively correlated with ecosystem primary productivity. At each location, plots with different levels of species-richness and functional group richness (i.e. communities either containing the functional groups 'herbs', 'legumes' or 'graminoids') or all possible combinations of these three groups were investigated.

Even with such interesting results, it remained unclear to which extent the basic results and lessons learned from short-term grassland experiments would to other, more long lived ecosystems such as forest communities. Thus, extending BEF experiments from grassland to forest ecosystems was considered an important step forward.

Forests, as plant communities with long lived individuals and slow generation turnover, are among the most important ecosystems. Similar to grassland ecosystems. forests provide ecosystem functions like carbon fixation, nutrient cycling and water purification (Vitousek and Reiners 1975; Ricketts *et al.* 2004; Obersteiner 2009). However, forest ecosystems cover approx 27% of the total land area but hold approx. 46% of the global total carbon stocks (IPCC, 2000). Therefore, an increasing number of forest-based BEF experiments have been established in all major biomes of the world (see e.g. Figure 1-2) to assess the effects of biodiversity on the functioning of forest ecosystems.

In general, the tendencies encountered in all BEF experiments (e.g. increasing species richness increases primary productivity) held across all studies. However, in studying plant communities across seven counties, Hector *et al.* (1999) found that, if the data for individual sites were analyzed separately, the results slightly differed from those obtained across all communities. The authors reported, that, depending on the county (i.e. study site), the results supported different hypotheses which were formulated to understand the connection between biodiversity and the functioning of ecosystems (for details see Hector *et al.* 1999). As all test sites however differed significantly in their climatic and ecological conditions, one may hypothesize that the mechanisms may vary over different climates or in different biomes. While this seems reasonable, Bengtsson *et al.* (2002) state that ecological studies are most relevant and thus powerful, when they are able to provide scale-independent results (e.g. reveal general principles that may explain the connection between biodiversity and ecosystem functioning).

In a joint approach, addressing the challenges to provide scale-independent principles as well as to test the transferability of results from short-term experiments (e.g. grassland communities, artificial microcosms) to long-term experiments (e.g. in forest BEF experiments), BEF scientists around the world decided to establish large databases. These databases, which contain information species specific traits as well as on ecosystem processes (e.g. primary productivity, litter decomposability), were also established to provide experiment-specific environmental factors such as e.g. climate, soil moisture and soil fertility. Sharing these information with other projects will increase the comparability across different experiments.

One example in which several BEF experiments joined together in a collective group is the TreeDivNet consortium (www.treedivnet.ugent.be). TreeDivNet currently comprises 17 forest-based BEF experiments situated in all major global biomes. With the exceptions of the sub polar and arctic climate zones, this consortium covers the most major climate zones (see Figure 1-2).

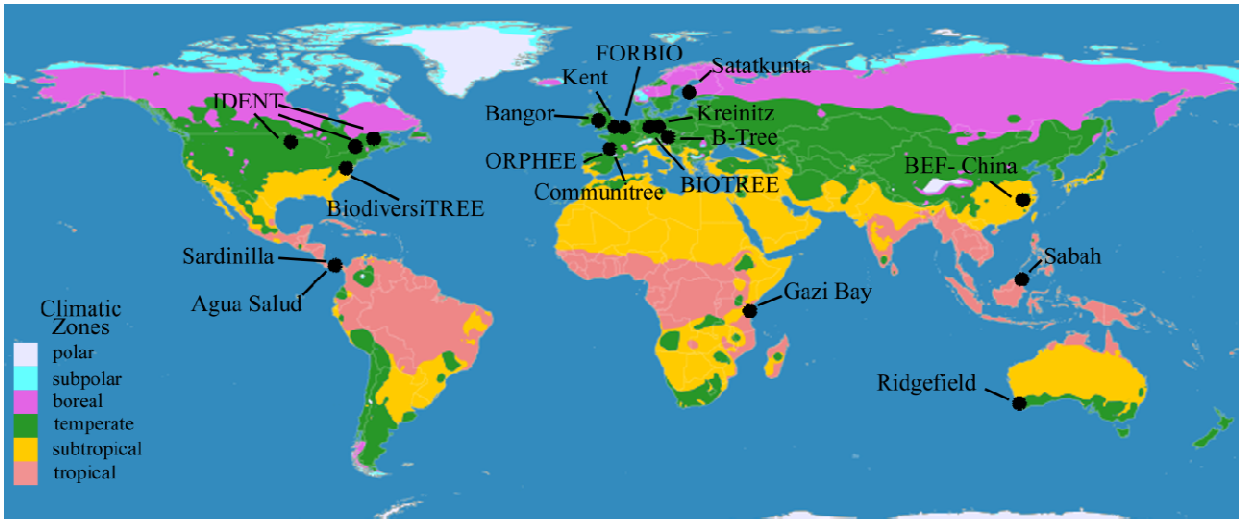


Figure 1-2: Location of the 17 forest-based BEF experiments cooperating in TreeDivNet. Color regions indicate global climate zones. Source: www.treedifnet.ugent.org; modified by Eichenberg

Recent meta-analytical approaches have increasingly utilized information provided by BEF databases (e.g. Koricheva *et al.* 2004; Wright *et al.* 2004; Vehviläinen *et al.* 2007; Freschet *et al.* 2010; Freschet *et al.* 2012; Moles *et al.* 2013). In most of these studies, climatic variables are used to either correct for climatic differences or incorporating these as covariates into their analyses. However, compiling trait information from different sources, e.g. databases, may not always be useful but may also bear some pitfalls. For example, traits may be collected or

determined according to different protocols in different projects. This may be obstructive when trait data from one experiment is mixed with trait information gathered in a different experiment according to different protocols and then the data is used to answer ecologically relevant questions. One of the manuscripts included in the present thesis addressed the problem of data comparability when samples have been collected according to different protocols (see Chapter 4.2).

2. The BEF-China project

As a member of the TreeDivNet consortium, BEF-China hitherto is the only BEF project situated in the subtropics. The project is located in southeastern China (see Figure 1-2 and Figure 2-1), approximately 400 km west of Shanghai.

With a mean annual temperature of 15.1 °C and mean annual precipitation accumulating to 1964 mm, the climate is characterized typical subtropical, exhibiting a wet season from May to June (Geißler *et al.* 2010). South-eastern China is recognized as one of the hotspots of terrestrial biodiversity with especially trees and shrubs featuring extremely high levels of diversity (Legendre *et al.*, 2009; Bruelheide *et al.* 2011, 2014). The BEF-China project was designed to investigate the role of tree and shrub diversity on important ecosystem functions such as primary production, element cycling (e.g. nutrients), species conservation as well as erosion control. Erosion control is especially important in wet, hilly terrains and is hitherto studied only in the BEF-China project.

The project has been established in 2008 near to the border area of the Jiangxi and Zhejiang provinces. It comprises two main platforms: on the one hand, experimental plantations were created, and on the other hand, plots were set up in a natural forest ecosystem close to the experimental sites (Figure 2-1). The forest plots were created in order to compare the results from the experimental set-ups to natural conditions.

2.1. The natural forest system: The comparative study plots

In 2008, the so-called comparative study plots (hereafter CSPs) were established within the boundaries of the Gutianshan National Nature Reserve (GNNR). In the Gutianshan region, some rainforest remnants in the vicinity of human settlements were declared as recreational feng-shui forests in 1975, prohibiting certain forms of land-use near rural settlements. In 2001, additional parts of the Gutianshan region were protected and now form a national nature reserve (see Figure 2-1). Due to this declaration and the implicit level of natural conservation, agricultural use in the periphery of the Gutianshan region ceased and left the forest to secondary succession. Nowadays, 57% of the total area of the GNNR is composed of natural forests (Legendre *et al.* 2009) that have never been under agricultural use, whereas other parts of the forest have undergone secondary succession since agricultural use has been abandoned.

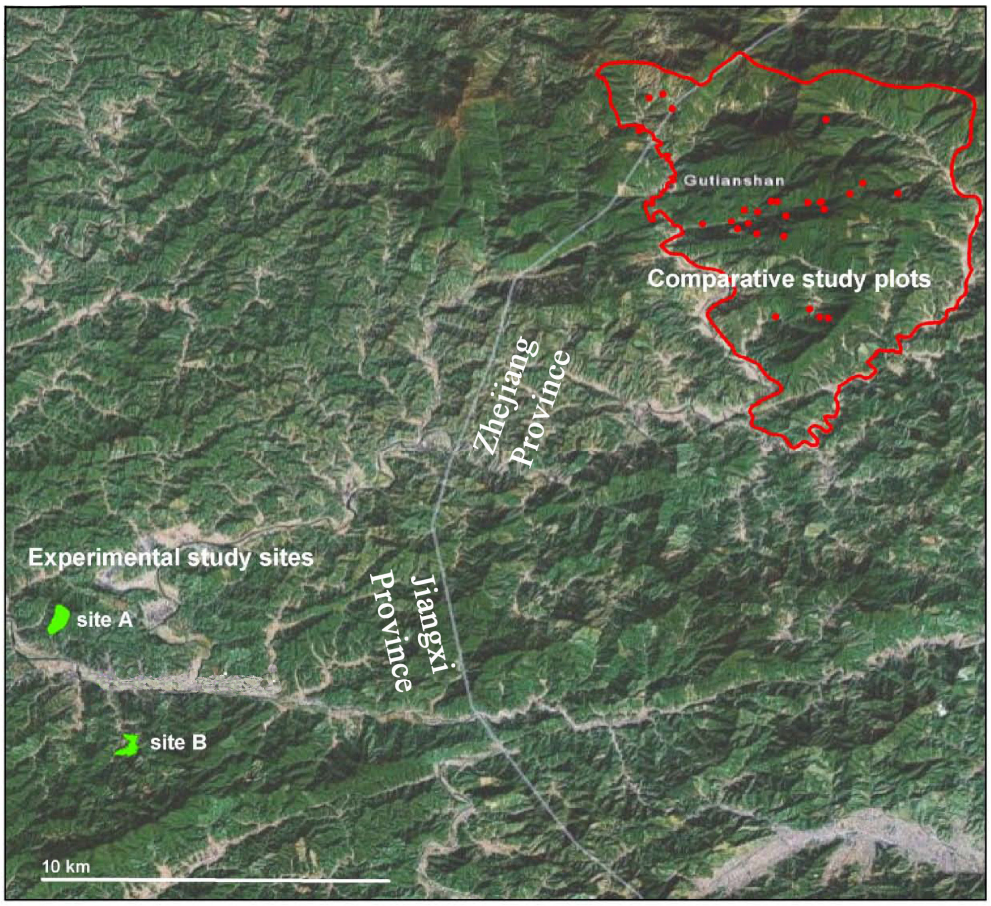


Figure 2-1: Study area of the BEF-China project; grey line: province borders. Red line marks the extent of the Gutianshan National Nature reserve. Red dots indicate the location of the 27 comparative study plots (CSPs). Green areas indicate the locations of the two experimental sites A and B.

Source: 'The role of tree and shrub diversity for production, erosion control, element cycling and species conservation in Chinese subtropical forest ecosystems", Bruelheide *et al.*, 2010, slightly adapted by Eichenberg

As a part of the global hotspots of biodiversity, the nature reserve comprises 1426 seed plant species from more than 600 genera and 149 families, of which more than 440 species are woody (Michalski and Durka, 2013). Within the GNNR, 27 plots of 30 x 30m (the CSPs) have been established in 2008, some of them on very steep slopes (up to 40°). The gradient covered within the CSPs follows a gradient of secondary succession from approx. 20 years to more than 80 years of natural forest succession. Moreover, the CSPs also span a gradient of species richness. Within these 27 plots, a complete species inventory detected a total of 148 woody tree or shrub species from 46 families (Bruelheide *et al.* 2011). The highest species richness encountered within a single plot accumulated to 69 species. Stand ages were

determined by counting tree rings from core drillings of the fifth largest tree in the respective CSP (see Bruelheide *et al.* 2011 for further details).

2.2. The experimental settings: Experimental sites A and B

In parallel to the establishment of the CPSs, two experimental sites (Site A and Site B) have been created on freshly clear-cut areas, previously used for agroforestry. The two experimental sites are located approx. 40 km west of the GNNR (Figure 2-1). In total, both sites comprise an area of approx. 50 ha, with site B being slightly larger in extent. In total, 60 woody species (42 tree species and 18 shrub species from the regional species pool) were planted within the experimental sites. Species were planted across sites A and B with different compositions in the site-specific species pool (see Table 2-1). Within the sites a total of 566 (271 and 295 in site A and B, respectively) plots of 1 mu (a traditional Chinese area unit; approx. 26 x 26 m) were defined. Digital elevation models were recorded for each site in order to obtain geographical information (e.g. inclination, height a.s.l.) for each of the 566 plots.

Trees were grown from seeds collected in the GNNR and raised in a local nursery. Between 2008 and 2009 the tree saplings were planted in equal densities of 400 individuals per plot. The plots were designed to contain different levels of species diversity (see Chapter 2.3). In total, approximately than 250.000 tree and shrub seedlings have been planted in defined species combinations with defined distances between each individual. Further details on the plot establishment, environmental conditions and planting schemes are given in Yang *et al.* (2013) as well as in Bruelheide *et al.* (2014).

Table 2-1: List of tree and shrub species planted in BEF-China as well as their location on the experimental sites.

Tree Species*	Experimental Site	Tree Species*	Experimental Site	Shrub Species*	Experimental Site
Acer davidii	A	*Daphniphyllum oldhamii*	A&B	*Ardisia crenata*	A
Castanopsis carlesii	A	*Diospyros glaucifolia*	A&B	*Camellia chekiangoleosa*	A
Choerospondias axillaris	A	*Lithocarpus glaber*	A&B	*Distylium buxifolium*	A&B
Cyclobalanopsis myrsinifolia	A	*Pinus massoniana*	A&B	*Distylium myricoides*	A
Koelreuteria bipinnata	A	*Schima superba*	A&B	*Eurya muricata*	A
Liquidambar formosana	A	*Ailanthus altissima*	B	*Raphiolepis indica*	A
Melia azedarach	A	*Alniphyllum fortunei*	B	*Syzygium buxifolium*	A&B
Nyssa sinensis	A	*Betula luminifera*	B	*Gardenia jasminoides*	A
Quercus acutissima	A	*Castanopsis fargesii*	B	*Itea chinensis*	A
Quercus fabri	A	*Celtis biondii*	B	*Loropetalum chinense*	A
Quercus serrata	A	*Elaeocarpus chinensis*	B	*Viburnum setigerum*	B
Rhus chinensis	A	*Elaeocarpus glabripetalus*	B	*Photinia hirsuta*	B
Sapindus saponaria	A	*Elaeocarpus japonicus*	B	*Itea chinensis*	B
Triadica cochnichinensis	A	*Idesia polycarpa*	B	*Rhododendron ovatum*	B
Triadica sebifera	A	*Machilus grijsii*	B	*Phyllanthus glaucus*	B
Castanea henryi	A	*Machilus thunbergii*	B	*Ficus erecta*	B
Castanopsis eyrei	A&B	*Machilus leptophylla*	B	*Rhododendron simsii*	B
Castanopsis sclerophylla	A&B	*Manglietia yuyuanensis*	B	*Hydrangea chinensis*	B
Cinnamomum camphora	A&B	*Meliosma flexuosa*	B		
Cunninghamia lanceolata	A&B	*Phoebe bournei*	B		
Cyclobalanopsis glauca	A&B	*Quercus phillyraeoides*	B		

* species nomenclature follows the taxonomy given in the 'Flora of China'. In addition, information on the taxonomic authors can be obtained from: http://www.efloras.org and from Bruelheide et al (2104)

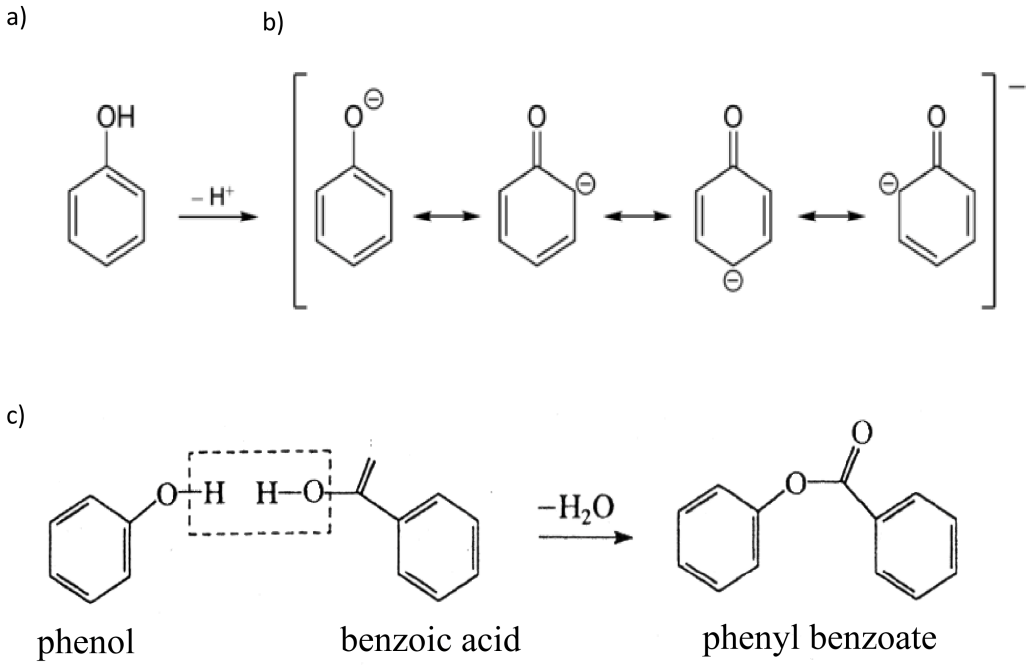
3. Plant Polyphenolics

Although the following paragraphs will be highly technical, their main purpose is to demonstrate the enormous variety and complexity of the group of plant secondary metabolites subsumed under the term 'polyphenols'. More general definitions for plant polyphenolics have been given by Bate-Smith and Swain (1962) and Haslam (1989), who state that polyphenols are water-soluble phenolic compounds with molecular weights between 300 and 20000 (note that relative molecular weights are normalized on $^{1}/_{12}$ of the mass of the ^{12}C atom and thus, *per definitionem*, have no unit). They bear chemical properties typical for phenolic substances, such as acidity and the propensity to be oxidized (Hagerman 2002). An additional important feature is their ability to precipitate alkaloids and proteins (Haslam 1989).

In contrast to Waterman and Mole (1994), Haslam (1989) used the term 'polyphenols' as a synonym for tannins. In order to avoid confusion between the definition of 'polyphenols' from Haslam (1989) and that of Waterman and Mole (1994), in the present thesis the terms 'non-tannin phenolics' will be used for low-molecular weight phenolic substances, whereas the term 'tannin' will be used for highly polymerized macromolecules (comprising condensed as well as hydrolysable tannins, see Chapter 3.2). The term 'polyphenols' will be used as a collective term for both, tannins and non-tannin phenolics. The term 'total phenolics' will be used to describe the combined quantity of non-tannin phenolics and tannins.

3.1. General characteristics of phenolic substances

Chemically, phenolic compounds are defined by the presence of at least one aromatic ring, bearing one or more hydroxyl (OH) substituents. The simplest phenolic molecule is Phenol (see Scheme 3-1a) with one aromatic ring and one hydroxyl group. Compounds that bear more than one hydroxyl substituent are often referred to as 'polyphenols' (Waterman and Mole 1994).



Scheme 3-1: Phenol and the phenolate ion (panels a and b) and phenol-esterification, a typical phenol reaction (panel c). Panel a) chemical structure the most simple phenolic molecule, Phenol; b) Phenolate-ion and its mesomers; c) esterification of phenol and an organic acid (here: benzoic acid).

Source: Waterman and Mole (1994)

A very important property of the phenolic hydroxyl group is their acidity, which clearly separates phenolic OH groups from alcohols (Waterman and Mole, 1994). Due to the dislocation of the double bonds within the aromatic ring, the bond between oxygen and hydrogen in phenolic substances has an increased propensity to break and form the negatively charged ion, the phenolate ion and its mesomers (Scheme 3-1b).

Phenolate ions, in turn, are the reactive forms of phenolic substances and can form esters with alcoholic OH groups (Scheme 3-1c), carboxylic acids or another phenolic OH group. In this way, phenolic monomers can polymerize to more complex molecules. In addition, phenolate ions may transfer their electron to oxygen molecules to form the negatively charged superoxide

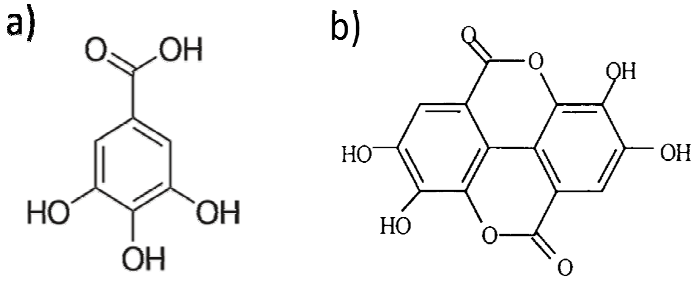
anion radicals ($O_2^{-\bullet}$). These superoxide anion radicals are highly reactive and very dangerous, e.g. to cells. These radicals may cause lipid peroxidation, enzyme inactivation or strand breaks in DNA (Appel 1993)

Based on the identity of the phenolic core molecule as well as the complexity of the corresponding molecule, highly variable macromolecules may be formed. While polyphenolic substances can be ubiquitously found in fungi, algae, bacteria, plants and animals, the present thesis will focus on plant polyphenols found in leaf tissue. Plant polyphenols are found in all major families of higher plants (Haslam 1989). Nowadays, a multitude of biological activities of this class of substances have been described (see Chapter 3.3) and a variety of methods for quantification and characterization of polyphenolic substances is available (see Chapter 3.4).

3.2. Groups of polyphenols

According to Hättenschwiler and Vitousek (2000) polyphenols can be roughly divided into two groups. The first group consists of compounds with low molecular weight, such as gallic acid. Gallic acid is one of the most important low molecular weight phenols in plants (Waterman and Mole 1994). Note that this rather simple molecule already bears three phenolic OH groups as well as one carboxylic acid group (see Scheme 3-2a). A typical and widespread polymer of gallic acid is ellagic acid (see Scheme 3-2b). In ellagic acid, two gallic acid molecules are double-esterified via their carboxyl groups. Low molecular weight (poly)phenolics were found to occur universally in higher plants, some of them common in a variety of plant species while others are highly species-specific.

The second group, with even more complex molecules of high molecular weight, has been found to be most abundant in woody plants (Swain 1979; Haslam 1989). More generally, these high molecular weight polyphenols are polymers of flavan-3-ols (Hagerman 2002). The flavan-3-ols are heterocyclic macromolecules that carry several phenolic OH groups. These high molecular-weight polyphenolics are mainly oligomers or polymers of catechin or epicatechin (see Schemes 3-3a and b).

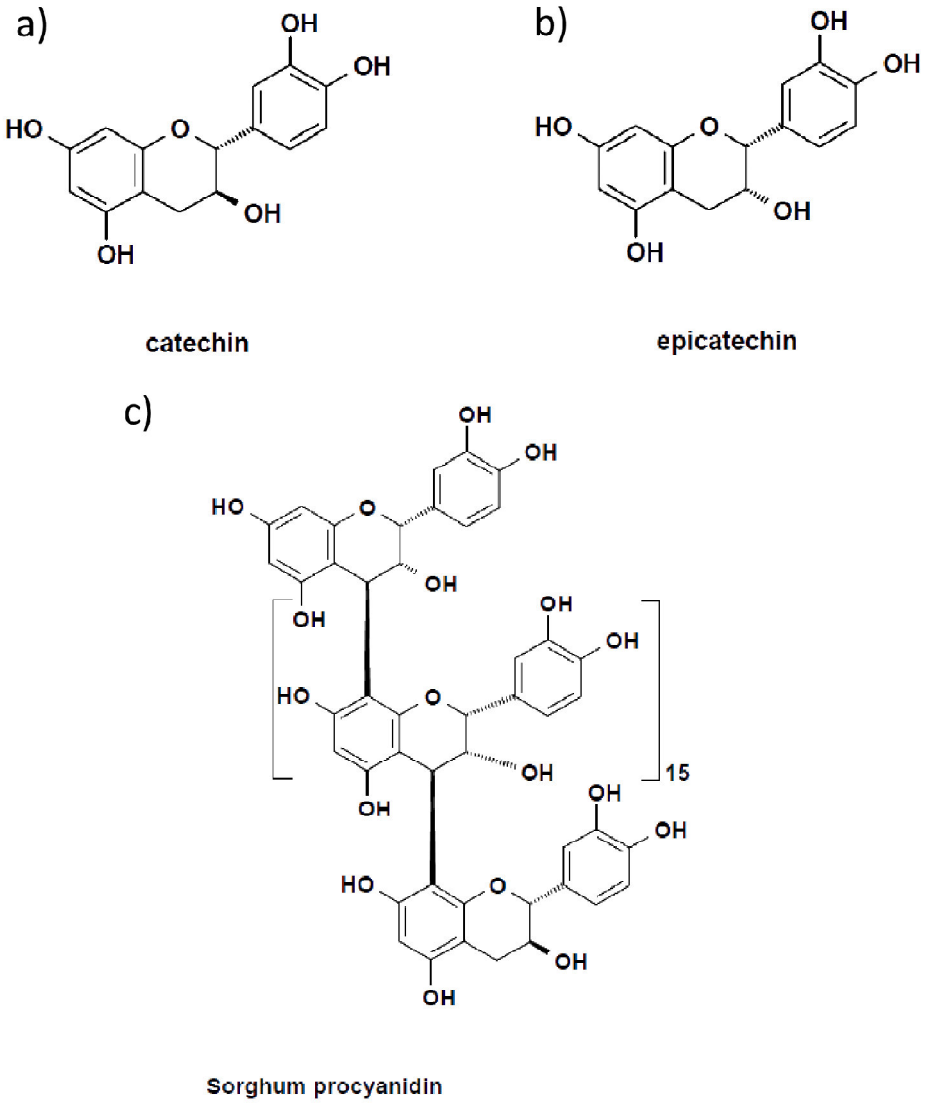


Scheme 3-2: Chemical structures of gallic acid (panel a) and its dimer ellagic acid (panel b)

Source: Hagerman (2002)

As it is the case for simple phenolic substances (low molecular weight phenolics) flavan-3-ols may form polymers with OH groups of alcohol, carboxyl groups from carboxylic acids or phenolic OH groups from other (macro-)molecules, too. Higher flavan-3-ol polymers are also known as 'proanthocyanidines' or 'condensed tannins'. One of the best characterized condensed tannins is sorghum procyanidin (found in *Sorghum spec.*, Hagerman 2002). Sorghum tannin is a high molecular-weight polyphenol composed by polymerization of 17 epicatechin molecules as the sole component substance. In addition, a huge variety of extremely diverse condensed tannins have been described (e.g. Haslam 1989; Waterman and Mole, 1994; Hagerman 2002).

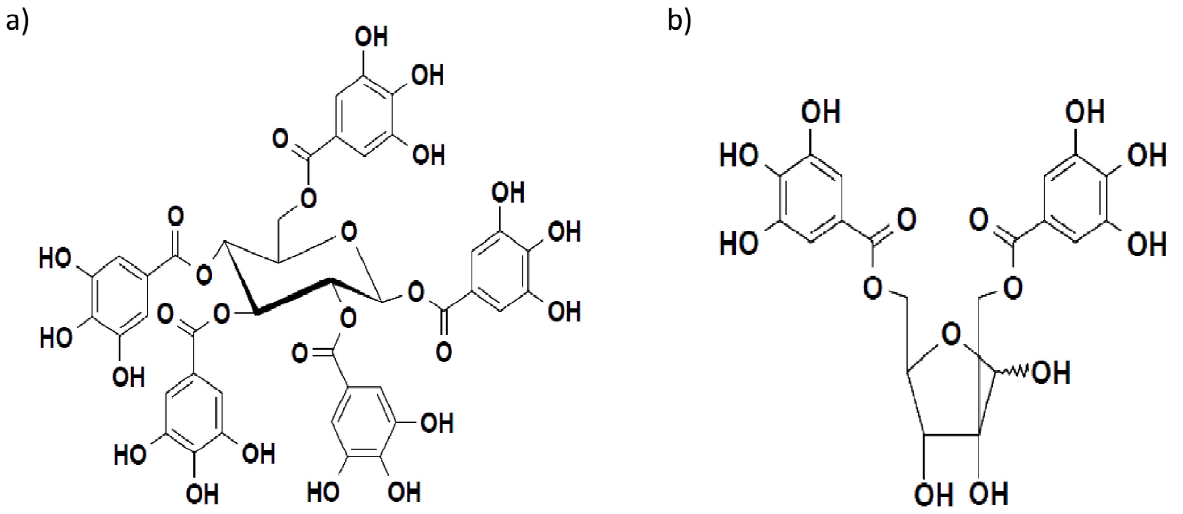
Besides condensed tannins, a second group of tannins has been described (e.g. Okuda *et al.* 1987; Okuda *et al.* 2009). The second group, the so-called 'hydrolysable tannins', is based on polymers of gallic acid (gallotannins) or ellagic acid (ellagitannins), coupled to glucose as a core molecule (see Scheme 3-4).



Scheme 3-3: Typical structures of the chemical group of flavan-3-ols (panels a and b) as well as one well characterized polymer (panel c). Note: catechin (panel a) and epicatechin (panel b) are isomers of basic flavan-3-ols, differing in the position of the aromatic ring and its adjacent OH group relative to the level of projection; Sorghum procyanidin (panel c) is a well characterized condensed tannin formed by polymerization of 17 flavan-3-ol monomers.

Source: Hagerman (2002)

Condensed tannins and hydrolysable tannins may polymerize into highly complex macromolecules. Examples for simple ellagitannins are penta-gallolyl-glucose (Scheme 3-4a) and hamamelitannin (isolated and characterized from *Hamamelis virginata* L. bark; see Scheme 3-4b). Hydrolysable tannins have been characterized from several herbaceous species but were found to be absent in most woody species (Swain 1979; Haslam 1989; Okuda *et al.* 2009). In common foodstuff their occurrence is restricted to a few fruit and nut species (Clifford and Scalbert 2000).



Scheme 3-4: Structures of simple hydrolysable tannins. Penta-gallolyl-glucose (panel a) is a widespread hydrolysable tannin encountered in various plant species; Hamamelitannin (panel b), a hydrolysable tannin isolated from *Hamamelis virginata* L.

Source: Hagerman (2002)

3.3. Ecological significance of polyphenols

The idea that polyphenolic substances may influence plant performance as well as interactions with other organisms through their chemical properties and biological activity slowly permeated ecological thinking in the 1960s and 1970s (Waterman and Mole, 1994). Even though this group of compounds was known before that time, they were found to have no direct role in the physiological processes of plants. These substances were classified as secondary metabolites which are produced during biosynthetic processes of the primary (vital) metabolism. However, humans have unknowingly utilized polyphenolic substances for centuries. Examples are the acetylsalicylic acid, which is a polyphenol extracted from willow bark as a medicine, or the tanning of animal skins in leather production, where polyphenolic substances are used in handcraft.

The roles polyphenols play in plant biology are various and often compound-specific (Waterman and Mole 1994; Kraus *et al.* 2003a). They have been found to act as protectants against environmental stresses. They may inactivate heavy metal ions by chelating them

(McDonald *et al.* 1996) and thus ameliorating their toxicity. They may protect photosynthetically active plant tissues against oxidative damage generated by UV-induced stress through their antioxidative activity (Close and McArthur 2002). Moreover, polyphenolic substances may scavenge toxic radicals such as the reactive oxygen species resulting from excess light (e.g. Kandaswami and Middleton 1994; Tattini *et al.* 2004). It has also been hypothesized that flavonoids (a group of plant condensed tannins) may be involved in protective processes that mitigate deleterious incidents during drought as well as frost events (Pizzi and Cameron 1986; Chalker-Scott and Krahmer 1989; Tattini *et al.* 2004).

Nowadays, various methods are available forthe quantification, isolation and identification of (plant) polyphenols (e.g. Hagerman 1987; Hagerman 1988; Waterman and Mole 1994; Hagerman 2002; Salminen and Karonen 2011). Once early experiments had elucidated potential functions of polyphenolic substances, a new field in ecology emerged. At that time, the 'chemical ecology' became one of the fastest growing fields in ecological research (Harborne 1989). Since then, a large array of biological activities of natural phenolic compounds has been detected. The possibility to relate some types of activity to readily quantifiable level of this class of secondary metabolites has further increased the pace of research.

The following chapter gives an overview of the current status of the roles of polyphenols in ecosystems and their functions.

3.3.1. Plant-plant interactions

Various polyphenolic substances (mainly flavonoids) have been reported to inhibit growth and seed germination of conspecific as well as heterospecific plant species (a feature termed 'allelopathy'). One of the best known allelopathic substances is juglon (e.g. Bahuguna *et al.* 2013), a polyphenol exuded from roots and leaves of the walnut tree (*Juglans regia)*. Other, more simple substances such as (-)-catechin were identified as allelopathic substances exuded by the roots of *Centaurea maculosa* (Bais *et al.* 2003). Allelopathy is thought to reduce competitive pressure for nutrients and light (Treutter 2005), thus directly affecting plant individual fitness. The allelopathic effect of polyphenolic substances may either result from direct impact on physiological processes during germination and growth (a field in which the mode of action is poorly understood), or by indirectly affecting growth via soil-related effects on microorganisms (see next chapter as well as reviews by Chou 1999 and Inderjit and Gross 2000). Through allelopathy, certain plant species have been discussed to increase their potential

to invade new habitats by e.g. reducing below-ground competition for nutrients by inhibition of seed germination or root growth of native species (e.g. Ridenour and Callaway 2001).

3.3.2. Plant-microbe interactions

The roles of polyphenols in plant-microbe interactions are manifold, reaching from positive interactions (e.g. stimulation of symbiont growth such as of nitrogen fixers or mycorrhizae) to defense against microbial pathogens (e.g. antibacterial and antifungal activities). It has been reported that several flavonoids exuded from plant roots may act as biochemical signal substances that induce the transcription of genes in N-fixing bacteria. Synthesis products of these genes were reported to be required to successfully infect plant roots (see e.g. Hungria and Stacey 1997). This group of secondary metabolites has furthermore been reported to influence the magnitude of nodulation of red alder (*Alnus rubra*) induced by the N-fixing actinomycete *Frankia spec.* (Benoit and Berry 1997). Moreover, antifungal activities of phenolic compounds from higher plants have also been frequently reported (e.g. Grayer and Harborne 1994). For example, Skadhauge *et al.* (1997) reported that polyphenolic substances are involved in the defense of barley species against the parasite fungi *Fusarium spec.* Other *in-vitro* studies provided evidence for a structure-activity related relationship between the anti-fungal activity and the growth-inhibitory effect on fungal pathogens such as the rice pest *Pyricularia oryzae* as well as the bacterial pathogen *Xanthomonas oryzae* (Padmavati *et al.* 1997). Colonization of plant roots by mycorrhizal fungi is also discussed to be affected by polyphenols, but different types of polyphenols were reported to have opposite effects (e.g. Leake and Read 1990; Nilsson *et al.* 1993). Interestingly, different pathogens responded differently to different polyphenolic compounds in these laboratory trials. This indicates a compound-specific as well as a compound-species-specific effect of polyphenols on plant pathogens.

3.3.3. Plant-soil interactions

In addition to plant mediated effects on sub-surface processes by affecting microbial communities, plant polyphenols have been recognized to directly regulate soil processes related to plant nutrient availability. It has been suggested that some polyphenols may inhibit nitrification (e.g. Baldwin *et al.* 1983; Hättenschwiler and Vitousek 2000) and evidence has been provided for their importance in litter decomposition (e.g. Hättenschwiler *et al.* 2005 as well as Chater 4.3 of the present thesis).

Leaf polyphenols are frequently related to altered litter quality in terms of decomposition, as polyphenol contents have been found to have a larger negative effect on litter decomposition than other frequently measured leaf and leaf litter traits such as nitrogen or phosphorous content or lignin (Palm and Sanchez 1990; Parsons *et al.* 2011).

Besides these rather indirect effects of polyphenols on nutrient cycling, these secondary metabolites can also directly interact with nutrient cycling in various other ways that go beyond simple negative correlations between phenol concentrations and decomposition rates.

Polyphenols have been reported to alter soil nitrogen availability by the formation of polyphenol-protein complexes, being resistant to most groups of decomposers (Northup *et al.* 1995; Hättenschwiler and Vitousek 2000). Thus, polyphenols may precipitate organic N compounds in dissolved organic matter, reducing the plant available organic N fraction in soils.

In addition, adsorption of phenols to clay minerals has been reported. Polyphenol adsorption may be antagonistic to adsorption of other micro- and macronutrients anions to soil clay minerals. This may increase nutrient loss through leaching (e.g. Qualls and Haines 1992). On the other hand, polyphenols have also been reported to retain exchangeable inorganic cations such as Ca^{2+}, Mg^{2+} and K^{+} in highly leached, acidic soils by providing sorption sites for these ions (e.g. Schnitzer *et al.* 1984; Zech *et al.* 1997).

3.3.4. Plant-insect interactions

Numerous types of plant-insect interactions based on polyphenolic substances have been reported. Polyphenols are found in flower pigments (so-called anthocyanidines) of many different plant species and have been discussed as attractants for pollinators (e.g. Harborne and Smith 1978; Saito and Harborne 1992; Abrol 2012).

Besides their role in stimulating pollination, plant polyphenols have often been discussed as a means of defense against herbivores (e.g. Feeny 1976). Again, the role of this group of secondary metabolites in this context is complex and often multifunctional (Treutter 2005). Plant phenolics may influence food preference of many herbivores, including insects (Hemingway *et al.* 1989), birds (e.g. Jakubas and Gullion 1990) and mammals (e.g. Robbins 1991). In this sense, effects of plant polyphenols may be both, detrimental as well as beneficial, for the herbivore. While phenolic substances have been reported to have negative effects on non-adopted generalist herbivores by reducing the survival rates in larvae of silk moths feeding on polyphenol-rich tissue (Matsuki and Koike 2006), other, more specialized herbivores (e.g.

some highly specialized Lepidoptera) may sequester phenolic compounds. These compounds may then serve as toxins in order to protect themselves against predators (Hesbacher *et al.* 1995) or as pheromones to attract mates (Simmonds 2003).

As already mentioned, rates of herbivory have frequently been reported to be negatively correlated with tissue polyphenolic contents (e.g. Eichhorn *et al.* 2007). Moreover, in addition to direct effects on insect herbivory, some plant species are reported to release polyphenolic substances that attract specialized parasitoids to the respective herbivore (e.g. Bruinsma *et al.* 2009). For example, the growth and survival of some parasitoid larvae were reported to be reduced when phenolics were included in the diet of the host insect (Bloem and Duffey 1990), thus having a negative effect on the parasitoid. This is an impressive example how tannins and non-tannin phenolics may also exert influence across several trophic levels. Thus, phenolics may have negative, positive or even neutral effects on herbivores. The type and mode of action is often dependent on the respective organism as well as the situation (Appel 1993). Phenolics may directly affect the herbivore through their influence on feeding preference (e.g. as repellents) or indirectly through its natural enemies or symbionts. In addition to their role as repellents, especially tannins are thought to affect herbivores through digestion inhibition. The inhibitory activity assumedly results from complexation of polyphenols with dietary proteins or digestive enzymes in herbivore guts. Thereby tannins reduce the nutritive value of the ingested food (Appel 1993).

Besides the reduction of the dietary value through complexation with proteins, phenolics were often reported to be toxic to the herbivore probably as an effect of oxygen radicals that are frequently formed during phenolic oxidation (Iason and Villalba 2006). These radicals are thought to disrupt membrane integrity in herbivore gut epithelia that are exposed to novel tannins (e.g. McArthur *et al.* 1991; Raubenheimer 1992). Another possibility to explain the toxic effects of polyphenols is the inhibition of the antioxidant enzyme system of herbivores, thereby further enhancing the detrimental effect of oxygen radicals (Lee 1991). Accordingly, as also stated in Chapter 3.1, the oxidized form of phenols, the phenolate-ion readily formed during diverse polyphenolic reactions, is the most reactive form of polyphenols. Thus, biological activity of phenols requires oxidation in many cases (Appel 1993). Owing to the structural complexity of polyphenol molecules and their various modes of action, the biological activity largely depends on the identity of the molecule as well as extrinsic factors such as the pH value which affects the oxidative status of the phenolic substance.

As demonstrated in the previous paragraphs, polyphenolic substances and their interaction with other ecosystem compartments are highly relevant in the context of biodiversity-ecosystem functioning. Albeit the role of polyphenols are manifold in different aspects of an ecosystem, one of the most proximate effects of this group of secondary metabolites is its effect on nutrient cycling via its effect on microbe, soil and herbivore interactions.

Focusing on plant leaves, being ephemeral plant organs that are continuously replaced, the present thesis investigates the role of tannins and non-tannin phenolics in plant leaves on nutrient turnover through their influence on leaf decomposability and leaf defense. In Chapter 4.3. the role of polyphenolic substances in nutrient turnover in communities through their influence on leaf litter decomposability is addressed. In Chapter 4.4 this thesis considers the role of polyphenols in the context of plant defense strategies together with leaf toughness as an additional aspect of leaf defense.

3.4. Challenges in interpreting polyphenolics in the context of BEF

Because tannins can play such variable, often contrasting biological roles and because of the enormous structural variation among tannins it has been difficult to develop models that allow accurate prediction of the over-all effects on tannins in any ecological system (Hagerman 2002). As stated in Chapter 3.2, even within one particular plant species, a high number of different polyphenolic substances can be found. Thus polyphenols of single plant species may affect ecosystem processes in various ways. To address this problem, numerous techniques have been developed for the isolation, characterization and quantification of polyphenolic substances.

Characterization of molecules often involves highly specific analytical techniques such as high performance liquid chromatography coupled to mass-spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) which allow the determination of the chemical structures of specific tannins (Kraus *et al.* 2003a). Given the high number of different molecule structures one may encounter within a single species, the preparatory and analytical effort is very high and even higher when considering a wide variety of different species. Thus, especially in the context of BEF-experiments, which are intrinsically tied to cover a high number of species, characterization of every single polyphenolic compound in every single species may not be feasible. Additionally, many aspects of biological activity, such as the relative propensity for oxidation as well as the power to precipitate proteins, are most likely related to structural differences among the polyphenols (Kraus *et al.* 2003b). To add more complexity to this field,

it has been widely recognized that different tannins do not react uniformly to the assays used to quantify them (e.g. Appel *et al.* 2001).

3.4.1. Methods to quantify total phenolics and tannins

Albeit the differences in reactivity and activity across the multitude of polyphenols, two types of measurements have become widely accepted in the ecological context: assays to quantify the total phenolics as well as total tannin contents in plant extracts (Hättenschwiler and Vitousek 2000).

Common methods to quantify the concentrations of total phenolics include the so-called Folin-Ciocaleu assay (FA). FA assays are based on defined colorimetric reactions typically evoked by polyphenols (Waterman and Mole 1994; Hagerman 2002). In FA, mixtures of two or more redox-based colorimetric reagents are employed (Appel *et al.* 2001). Through the oxidative capacity of polyphenolic substances, the colorimetric compounds become reduced, forming colored complexes that can be quantified by UV-Vis spectrometry. However, as pointed out by several studies, the intensity of the redox activity may be affected by structural variations within and among different plant species (e.g. Appel *et al.* 2001). Thus, differences in color intensity produced by FA may be due to either higher concentrations in polyphenolics or due to differences in their oxidative capacity as a direct consequence of structural differences. Consequently, the quantification of polyphenols using FA has been discussed to be inappropriate for the comparison of total phenolic contents between species due to the many different types of polyphenolics encountered among different species (e.g. Mole and Waterman 1987; Appel *et al.* 2001).

Methods to determine tannin contents are often based on protein-tannin interactions (see reviews e.g. by Hagerman 1987; Hagerman 2002). Although the ability to precipitate proteins is one of the defining characteristic of tannins (Haslam 1989), the detailed chemistry of these interactions is still only partly understood. According to Hagerman (2002), the type of interaction and the strength of interaction are predetermined by the chemistry of the tannin as well as the chemistry of the precipitated protein. Numerous methods for determining tannin concentrations based on the interaction between tannin and protein have been devised. One of these methods is the 'radial diffusion method' (Hagerman, 1987). Here, plant extracts are applied to an agarose-gel plate inoculated with a purified and standardized protein (bovine serum albumin, fraction V; BSA fraction V). The plant extracts diffuse into the gel, and form a visible ring of precipitated protein. The area of the precipitate-ring is proportional to the

amount of tannins in the extract. However, as for total phenolic determination using e.g. Folin-Ciocalteu assays, comparison of concentrations determined from different species is not straightforward. Different tannins may have different precipitative activity due to structural differences in the macromolecules.

To overcome the problem of incomparability, it has been proposed that comparisons of polyphenolic concentrations across different species require standardization on the basis of a known and well characterized standard substance. However, selecting appropriate standards for quantification purposes is not a trivial issue. Frequently researchers use tannic acid or similar commercially available standards (Hagerman and Butler 1989). In these cases, tannin concentrations are reported in e.g. tannic acid equivalents (TAE). However, even commercial polyphenols are not fully standardized and may slightly vary in their purity and composition, depending on the source of the respective tannin. Thus, in addition to convert results from polyphenolic assays e.g. into tannic acid equivalents, it has been recommended to report the manufacturer as well as the batch number of the standard substance used in the respective assay (Hagerman and Butler 1989).

As an alternative to the use of a commercial standard, Stewart *et al.* (2000) proposed to produce species-specific standards by purifying tannins from the species of interest. Owing to the large preparatory and analytic effort, this method seems inappropriate in the early phases of BEF experiments. However, once preliminary research allows formulating specific hypotheses, it may become relevant to carry out directed species-specific characterization of the full polyphenolic profile of certain species also in the context of BEF research. The present thesis, however, will focus on total phenolic concentrations as well as tannin concentrations in plant leaf tissue.

3.4.2. Extraction from plant material

Most assays for polyphenol quantification require extraction of these secondary metabolites from plant material prior to the analysis. Typically, the plant material is dried and subsequently ground to a fine powder to increase the surface which is accessible to the respective solvent. Although one of the main characteristics of polyphenols is their solubility in water (Haslam 1989), some phenolics were found to be less soluble in water, but soluble in non-polar solvents (Waterman and Mole 1994). Depending on the number of OH groups and their relative position within the macromolecule, phenolic substances exhibit higher or lower polarity. Thus, some polyphenols were found to be soluble in less polar substances than water. In this respect

methanol and acetone, pure or in aqueous dilutions, have frequently been used for extraction (Hagerman 1988; Hagerman and Butler 1989; Waterman and Mole 1994). In comparative studies, aqueous dilutions of the solvents (i.e. a mixture of polar and non-polar solvents) were found to be more effective. Several studies reported that aqueous acetone extracted higher amounts of polyphenols from leaf powder than aqueous methanol (e.g. Hagerman 1988; Salminen 2003).

Besides the solvent applied in extraction, investigations have reported that a significant proportion of tannins remain non-extractable (5-50%; Makkar and Singh 1991; Matthews *et al.* 1997; Preston 2000). This is presumably due to covalent binding of polyphenols to the cell-wall. The proportion of extractable vs. non-extractable tannins has been reported to be dependent on the extraction method used (Cork and Krockenberger 1991). Torti *et al.* (1995) could show that the application of a homogenizer (rotor-stator principle) during the extraction of plant powder increased the amount of extractable polyphenols significantly as compared to extraction using sonication. Homogenization has been found to reduce the non-extractable fraction of polyphenols bound to the cell wall to approx. 25%. The increase in extractability was attributed to the efficient physical rupture of the cell-wall caused by the shearing forces resulting from homogenization.

3.4.3. Drying methods and storage conditions

Besides extraction methods and choice of an appropriate standard for comparable quantification there are other factors influencing the quantifiable amount of plant polyphenols. Drying methods, the conditions during grinding as well as the sample storage conditions and duration were reported to affect the structure as well as the amounts of extractable polyphenols detected in plant material (Dalzell and Shelton 1997; Stewart *et al.* 2000; Yu and Dahlgren 2000; Julkunen-Tiitto and Sorsa 2001).

Investigations on the effect of drying conditions indicated that the amount and activity of extractable polyphenols was strongly affected by elevated temperatures during the drying process in comparison to extraction from fresh leaf material (e.g. Cork and Krockenberger 1991; Makkar and Singh 1991; Julkunen-Tiitto and Sorsa 2001). Frequently, lyophilization (freeze drying) and vacuum drying were found to be the most appropriate drying methods (e.g. Cork and Krockenberger 1991; Salminen 2003).

It has also been reported that elevated temperatures during the grinding process may negatively affect the amount as well as activity of extractable polyphenols and grinding material in liquid

nitrogen has been proposed (e.g. Makkar and Singh 1991). Alternatively, avoidance of elevated temperatures during the grinding process may also be achieved short grinding times.

Likewise, elevated temperatures during storage, light exposure and the duration of the storage period of the plant material have been reported to affect the amount of extractable polyphenols (Salminen, 2003). It has been proposed to store the samples under dry conditions at temperatures of maximum 4°C in non-transparent vessels in order to mitigate the adverse effects of sample storage (e.g. Cork and Krockenberger 1991). However, when leaves are stored over long periods (e.g. one year or more), Salminen (2003) suggested to store them at -20°C.

3.4.4. Effect of seasonality and tissue maturation

In addition to drying conditions, the concentrations of polyphenolics depend on plant tissue age, and thus on phenology. This further complicates the comparability of polyphenol concentrations within and across different species.

For *Quercus macrocarpa* (Bur oak), *Acer saccharum* (Sugar maple) and in *Carya ovata* (Shagbark hickory) higher amounts of polyphenols in plant leaf material collected in the early season than in the late season were reported by Hagerman (1988). Brunt *et al.* (2006) reported differences in the polyphenolic contents of two species (*Toona ciliata* and *Nothofagus moorei*) sampled at five stages of leaf maturation. Responses due to maturation were different between the two species: *Toona ciliata* exhibited an increase in polyphenols, whereas for leaves of *Nothofagus moorei* the authors reported a decrease in polyphenolic concentrations during maturation. However, as stated by Koricheva (1999), concentrations reflect the over-all distribution of the investigated metabolites across the tissue, and therefore is a function of biomass. Thus, changes in polyphenol concentrations in plant tissues (e.g. throughout maturation) may not reflect changes in absolute amounts of the secondary metabolites but may rather reflect changes in other constituents of biomass (e.g. protein content, or structural compounds). Whereas Koricheva (1999) discussed this problem in the context of nutrient availability to plants (i.e. general facets of plant metabolism based on *in-situ* growth conditions), dilution due to tissue maturation may also obscure changes in concentrations of polyphenols during leaf maturation, as leaves get e.g. tougher during leaf expansion (Brunt *et al.* 2006) and thus increase the relative proportion of structural compounds in the tissue.

3.5. Specifying the research questions

Summing up all the obstacles listed in the previous paragraphs, the general use of concentrations in secondary metabolites has been discussed to possibly lead to erroneous conclusions. These challenges must be met, overcome and taken into consideration when investigating the role of polyphenols in ecosystem functioning. Therefore a standardized procedure for the sample collection, storage conditions and quantification methods was established as a part of the present thesis (see Chapter 4.1).

One of the questions that is addressed by the studies presented in Chapter 4 is: 'How reliable are ecological conclusions on the effect of plant polyphenolics on ecosystem functions if the information on leaf polyphenolic concentrations are derived from samples collected and handled under sub-optimal conditions?'. In the first investigation included in this thesis, the effect of sub-optimal sample treatment on the deduction of ecological conclusions was assessed. In this study, two sample-sets, sampled according to two different sampling protocols and at different times of the year, were compared. One sample set was collected according to the standardized protocol for the sampling and measurement of functional leaf traits established by Cornelissen *et al.* (2003), while the second sample set was collected according to the optimized sampling protocol presented in Chapter 4.1. The concentrations of leaf polyphenolics of 20 tree species from the BEF-China species pool were compared. Polyphenolic substances were found to be highly diverse even within closely related plant species (Salminen 2003; Salminen *et al.* 2004). The high variability in the identity as well as the quantity of polyphenolics between species may be more important than the variability between these concentrations evoked by the two sampling and quantification protocols. The effects of biodiversity (i.e. the taxonomic extent) were compared to the effects of sub-optimal sample treatment. The amount of extractable leaf polyphenols of the two sample sets were assessed and compared. It was hypothesized that i) the variance in the extractable concentrations of total phenolics as well as tannins brought about by the sub-optimal sample treatment precludes the transfer of results from studies with different sample handling. Therefore, the extent of phylogenetic conservation of the polyphenol concnetrations for all investigated species was assessed. In addition, six other functional leaf traits known from the investigated species were incorporated into the analysis. This was done in order to assess the relative importance of taxonomy on polyphenolic substances in comparison to the other functional traits. It was hypothesized that ii) the variance of the two groups of secondary metabolites shows a similar distribution across taxonomic levels as the variances known from other leaf traits. Finally, to

qualitatively assess the effect of sub-optimal sample handling on ecological conclusions drawn from these two sample sets, multivariate analytical approaches were used to examine and compare ecological conclusions drawn from the two sample sets.

The second investigation included in this thesis investigated the effect of plant polyphenols on the dynamics of forest ecosystem nutrient turnover. Litter decomposition is one of the most important ecosystem processes, ensuring the sustainable turnover of nutrients (Aber *et al.* 1991). Leaf polyphenols have been reported to be important an aspect in litter decomposability (e.g. Hättenschwiler and Vitousek, 2000). Community weighted mean traits (CWMs) for total phenolics and tannin concentrations as well as 12 additional functional leaf traits were calculated for all 27 CSPs. These additional traits comprised aspects of leaf nutritional value (e.g. leaf nitrogen and phosphorous concentration), traits related to leaf physical (e.g. leaf toughness) and traits related to tissue productivity (e.g. specific leaf area, leaf calcium). All of these traits have been reported to relate to leaf litter decomposition in previous studies (e.g. Hättenschwiler *et al.* 2005; Kraus *et al.* 2003). Moreover, leaf litter decomposition rates (k-rates) based on *in-situ* litter mixtures were calculated for all 27 stand communities covered by the CSPs. Several previous studies reported directional changes in the composition of community leaf functional traits, and that these changes were related to a shift in the resource-use strategy of plant communities along secondary succession (Garnier *et al.* 2004; Caccianga *et al.* 2006; Raevel *et al.* 2012). These investigations often reported a shift from high nutrient acquisition in young forest stands to high nutrient retention in old forests (e.g. Vile *et al.* 2006b). In the second study included in this thesis it was hypothesized that i) along secondary succession there is a shift from communities dominated by species with high productivity, high leaf nutrient contents and short-lived leaves with low physical or chemical resistance to communities consisting of species with low productivity, low leaf nutrient contents and more persistent, better defended leaves. It was further hypothesized that this holds true irrespective of plot species richness. As chemical or physical resistance traits are known to affect litter decomposition rates, it was hypothesized that, ii) as a consequence of a shift in the CWM leaf traits, community leaf litter-decomposition rates decline with successional age, irrespective of species richness. In addition, iii) a causal relationship between shifts in CWMs of leaf functional traits and shifts in community k-rates was hypothesized.

In the third investigation included in this thesis, leaf polyphenolic substances were investigated in relation to leaf toughness. Here, polyphenolic substances and leaf toughness were considered as two different aspects of leaf defense, namely chemical and physical defense. The

development of both aspects is dependent on the provision of carbon for their biosynthesis. In the recent literature, aspects of leaf defense have been discussed to trade-off each other (e.g. Koricheva *et al.* 2004, Moles *et al.* 2013). In most cases, this was investigated as negative trait covariations caused by constraints in resource allocation. The bulk of these studies has been carried out using species mean trait values. However, the application of species trait means neglects possible effects of intraspecific variability (ITV). As reported by Albert *et al.* (2010, 2011), it is advisable to assess the relative importance of ITV, because, if ITV is higher than the variability within a respective species, species trait means may be less informative. The third study enclosed in this dissertation comprised a total of 186 individuals sampled from 51 species from the local species pool in the Gutianshan National Nature Reserve. Each species was sampled with at least three individuals. Each individual was sampled with three to ten leaves. Thus, the relative importance of ITV as well as the impact of the incorporation of ITV in studies on trade-offs between leaf defense traits could be, and was assessed. However, trade-offs are also frequently discussed to be based on trait evolution and phylogenetic constraints (see Agrawal 2007). This thesis presents the first study that addressed the question of allocational constraints and evolutionary trait conservatism in the same investigation. To do so, phylogenetic information on all 51 species (Michalski and Durka 2013) was included as essential information in the analysis. It was hypothesized that i) trade-offs between chemical and physical carbon-based defense aspects are ubiquitous, i.e. consistent in all 51 species. It was further hypothesized that ii) in cross-species analyses, trade-offs will be encountered when carried out using species trait means and iii) intraspecific trade-offs based on single sample trait values (thus, including ITV) are matched by interspecific trade-offs based on species trait means. In other words it was hypothesized that the incorporation of ITV will not significantly alter the results in trade-off analyses when compared to the results based on species mean trait values. In addition, it was hypothesized that iv) across all species leaf polyphenol concentrations and leaf toughness show a strong phylogenetic signal (i.e. the traits are phylogenetically conserved). Finally, it has been tested that v) chemical and physical defense traits show similar phylogenetic signals along trait evolution, indicating that the two traits have experienced similar selective pressure during their evolution.

4. Challenges met in this thesis concerning the interpretation of the role of plant polyphenolics in the context of BEF

The previous chapters gave an introduction into the general settings, theory and rationale of BEF investigations as well as the chemistry and ecological significance of plant polyphenols. In the following, specific procedures and investigations carried out to increase the knowledge on the role of plant polyphenols in the setting of the BEF China project are presented. The chapters summarize the established, modified procedures for the quantification of polyphenolic substances and outline the most findings of three specific investigations which are the central aspect of this thesis

4.1. Establishing an optimized protocol for sampling, processing and storage of plant material to assess polyphenol concentrations in BEF experiments

A detailed laboratory protocol for both quantification methods and the necessary equipment and solutions, including instructions for the preparation of the gels, is provided on the electronic supplementary material available in the electronic appendix enclosed in this thesis (compact disc).

Owing to the multitude of aspects presented above, it is difficult to draw ecological conclusions from within and across species studies on plant phenolic substances. All of the factors mentioned in the previous paragraphs were found to affect the extractability, stability and quantifiability of polyphenols in plant tissue. For ecological studies this carries the problem that the amount of detectable phenolics in plant tissue might be affected by both, ecologically relevant factors due to in-situ growth conditions as well as due to suboptimal sample preparation prior to their analysis. Thus, in order to disentangle ecologically significant effect of polyphenol concentrations from methodological defects due to the sample treatment, it is of major importance to establish a protocol that prevents loss of polyphenols in plant material during sample processing and storage. Moreover the protocol should minimize variability in leaf concentrations evoked by seasonality as well as the ontogenetic stage of leaves.

As mentioned in Chapter 1.3, as soon as in the early phases of BEF studies, standardized protocols have been developed to minimize variations in functional traits resulting from ecologically insignificant factors. These standard protocols involve sampling of only fully expanded, sun-exposed leaves, preferably without any signs of herbivory (e.g. Cornelissen *et*

al. 2003). However, besides these protocols do not imply general recommendations for quantification of polyphenolics, the proposed drying methods also frequently do not account for the liability of polyphenolic substances. Thus, the first objective of this thesis was to establish a protocol for optimized sampling storage and quantification procedures that can be generally applied in studies on polyphenols in BEF-experiments.

Often the optimal conditions proposed for quantification of polyphenols in other biological fields such as in the chemical ecology (e.g. species-specific interactions between plant and pollinators, herbivores and parasitoids) cannot be achieved in BEF-experiments. Such experiments are often established in remote locations with sometimes rather limited access to adequate laboratory equipment. In many BEF-experiments, quantification is carried out days, weeks or even months after sample collection due to the lack of suitable equipment in the field labs. This, for example, highlights the importance of appropriate procedures for drying and conditions for sample storage. As the present thesis will concentrate on the effects of polyphenolics on nutrient cycling within the ecosystem studied in the BEF-China experiment, the main focus will be on the effect of tannins and non-tannin phenolics on litter decomposability and herbivory. Anti-herbivore effects have been attributed to the role of plant phenolic substances as repellents as well as the reduction of the nutritive value of tissues rich in polyphenols (e.g. Coley and Barone 1996; Hättenschwiler and Vitousek 2000).

While the activity as a herbivore repellent has been attributed to the ability of polyphenols to be oxidized, the latter aspect has been related to the ability of tannins to form precipitative complexes with proteins (e.g. Appel 1993; Hättenschwiler and Vitousek 2000; Kraus *et al.* 2003a). Nutrient dynamics based on soil processes and microbial interactions have been attributed to both, the capacity to be oxidized as well as the potential to form complexes with organic matter in the soil but also liable minerals (Hättenschwiler and Vitousek 2000; Kraus *et al.* 2003a; Hättenschwiler *et al.* 2005).

As described in Chapter 3.5.1. these intrinsic characteristics of polyphenols can be assessed with methods that were designed to quantify the concentrations of total phenolics and tannins within plant material. Thus, I decided to use an FA based method for the quantification of the total phenolic concentrations and a method based on protein precipitation capacity of leaf extracts. Based on screening a wide set of articles related to sampling, drying, extraction and quantification procedures, an optimized protocol was established. This procedure was applied throughout all the investigations presented in this thesis. This protocol is tailored to preserve

most of the extractable polyphenols during sampling, drying and storage. Moreover it is designed to allow for a high sample throughput by reducing the time for sample quantification.

The protocol comprises leaf sampling of only fully expanded, sun exposed leaves with no signs of herbivory. Directly after collecting the leaves from the individual, the samples are stored in a light tight cooling box and transported to the laboratory. Samples are then immediately deep frozen at -20°C, until they are lyophilized. Sample storage is conducted in light tight bags at 4°C until further analysis. Prior to quantification, samples are ground to a fine powder using a ball mill with consecutive grinding pulses of 1 min until the material was sufficiently disintegrated.

Aqueous acetone has been found to be the most effective solvent, and thus extracts are prepared using 50% aqueous acetone as a solvent. Extraction is carried out using a homogenizer in four subsequent extraction steps as suggested by Torti *et al.* (1995).

Total phenolics are assayed using the Prussian-Blue method (Price and Butler 1977; Hagerman 2002), modified in a way that stabilizes the ephemeral color complex formed throughout the colorimetric assay. According to the present protocol, a stabilizer solution is prepared from gum arabic and 85% phosphoric acid as described by Graham (1992) and applied after a defined reaction time. The volumes given in the original procedure (Price and Butler 1977) have been adjusted to a total volume of 1 ml. This allows the use of disposable half-micro cuvettes and thus allows for a higher sample throughput. The respective concentrations determined through the assay are standardized against solutions with known concentrations prepared from tannic acid as a commercial standard (Charge Nr. 250153788; Carl Roth, Germany).

Tannin concentrations are assessed using the radial diffusion method (Hagerman 1987) slightly modified for increased sensitivity as described in Hagerman (2002). This procedure requires the preparation of a 0.8% agarose gel with uniform thickness. After cooling the solution to 40°C, the gel is then inoculated with bovine serum albumin (BSA), fraction V. The gel solution is applied to a specially tailored vertical gel with plate (approx 30 x 21cm) to ensure uniform gel thickness. After the gel has hardened, wells of a known diameter (here: 4mm) are punched into the gel and crude extracts are applied (Figure 4-1). The gel plates are then covered sealed with parafilm and incubated for 96h to allow a complete permeation of the extract into the matrix. Standard solutions with known concentrations of tannic acid are applied within the respective gel, allowing to quantify the tannin concentrations of each extract. After incubation,

the gels are scanned on a desk scanner and the areas of the precipitation rings are determined using the open-source digital image processing software ImageJ (Abramoff, 2004).

The original assay as presented in Hagerman (1987) has been optimized in several ways to reduce possible sources of error. In the original assay, the gels were prepared in commercially available petri dishes of approx. 9 cm diameter. The usage of the customized vertical gel plate in the optimized protocol allow to apply up to 30 samples to one gel plate simultaneously (in contrast to approx. 6 samples in the original assay). This allows for a high sample throughput. Using e.g. several vertical gel plates, the optimized protocol allows for the simultaneous processing of hundreds of samples. Following Hagerman (1987), the standards would be applied to a separate petri dish, and all other dishes would be standardized against the calibrations prepared from this plate. This however may introduce errors in tannin quantification due to differences between the different gel plates such as the gel thickness. In the original protocol, uniform gel thickness was assured by applying the same volumes of gel solution to every petri dish, with the dish placed on a level surface. However, exact volume deposition and exact levelness is difficult in some practical situations. The present assay circumvents this source of error by using vertical gel plates with a top glass plate using spacers of 1.7mm width (Figure 4-1). Moreover, applying the standard solution in the same gel as the plant extracts reduces calibration errors across plates as this allows standardizing each gel within itself.

According to the original protocol, the area of the precipitation rings is determined using a ruler and calculating the radius according to established mathematical relations. However, the precipitation rings are not completely circular in every case. Hagerman therefore suggested to measure the radius of each ring two times, rotating the axis of measurement for 90°. The mean value calculated for the radius is then used to correct for non-circularity. This introduces an additional source of error which the present protocol circumvents by the usage of modern digital techniques.

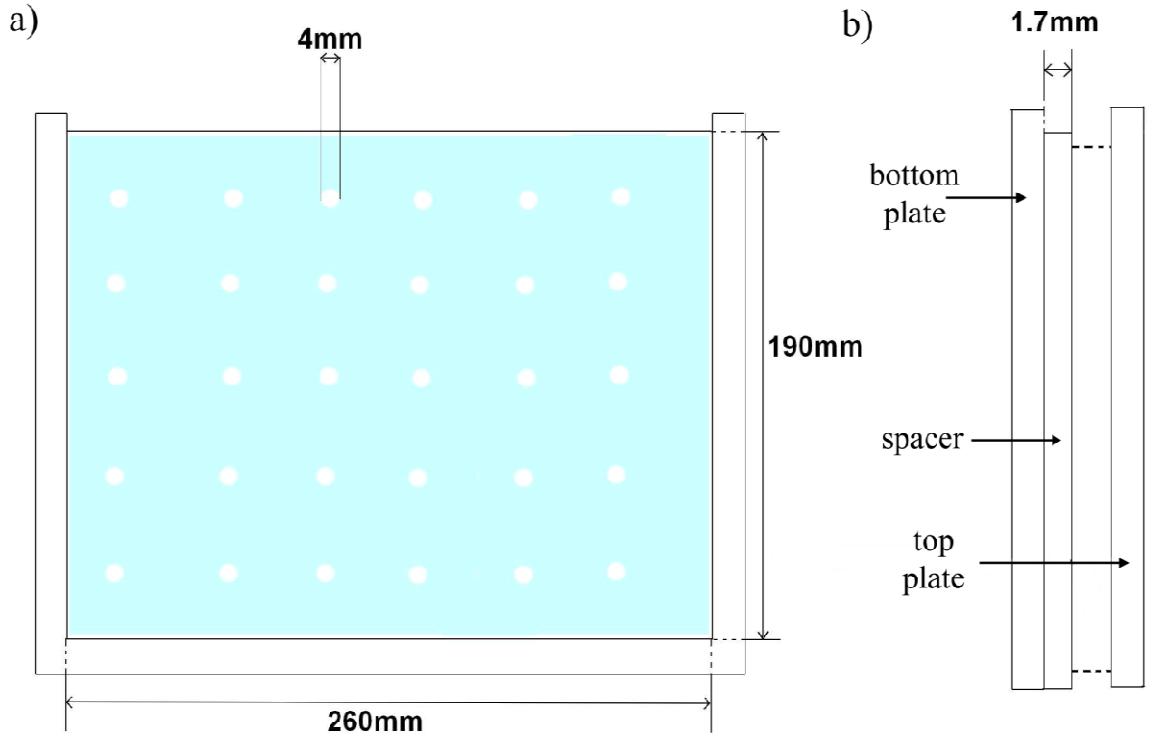


Figure 4-1: Sketch of the customized gel plates used in the determination of tannin content. a) top view; light blue: 0.8% agarose-gel, white spots: wells of 4mm diameter. b) side-view; the spacer plates of 1.7mm are fixed to the bottom plate using silicone glue. The removable top plate is fixed during the gel-preparation using removable clamps.

Source: Ristok 2011; slightly modified by Eichenberg

As the optimized protocol presented above deviates from other protocols frequently used in the assessment of plant traits (e.g. in sampling procedures and drying conditions), the effect of methodological differences in these protocols will be addressed in the first manuscript incorporated in this thesis.

4.2. Plant polyphenols- Implications of different sampling, storage & sample processing in BEF experiments

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Abstract- Plant polyphenols are involved in important ecosystem processes and may affect nutrient cycling. Previous investigations have demonstrated detrimental effects of suboptimal sample treatment on the quantity of extractable plant polyphenols. We compared leaf polyphenol concentrations of 20 tree species from East China in two sample sets collected under different conditions: a) according to established protocols and stored more than three years, b) under conditions optimized for leaf polyphenols. We investigated the variance brought about by suboptimal sample handling as compared to the variance caused by the taxonomic range of species. Family-affiliation explained the largest proportion of variance while sample handling had only minor effects. Reducing the taxonomic range increased the impact of differences in sample handling. Additionally, we showed that the concentrations of leaf polyphenols were phylogenetically more conserved than other leaf traits. Non-metric-multi-dimensional scaling revealed similar ordination patterns for leaf polyphenol concentrations in both sample sets with both ordinations being closely correlated. Finally, we computed separate ordinations including an extended set of leaf traits and found that both analyses led to similar ecological conclusions. Consequently, in studies comprising a wide taxonomic range, the adverse effects of suboptimal sample handling may be overridden by the variation brought about by phylogeny.

Key Words- phylogenetic trait conservatism; plant functional traits; plant polyphenols; procrustes analysis; sampling conditions; variance components analysis

4.2.1. Introduction

The increasing interest in the role of biodiversity in natural ecosystems has triggered the setup of biodiversity-ecosystem functioning (BEF) experiments in all major biomes of the world Balvanera *et al.* (2006). These experiments, covering boreal to tropical biomes, have the aim to assess the effects of biodiversity on ecosystem sustainability and functioning (Bruelheide *et al.* 2011; Yang *et al.* 2013). In this respect, functional traits have become a powerful tool to

characterize the functions of plant species within a certain ecosystem (Ackerly and Cornwell 2007; Webb *et al.* 2010). In particular, leaf traits have often been linked to ecosystem functioning (e.g. Mason *et al.* 2011; Kröber *et al.* 2012) as they can be universally applied and are assessed relatively quickly. However, often only soft leaf traits have been used and chemical leaf traits such as tannins and non-tannin phenolics, which are known for their function as deterrents of herbivory (Whittaker and Feeny 1971; Barbehenn *et al.* 2006), antibacterial active compounds (Sofidiya *et al.* 2009, Xia *et al.* 2011), radical scavengers (Touré *et al.* 2010) as well as determinants of decomposition rates (Hättenschwiler and Vitousek 2000; Mason *et al.* 2011), have been largely neglected in BEF studies. The reluctance to consider these secondary metabolites in BEF research is partly explained by doubts about the feasibility to analyze them in huge field trials with many species and thousands of individuals.

In recent literature there is an increasing number of publications relating plant polyphenols to ecosystem functions. For example, Mason *et al.* (2011) investigated the shift in community-weighted aggregated mean trait values in a New Zealandian secondary forest succession series. With ongoing succession they observed a decrease in community mean leaf polyphenol concentrations as well as an increase in leaf nutrient concentrations and concluded that leaf palatability and decomposability might increase during secondary succession. In a study in the Australian tropics, Parsons *et al.* (2011) collected leaves over two years and, among other traits, related polyphenolic concentrations to the decomposition rates of leaf litter. Using near infrared spectroscopy to determine the *in-situ* decomposability of leaf material the authors found that polyphenols were negatively correlated with leaf persistence.

Despite their usefulness in functional ecological research, an obstacle is seen in the sensitivity of extractable tannins and non-tannin phenolics in plant tissues to differences in sampling, sample storage and sample processing conditions (see Hagerman 1988; Salminen 2003). Mueller-Harvey (2001) noted that leaves are best stored on ice or cooled directly after collection; conditions that in some biomes, such as tropical or subtropical regions, can hardly be met when collecting samples in the field. Makkar and Singh (1991) demonstrated the rapid decay of phenolic compounds when exposed to light as well as to elevated temperatures. Moreover, Salminen (2003) described a significant decay of hydrolysable tannins at low temperatures when stored over long periods. Julkunen-Tiitto and Sorsa (2001) demonstrated detrimental effects of different drying methods, which affected the structure of some polyphenolic compounds, but not others. Some attempts have been made to establish optimized protocols for collecting and storing samples to conserve the majority of these metabolites (e.g.

Hagerman 1988; Orians 1995). These procedures require high expenditure of labour and material, which under most field conditions cannot easily be met. Apart from factors related to sample treatment, the concentrations of tannins are also dependent on abiotic conditions such as elevation, precipitation regime and light environment as well as on biotic factors such as herbivory (Alonso-Amelot *et al.* 2007; Nabeshima *et al.* 2001). Additionally, polyphenol concentrations of leaves were found to be dependent on leaf development (Brunt *et al.* 2006; Makkar *et al.* 1991) and on season (Salminen *et al.* 2001; Salminen *et al.* 2004), and have shown large species-specific differences in temporal variation (Ammar *et al.* 2004). It is also well known that many traits exhibit a large phenotypic plasticity across different environments (Bruelheide *et al.* 2014; Pigliucci *et al.* 2006).

Westoby *et al.* (1995) emphasized that cross-species trait comparisons require a phylogenetic correction, and in particular when analyzing traits within taxonomically widespread data-sets, because traits tend to be phylogenetically conserved. Phylogenetic conservatism can be illustrated by comparing the variance explained by different taxonomic levels. Similarly, trait relationships can be taxonomically corrected by incorporating information on family, genus and species identity in the analyses. Investigating tannins and non-tannin phenolics in plant material from different taxa, Villar *et al.* (2006) reported that about 50-80% of the variation was explained by family affiliation. Similarly, in a dataset of eight leaf traits on 142 species, which also comprised our study species, Kröber (unpublished data) found that the family level accounted for the highest amount of variation explained. Nevertheless, especially in the context of BEF studies, knowledge on the variation at the species and genus level is important to assess the degree of redundancy of con-family genera and con-generic species.

Given the potentially severe impact of sample treatment on the amount of polyphenolics in leaves caused by differences in biotic and abiotic growth conditions as well as of the season when the sampling was carried out, the question arises whether conclusions drawn from analyses such as those of Mason *et al.* (2011) and Parsons *et al.* (2011) are reliable. The issue of comparability of polyphenolic concentrations may become more severe when concentrations in plant polyphenols are compared in cross-experimental studies, incorporating data from different experiments into the analyzed data, e.g. from databases.

To evaluate the severity of this problem, we compared a set of 20 species from 14 genera and 10 families for their total phenolics and tannin concentrations. Leaf material of each species was i) collected and processed under standardized conditions and analyzed after storage in the lab for three years under ambient conditions (hereafter referred to as sample set I) and ii)

collected under optimized conditions, stored at cool temperatures and analyzed less than three months after sampling (termed sample set II).

In summary, we tested the following hypotheses: 1) The variance in the extractable concentrations of the two secondary metabolites brought about by the inclusion of two different sample sets (collected and processed under substantially different conditions) precludes the transfer of results from studies with different handling of samples; 2) The variance of the two groups of secondary metabolites shows a similar distribution across taxonomic levels as the variances known from other leaf traits. Comparing separate non-metric multi-dimensional scaling (NMDS) ordinations from the different sample sets, we discuss the implications of our findings for BEF studies.

4.2.2. Materials and Methods

Study Sites and Sample collection

Site description

Two sets of samples (set I and II) were collected in different locations within sites of the BEF-China project situated in the species-rich subtropics of southeast China. Set I was sampled in the so-called 'comparative study plots' (CSPs) of the BEF-China experiment, established within a subtropical rainforest that is undergoing secondary succession (see Bruelheide *et al.* 2011 for detailed information). These samples were collected randomly in 27 CSPs in August 2008. Each species was only sampled once within one plot, thus covering a broad range of the biotic as well as abiotic conditions (see Kröber *et al.* 2012).

Set II was sampled in 2011 from May to June on the experimental sites (Site A and Site B, see Bruelheide *et al.* 2014 for detailed information) of BEF-China, located about 30 km southwest of the CSPs. On the experimental sites approx. 350.000 single trees of 42 tree species and 20 additional shrub species were planted in 566 plots varying in levels of species richness (further details are given in Bruelheide *et al.* 2014 as well as on www.bef-china.de). Tree saplings had heights between 0.3 m and 5 m, depending on the species, at the date of sample collection. In these experimental plantations we sampled the same species as those collected in the CSPs.

Sample collection

For both sample sets leaf sampling in the field followed a standardized protocol for the assessment of leaf traits (Cornelissen *et al.* 2003), with only fully expanded and the most sun-exposed leaves from the present vegetation period being collected. We avoided leaves with

considerable herbivore damage as well as those colonized with epiphyllic cryptogams. However, collection of leaves from completely herbivory-free trees could not be achieved in every case (e.g. canopy herbivory). Thus, the field data also included the condition of the sampled tree as an additional source of variance. Small branches were collected with leaves (four leaves per individual, up to 40 for species with needle shaped leaves) and leaves were separated from branches in the lab. Moreover, sample processing and storage conditions differed substantially between the two sample sets (see below).

Sample processing and storage conditions

Samples of set I were cut from the tree and brought to the lab without further precautions (e.g. cooling) and were dried for 48h at 80°C in a drying oven. For chemical analyses the samples were ground to a fine powder using an orbitary mill (Scheibenschwingmühle-TS, Siebtechnik, Germany). This sampling procedure meets the requirements for general leaf trait analyses as proposed in the protocol of Cornelissen *et al.* (2003). The specimens were stored in translucent glass vials for approx. 3 years under ambient room conditions before total phenolics as well as tannin concentrations were analyzed.

Samples of set II were stored in a cooling box (approx. 12 - 18°C, depending on ambient temperature) directly after cutting from the trees and on the same day deep-frozen at -18°C and lyophilized at 1 Pa for 24 h. Subsequently, the specimens were ground to a fine powder using a ball mill (Mikro Dismembrator U, B. Braun Biotech International, Germany) and stored for approx. 2 months in UV-impermeable vials at 4°C until the analysis of polyphenolics. This procedure is a combination of several recommendations to prevent loss of total phenolics as well as tannins from plant material (c.f. Granito *et al.* 2008, Xu 2005) and can be considered as optimized sample processing conditions.

Quantification of secondary metabolites

Approximately 50 mg of each sample were subjected to four subsequent extractions in 50% aqueous acetone following the protocol of Torti *et al.* (1995). Total phenolics were determined as the amount of bulk non-tannin phenolics using the Prussian-blue method by Price and Butler Price and Butler 1977 as modified by Graham (1992). To determine the total amount of tannins we made use of the protein-precipitation based radial diffusion assay for increased sensitivity Hagerman (2002) with the following modifications: we used a 0.8% agarose-gel, containing 0.01% BSA fraction V (Roth, Germany, Charge Nr. 360160110) as standard protein instead of a 1% agarose-gel as proposed in the original protocol. The protein-containing gel (30 wells, Ø

4 mm; distance between each well: 50 mm) was inoculated with crude extract, incubated for 96h and stained with the Prussian-blue reagent. Subsequently, gels were digitized using a flat bed scanner. Data evaluation was carried out by ImageJ (Abramoff *et al.* 2004), using the extension package 'chart white balance' (Haeghen 2009). Diameters of precipitation rings for each well were calculated as the mean of four measurements per well, each rotated by 45°. Both, total phenolics and tannins, were quantified by standardization against tannic acid (Roth, Germany, Charge Nr. 250153788); consequently all results are given in tannic acid equivalents (TAE). Total phenolics as well as total tannin concentrations were expressed on a dry mass basis in mg/g_{dw}.

Statistical analyses

To evaluate whether total phenolics as well as tannin concentrations show a phylogenetic signal in the investigated species (i.e. phylogenetic trait conservatism, see Pagel 1999 as well as Freckleton *et al.* 2002), we calculated Pagel's λ for both traits. The phylogeny was established using sequence information on the matK, rbcL and ITS region including the 5.8s genes retrieved from GenBank or using standard barcoding protocols (Fazekas *et al.* 2012) for all investigated species. Alignment was carried out using MAFFT v6 (Katoh *et al.* 2002) for each marker, separately. Detailed information on the phylogeny is available from the online database of BEF-China (www.china.befdata.biow.uni-leipizg.de) as well as in Barrufol *et al.* (2013). Under the assumption that trait evolution follows Brownian motion Pagel's λ tests to which extent correlations in traits reflect species' shared evolutionary history. Values of λ near 1 indicate that trait evolution closely follows Brownian motion model. Significance of phylogenetic trait conservatism was tested by shuffling species names at the tips of the phylogeny for 9999 times and testing these trait-species combinations expected from a random distribution against the observed trait-species distribution. To test for trait conservatism we used the phylosig routine available in the R package phytools (Revell 2013a). All statistical analyses were conducted in R 2.13.1 (R Development Core Team 2011).

Model considerations

For statistical evaluation both variables (i.e. total phenolics as well as tannin concentrations) were log10-transformed to meet the requirement of normality of data-distributions. To compare species-specific effects of sample treatments we conducted Mann-Whitney U-tests for tannins and non-tannin phenolics separately by species. This non-parametric test was chosen instead of the t-test because of a higher robustness to small sample sizes (McKnight and Najab 2010). To assess the strength of effects related to phylogeny and effects brought about by sample

treatment, we conducted a variance components analysis as described in Crawley (2007). Calculating a mixed effects model without fixed factors but including *family*, *genus* and *species* as well as *sample set* as hierarchically nested random factors, the proportion of variance explained by every factor was estimated using the lme (package nlme, Pinheiro *et al.* 2011) and varcomp procedures (package ape, Paradis *et al.* 2004). We also used univariate linear models to test for correlations between tannins and total phenolics in the two datasets separately as well as in the combined dataset. The combined dataset comprised all individual values for total phenolics and tannin concentration of all species from both datasets.

Variance components analysis may be sensitive to the number of observations included in the different categories. As our dataset was not balanced, with some families only being presented with one genus and one species each, we tested how this bias might influence the results from the variance components analysis. To this end, we additionally ran analyses using stratified datasets containing subsamples. In Table 4-1 we present different datasets that were subsequently analyzed and mention potential biases with respect to balance across families. In Table 4-2 we, inter alia, present a list of species included in the present study.

The first subset comprised only those families with two or more species (i.e. Elaeocarpaceae, Fagaceae and Lauraceae). Furthermore, a considerable number of our study species were members of the Fagaceae family. This allowed us to test the effect of a reduced taxonomic extent of species included in our study by assessing to which degree the exclusion of this family affected the impact of phylogeny and *sample set*. Thus, we additionally split our analysis into a dataset that contained only genera of the Fagaceae and into one that consisted of all other non-Fagaceae species. Additionally, within the Fagaceae, there were three different species from the genus *Castanopsis*, each of them being sampled in both datasets from three individuals each. This allowed us to test the variance component brought about by *sample set*, e.g. differences in sample collection, storage and processing, also within a single genus.

Table 4-1 Dataset stratification of subsets to investigate the effects of dataset balance in the data structure on variance components analysis

Dataset	taxonomic extent	dataset balance
Original dataset	20 species from 10 families	biased across families
Two or more species per family	11 species from three families (Elaeocarpaceae, Fagaceae, Lauraceae)	less biased across families
All species without Fagaceae	11 species without Fagaceae	less biased across families
two species each family (Elaeocarpaceae, Fagaceae, Lauraceae)	11 species (2 per combination; five possible combinations)	fully balanced across families
two species from each one genus	All species (2 species per combination, 27 possible combinations)	fully balanced across genera
Fagaceae only	Nine species within Fagaceae	Moderately biased within Fagaceae
Castanopsis	three species within genus *Castanopsis*	fully balanced within *Castanopsis*

Julkunen-Tiitto and Sorsa (2001) and Harbourne *et al.* (2009) reported that individual phenolic or tannin compounds might be differently affected by storage and handling conditions. Thus, our approach with a simple reduction of taxonomic extent might solely reflect the presence of tannins and non-tannin phenolics with similar activity but different structure in distinct species, and results from the variance components analysis with reduced taxonomic extent might solely reflect the behavior of similar groups of tannins and non-tannin phenolics that can be present in taxonomically distinct species. To account for the impacts of species-specific compositions of phenolics or tannins, we repeated the variance components analysis with all possible combinations of samples from families with at least two species available in our dataset (i.e. Elaeocarpaceae, Lauraceae and Fagaceae). We generated all possible combinations of datasets including two species from one genus, within each of these families, resulting in five datasets. Additionally, we generated all possible combinations of datasets containing two species within each family from different genera. This resulted in 27 different sample sets each containing two species per family. If chemically similar compounds within taxonomically distinct species are differently affected by storage conditions, there should be at least some runs with a high proportion of variance explained by *sample set*. Thus, we report the results of the most extreme run over all five 'one genus, two species each' as well as all 27 'two species each family' combination of sample sets, i.e. that run with maximum proportion of variance explained by

sample set. In total, we conducted 32 variance components analyses from 32 different sample combinations.

Phylogenetic conservatism of leaf polyphenolics concentration compared to other traits in the leaf-economics spectrum

To evaluate the second hypothesis we assessed the effect of phylogenetic relatedness on tannin and non-tannin phenolics concentrations in comparison to other traits known from our studied species, taken from Kröber *et al.* (2012). The additional traits include important leaf characteristics known from the leaf economics spectrum (LES, sensu Wright *et al.* 2004), namely leaf dry matter content (LDMC), specific leaf area (SLA), leaf nitrogen (LNC) and phosphorus (LPC) concentration as well as stomatal density (StoD). In addition we included information about carbon to nitrogen ratio (C/N) and leaf sulfur concentration (LSC) obtained from Kröber *et al.* (2012). Matsuki and Koike (2006) found the carbon to nitrogen ratio (C/N) to be positively correlated with leaf life-span for seven broad-leaved tree species; thus, we used this trait as a proxy for leaf longevity. Moreover, StoD is generally considered to be positively related to a plant's ability to regulate transpiration, with higher densities of stomata associated to xeric leaf morphology (Carpenter and Smith 1975, Gindel 1969). For the subtropical species in our dataset, Kröber *et al.* (2012) pointed out that StoD did not covary with the leaf economics spectrum but represented a trait that conveyed additional information on regulation of transpiration. These additional traits were available for only 17 out of 20 studied species, thus, species with no additional information on other traits were excluded from this particular analysis (c.f. Table 4-2). We had no additional information on the effect of differences in the sample sets for these other traits in this particular analysis. Here we also made use of the mixed effects model without fixed factors as described above. As we calculated the explanatory power of the variance components in a hierarchical model, omitting the factor *sample set* from the model did not change the overall variance in the dataset for total phenolics and tannin concentrations used in this analysis. Removing this random factor from the model distributed the variance previously explained by *sample set* over the remaining random factors in the model.

Effects of sample treatment on ecological conclusions

To compare the differences between the two sample treatments, we conducted ordinations that are typically found in analyses of BEF-experiments when investigating, for example, trait inter-relationships. We first assessed the differences between the two sample sets as shifts between two separate two-dimensional NMDS ordinations (R-package ecodist, Goslee and Urban 2007)

based on Euclidean distance, solely incorporating the information about total phenolics and tannin concentrations. We used Procrustes rotations to check for randomness in the shifts caused by the different sample treatments between the two ordinations. Subsequently, these rotations were tested for non-randomness using the *protest* command (R- package vegan, Oksanen *et al.* 2011). A Procrustes significance test results in three parameters: a) the m^2_{12}-value (also called Procrustes sum of squares) which varies between 0 and 1 and shows the similarity of the two matrices. Values near to 0 and 1 indicate very similar and dissimilar matrices, respectively (Leyer and Wesche 2008). b) The *r* value can be interpreted as correlation between the target and the rotated matrix. c) The probability level *p* indicates the significance of non-randomness of matrix similarity. Moreover, we also calculated the absolute shift between rotated and target matrix by summing up the Euclidean distances between the positions for each corresponding pair of species in the two ordinations. From this we calculated the percentage of contribution to the total shift for each species.

Finally, to test for robustness in trait interrelationships we used a set of 20 further traits assessed on the same species by Kröber *et al.* (2012) and run two NMDS for analyses of trait interrelationships. The additional trait set comprised the leaf traits of the LES as described above. We considered several leaf characteristics that have been used in models of leaf construction costs (Brunt *et al.* 2006; Villar *et al.* 2006), comprising a wide range of minerals and micronutrients. For a description of all used traits see the caption of Figure 4-3. Finally, to assess the significance of particular trait relationships we calculated univariate Pearson correlations.

4.2.3. Results

The effect of phylogeny and sample set

According to Pagel's λ- statistics, both tannins and non-tannin phenolics showed significant trait conservatism in the investigated species. We found that with λ-values close to 1 ($\lambda = 0.995$, $p < 0.001$ and $\lambda = 0.999$, $p < 0.001$ for total phenolics and tannin, respectively) trait evolution of both traits was close to the expected Brownian motion model for all species included in this study.

The general tendencies in concentrations of the two secondary metabolites is shown in Table 4-2. We found that tannin concentrations can amount up to approx. 28% of total leaf biomass, as was the case for *Eleaeocarpus japonicus*. For most species, no significant differences in the concentrations of total phenolics were detected between the two sample sets. Exceptions were

Elaeocarpus chinenis, *E. japonicus* and *Idesia polycarpa*, as well as *Schima superba*, for which phenolic concentrations were higher and lower in sample set 1, respectively. In contrast, tannin concentrations were significantly different in 14 out of 20 species (see Table 4-2). For these species we also found higher concentrations mostly in set I. However, concentrations of tannins and total phenolics were strongly correlated in the two single sample sets (R^2= 0.88, p< 0.001 and R^2= 0.80, p< 0.001 for set I and set II, respectively) as well as in the combined dataset that comprised all species of both sample sets (R^2= 0.83, p< 0.001, data not shown).

Table 4-3 summarizes the results of the variance components analysis for the different datasets, including all species, excluding the Fagaceae, including only Fagaceae or including only *Castanopsis*. Moreover, it shows the variance components for the more balanced dataset comprising all species of the families Elaeocarpaceae, Fagaceae and Lauraceae. Additionally, Table 4-3 shows the variance components calculated from the dataset of all possible two-species combinations within one genus with the highest explanatory power of the factor *sample set*. Variance components analysis of the complete dataset revealed that family affiliation explained the highest proportion of variance, followed by *genus*. The proportion of variance in total phenolics explained by *within*-species variability was larger than the variation among species. However, for total phenolics, the effect of sample treatment accounted for a larger amount of explained variance than the *among species* level but less than the *within* level. In the case of tannins, both taxonomic levels, *among species* and *within*, explained the same amount of variance. Here, *sample set* accounted for a lower amount of explained variance than the former two factors.

Table 4-2: Concentrations in total phenolics and tannin of the investigated species; Species and Family: species taxonomic affiliation; Code: short codes used in Figure 3; Leaf habit: eve=evergreen, dec= deciduous; All concentrations are presented as tannic acid equivalents, and calculated as mean values ± standard deviation of all specimens for the sample sets I and II, respectively. Letters indicate species-specific significant differences in total phenolics and tannin concentrations between the two datasets, respectively, based on Mann-Whitney-U tests.

Species	**Family**	**Code**	**Leaf habit**	**Phenolics ± SD [mg/g] Sample-set I**	**Phenolics ± SD [mg/g] Sample-set II**	**Tannin ± SD [mg/g] Sample-set I**	**Tannin ± SD [mg/g] Sample-set II**
Liquidambar formosana	Hamamelidaceae	Liq for	dec	102.67 ± 50.42A	86.21 ± 16.84A	64.21 ± 44.33a	40.99 ± 7.27a
*Choerospondias axillaris**	Anacardiaceae	Cho axi*	dec	102.59 ± 19.09A	79.59 ± 4.64A	41.4 ± 5.71a	27.12 ± 7.52b
Daphniphyllum oldhamii	Daphniphyllaceae	Dap old	eve	5.94 ± 2.11A	10.07 ± 1.34A	0.11 ± 0.07a	0.09 ± 0.04a
Elaeocarpus chinensis	Elaeocarpaceae	Ela chi	eve	186.01 ± 31.82A	169.58 ± 26.61B	256.99 ± 80.46a	190.37 ± 25.86b
Elaeocarpus japonicus	Elaeocarpaceae	Ela jap	eve	208.72 ± 32.12A	151.33 ± 62.48B	278.01 ± 46.60a	153.88 ± 54.57b
Castanea henryi	Fagaceae	Cas hen	dec	91.65 ± 6.76A	65.22 ± 0.71A	103.54 ± 21.85a	81.42 ± 25.87a
Castanopsis eyrei	Fagaceae	Cas eyr	eve	76.25 ±20.68A	62.99 ± 13.20A	61.35 ± 41.05a	96.11 ± 11.06a
Castanopsis fargesii	Fagaceae	Cas far	eve	64.23 ± 11.47A	45.44 ± 11.92A	16.91 ± 5.65b	39.34 ± 12.78a
*Castanopsis sclerophylla**	Fagaceae	Cas scl*	eve	58.03 ± 5.17A	40.53 ± 11.63A	28.8 ± 6.77a	17.56 ± 4.18b
Cyclobalanopsis glauca	Fagaceae	Cyc gla	eve	41.85 ± 24.99A	36.85 ± 10.27A	22.07 ± 19.49a	5.25 ± 1.58b
Cyclobalanopsis myrsinifolia	Fagaceae	Cyc myr	eve	22.53 ± 2.34A	36.82 ± 6.44A	4.12 ± 3.76b	8.56 ± 5.05a
Lithocarpus glaber	Fagaceae	Lit gla	eve	33.08 ± 7.60A	38.65 ± 9.93A	5.69 ± 2.20b	7.18 ± 1.19a
Quercus phillyraeoides	Fagaceae	Que phi	eve	82.32 ± 4.83A	65.95 ± 6.44A	104.5 ± 6.28a	77.28 ± 11.78b
Quercus serrata	Fagaceae	Que ser	dec	107.37 ± 23.63A	141.64 ± 87.06A	204.38 ± 37.93a	136.59 ± 70.44a
*Idesia polycarpa**	Flacourtiaceae	Ide pol*	dec	9.07 ± 1.79A	8.36 ± 1.28B	0.49 ± 0.38a	0.25 ± 0.19b
Machilus grijsii	Lauraceae	Mac gri	eve	26.12 ± 5.47A	20.09 ± 3.88A	3.24 ± 0.37a	0.91 ± 0.13b
Machilus thunbergii	Lauraceae	Mac thu	eve	20.14 ± 5.75A	14.79 ± 6.29A	0.84 ± 0.62a	0.34 ± 0.13b
Meliosma flexuosa	Sabiaceae	Mel fle	dec	17.31 ± 4.25A	32.43 ± 11.26A	15.77 ± 5.01a	4.74 ± 2.55b
Alniphyllum fortunei	Styracaceae	Aln for	dec	125.31 ± 12.96A	142.42 ± 57.47A	29.41 ± 3.57a	28.17 ± 12.07a
Schima superba	Theaceae	Sch sup	eve	49.73 ± 1.95B	107.65 ± 3.69A	25.81± 1.40b	31.22 ± 4.36a

*: species excluded from the analyses shown in Figure 1 as well as in the NMDS ordinations in Figure 2 and Figure 3

This relative structure of explanatory power in variance (family> genus> among species > among sample sets) remained stable even after reducing the data bias by only including families with at least two species into the analysis. The proportion of variance in total phenolics and tannin was similarly low as for the factor *species*.

Table 4-3: Results of the variance components analyses. 'Dataset' describes the taxonomic extent in the different variance components analyses, 'compound' refers to the metabolite analyzed. Family, Genus, Species: variation among investigated families, genera and species, respectively. Sample set contains the variation introduced by differences in sampling and processing conditions. Within: residual variance within species.

Dataset	**Compound**	**Relative amount of explained variance [%]**				
		Family	Genus	Species	Sample set	Within
All Species	Total Phenolics	76.1	15.0	0.8	2.8	5.3
	Tannin	63.7	27.3	3.3	2.4	3.3
All species of Fagaceae, Lauraceae, Elaeocarpaceae	Total Phenolics	75.3	15.7	1.6	1.4	5.8
	Tannin	68.2	23.9	2.7	2.5	2.5
Fagaceae, Lauraceae, Elaeocarpaceae, each one genus, sampled with two species each*	Total Phenolics	46.5	46.5	<0.01	1.8	5.2
	Tannin	45.5	45.5	1.7	3.8	3.5
Fagaceae, Lauraceae, Elaeocarpaceae all, two species each*	Total Phenolics	49.6	43.3	<0.01	2.7	4.4
	Tannins	89.4	1.4	2.6	4.3	2.3
All species without Fagaceae	Total Phenolics	46.0	46.0	< 0.01	3.4	4.6
	Tannin	45.4	45.4	4.0	2.3	3.0
Fagaceae	Total Phenolics	--	60.7	8.9	5.4	25.0
	Tannin	--	75.6	8.1	6.5	9.8
Castanopsis	Total Phenolics	--	--	7.2	34.9	57.9
	Tannin	--	--	46.5	28.1	25.4

***** Values of the dataset containing the combination of species that have highest explanatory power for the factor 'sample set'

Examining the proportions of variance calculated from the most balanced datasets (containing all possible combinations of two species from one genus within the families of Elaeocarpaceae, Fagaceae and Lauraceae) showed that *family* and *genus* accounted for equally high proportions of variance in total phenolics as well as tannin concentrations. In the dataset with the highest explanatory power of *sample set* to predict total phenolics concentrations *species* explained only a marginal proportion of variance. However, in the case of tannin concentrations *species* accounted for approx. 2% of explained variance within this dataset. Interestingly, *sample set* here accounted for 3.8% whereas within-species variability explained only 3.5% of variance.

In the case of total phenolics, the same pattern was encountered when analyzing the 27 combinations of two species from each family, even when only considering the run with the highest proportion of variance explained by *sample set* (Table 4-3). Regarding tannin concentrations, highest proportions of variance were also found to be explained by the factor *family* in the dataset exhibiting the highest explanatory power of *sample set*. Again, tannin concentrations were more affected by *sample set* than total phenolics concentrations

Excluding the family Fagaceae from the analysis considerably decreased the explanatory power of the *family* level on variance explained for total phenolics and tannin concentrations (Table 4-3), indicating that this particular family carried a large proportion of overall variance in the dataset. However, after excluding Fagaceae from the analysis, *family* and *genus* jointly still accounted for more than 90% of variance in both secondary metabolites. Similar to the complete dataset, variance explained by the effect of *sample set* was larger than variance among species in the case of total phenolics but smaller than for tannin concentration.

Within the family of the Fagaceae, the highest taxonomic level included in the analysis (*genus*) accounted for the largest amount of explained variance in phenolics and tannin concentrations. The explanatory power of the within-species variability was much higher than that of the variability among species in the case of total phenolics but only slightly higher in the case of tannin concentration. Within this family, the effect of *sample set* never outweighed the *species* level, neither for total phenolics concentration, nor for the amount of extractable tannin.

The analysis of the impact of sampling conditions on the variance components of the dataset that contained only species from the genus *Castanopsis* revealed that the variance explained by *sample set* drastically increased more than 6-fold with respect to total phenolics and more than 4-fold in the case of tannins when compared to the explanatory power of this factor in the dataset containing the whole sample set of the Fagaceae. It even increased more than 10-fold when compared to the dataset containing all specimens sampled within this study. In the case of total phenolics concentrations, the within-species variability was much larger than the among-species variability. In contrast, for tannin concentration we found the among-species variability to be most explanatory.

Levels of phylogenetic conservatism in plant polyphenols compared to other traits in the leaf economics spectrum

Comparing the relative amount of explained variance among all analyzed traits studied from the leaf economics spectrum (see Figure 4-2), leaf dry matter content (LDMC) and specific leaf area (SLA) were characterized by a large proportion of variance explained by *family* and *within* individuals, whereas the *genus* level in both cases carried marginal explanatory information. Leaf nitrogen (LNC) and phosphorus concentrations (LPC) *family* and *species* explained similar proportions of variance. *Genus* had a considerable explanatory power in the case of LNC. For both LNC and LPC, the *within*-individual variation accounted for the majority of variance in the data. Similarly, *within*-species variation explained the largest amount of variance in StoD, while family and genus together only accounted for about 40%.

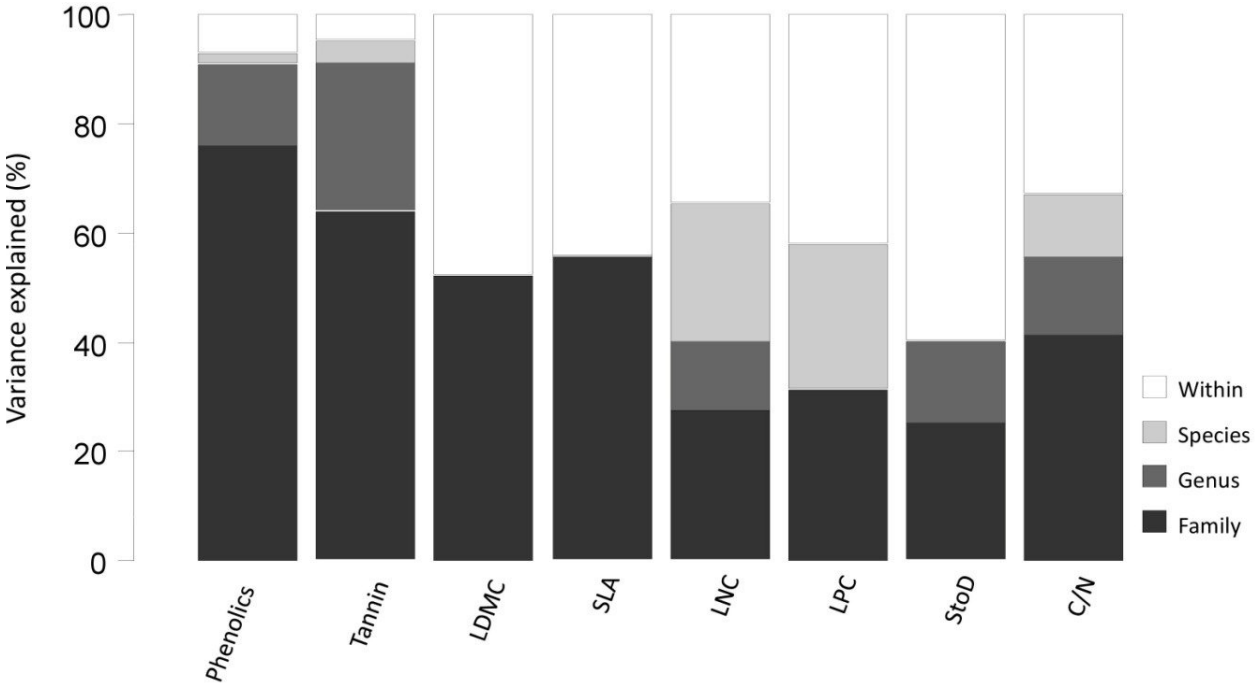

Figure 4-2: Percentage of variance explained by family, genus, species and individuals within species for total phenolics and tannin concentration as well as selected traits from the leaf economics spectrum. LDMC: leaf dry matter content, SLA: specific leaf area, LNC: leaf nitrogen concentration, LPC leaf phosphorus concentration, StoD: stomatal density, C/N: carbon: nitrogen ratio. Data on additional leaf traits (i.e. LDMC, SLA, LNC, LPC, StoD and C/N) were obtained from [11].

In contrast, and in accordance to the results presented in section '*The effect of phylogeny and sample set'*, the bulk of variance for total phenolics and tannin was explained by the level of *family*, followed by *genus*. No other trait carried more information at these highest taxonomic

levels. Moreover, the *within*-species variability was much lower in the two secondary compounds than in the other investigated traits.

Effect of different sample sets

The Procrustes analysis showed a high similarity of the two ordinations for the two different sample sets as indicated by the Procrustes sum of squares m^2_{12}=0.35. This result was found to be highly significant in a Procrustes significance test based on 10.000 permutations (R^2= 0.81, p< 0.001). Calculated from the sum of Euclidean distances of all 20 corresponding species pairs, the absolute shifts between target and rotated matrix summed up to a total of 2.21. These shifts could be mainly traced back to only three species, namely *Quercus serrata*, *Castanopsis fargesii* and *Meliosma flexuosa* (see Figure 4-3).

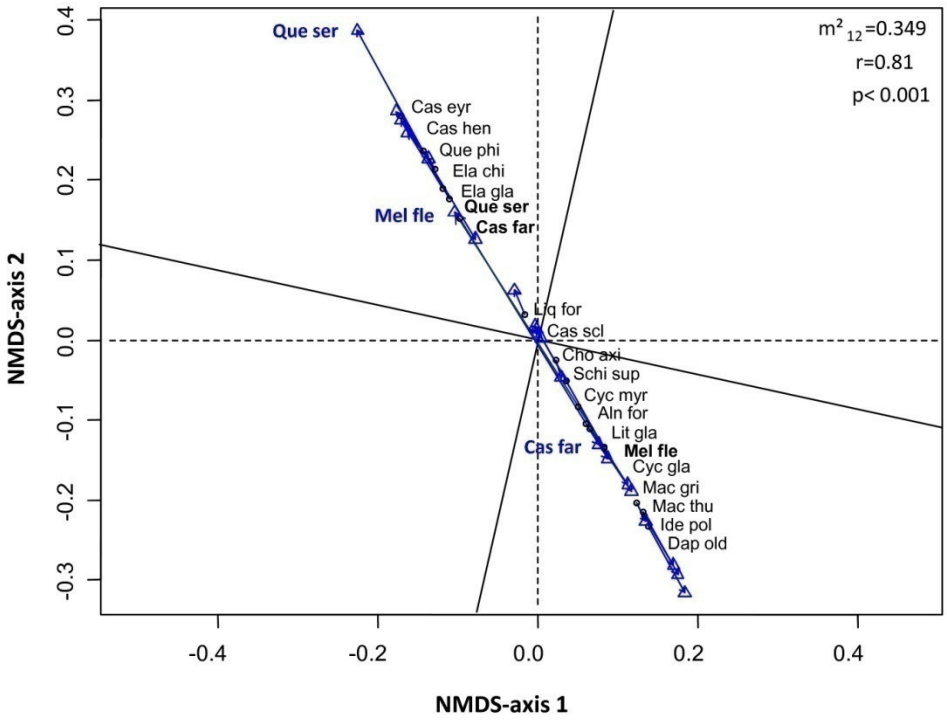

Figure 4-3: Plot of the Procrustes rotations between matrices of sample set I (black letters, dotted axes) and set II (blue letters, solid axes); note that only species that each contributed to more than 10% of the total shift are drawn in blue. For abbreviations see Table 4-2.

The latter three species each contributed to more than 10% of shift in the matrix rotation, i.e. their Euclidean distance between rotated and target matrix was more than 10% of the total Euclidean distance of all shifted species (Table 4-4).

Table 4-4: Relative contribution of each analyzed species to the total shifts in the Procrustes rotation. Data is based on the sum of the Euclidean distances across all species between the NMDS scores in data set I and II. Species with more than 10% of relative contribution to overall shifts are printed in bold letters.

Species	**Relative shift (%)**	**Species**	**relative shift [%]**
Alniphyllum fortunei	2.3	*Elaeocarpus japonicus*	4.5
Castanea henryi	0.5	*Idesia polycarpa*	3.5
Castanopsis eyrei	8.2	*Liquidambar formosana*	1.5
Castanopsis fargesii	15.0	*Lithocarpus glaber*	4.2
Castanopsis sclerophylla	0.7	*Machilus grijsii*	1.2
Choerospondias axillaris	1.0	*Machilus thunbergii*	3.8
Cyclobalanopsis glauca	8.0	***Meliosma flexuosa***	15.7
Cyclobalanopsis myrsinifolia	5.2	*Quercus phillyraeoides*	2.1
Daphniphyllum oldhamii	4.3	***Quercus serrata***	10.8
Elaeocarpus chinensis	3.9	*Schima superba*	3.4

The NMDS analyses show the inter-relationships of traits including total phenolics and tannin concentrations of sample set I (Figure 4-4a) or II (Figure 4-4b). Stress levels were lowest in both ordinations when six dimensions were used. Both NMDS ordinations showed very low levels of stress (0.049 and 0.047 for ordinations based on sample set I and II, respectively). These stress levels indicate a reliable spread of traits and species in the six dimensions calculated (MacCune *et al.* 2002), permitting reasonable conclusions from the plots. For the sake of clarity we only present the first two axes of both ordinations. The qualitative outcome was found to be similar for both analyses concerning both, the spread of the species in trait space as well as the post-hoc correlations of the trait vectors in species space. In particular, deciduous species clearly segregated in both panels of Figure 4-4, as also indicated by the corresponding trait vector 'deciduous'. In both cases phenolics and tannins were strongly correlated with each other as well as with leaf nutrient concentrations such as leaf nitrogen (LNC) and sulfur concentrations (LSC). This is also seen in the univariate correlations presented in the four panels of Figure 4-5. In the case of tannin concentrations we found a logarithmic increase of tannin concentrations with increasing nitrogen concentration.

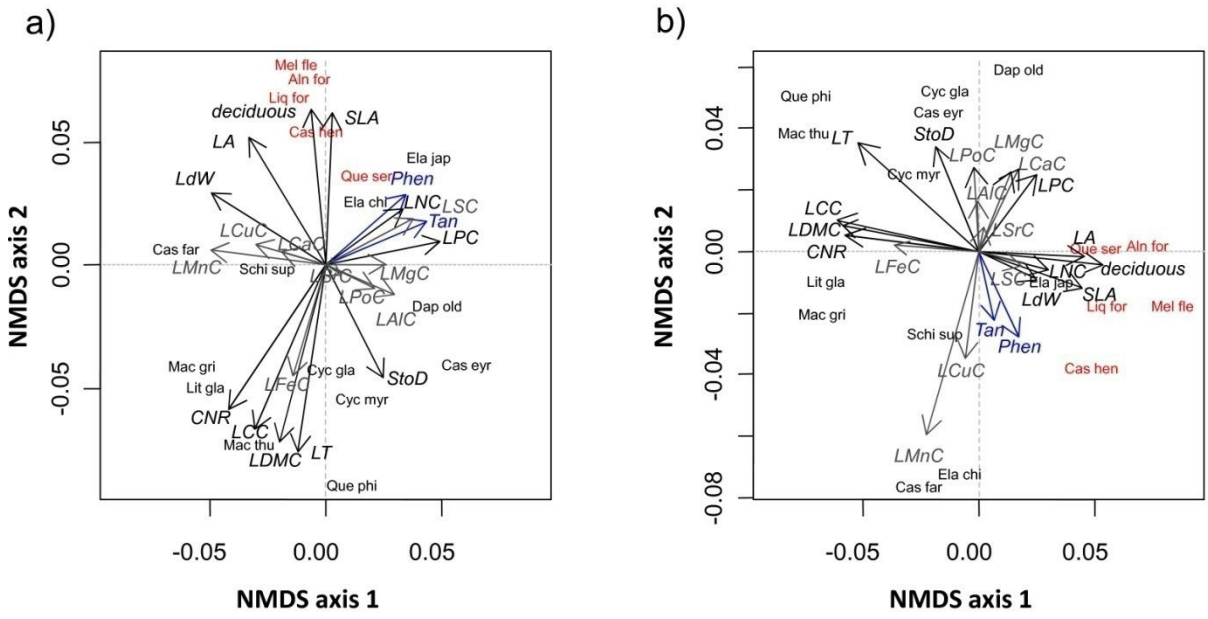

Figure 4-4: NMDS plots of the first two axes of the NMDS ordinations based on polyphenolic concentrations in sample set I (a); or II (b); black fonts: evergreen species, red fonts: deciduous species; blue arrows: secondary metabolites investigated in this study, Phen: total phenolics concentration; Tan: tannin concentration; black arrows: additional traits as determined [11], SLA: specific leaf area, LDMC: leaf dry matter concentration, C/N: carbon: nitrogen ratio, LT: leaf toughness (Kröber, unpublished data), LCC: leaf carbon concentration, LNC leaf Nitrogen concentration, LSC: leaf sulfurous concentration, LPC: leaf phosphorus concentration, LdW: leaf dry weight, LA: leaf area, StoD: stomatal density, deciduous: deciduous leaf habit, gray arrows: mineral contents: LAlC: leaf aluminum concentration, LCaC: leaf calcium concentration, LCoC: leaf cobalt concentration, LCuC: leaf copper concentration, LFeC: leaf ferric concentration, LPoC: leaf potassium concentration, LMnC: leaf manganese concentration, LSrC: leaf strontium concentration; for species abbreviations see Table 4-2.

Apparently, the direction of the vectors for phenolics and tannins were dominated by the species that had the highest amounts of these secondary metabolites, namely *Elaeocarpus chinensis* and *E. japonicus* (see also Table 4-2). Specific leaf area (SLA) was collinear to the 'deciduous' leaf habit in both panels of Figure 4-4. Moreover, leaf dry matter content (LDMC) was strongly correlated to leaf toughness (LT) as well as carbon-to-nitrogen ratio (C/N), but negatively related to tannins and total phenolics, as seen in opposing vector directions. The vector for the deciduous leaf habit pointed to the opposite direction of the vector for stomatal density (StoD). These results were similar in both ordinations of the two datasets.

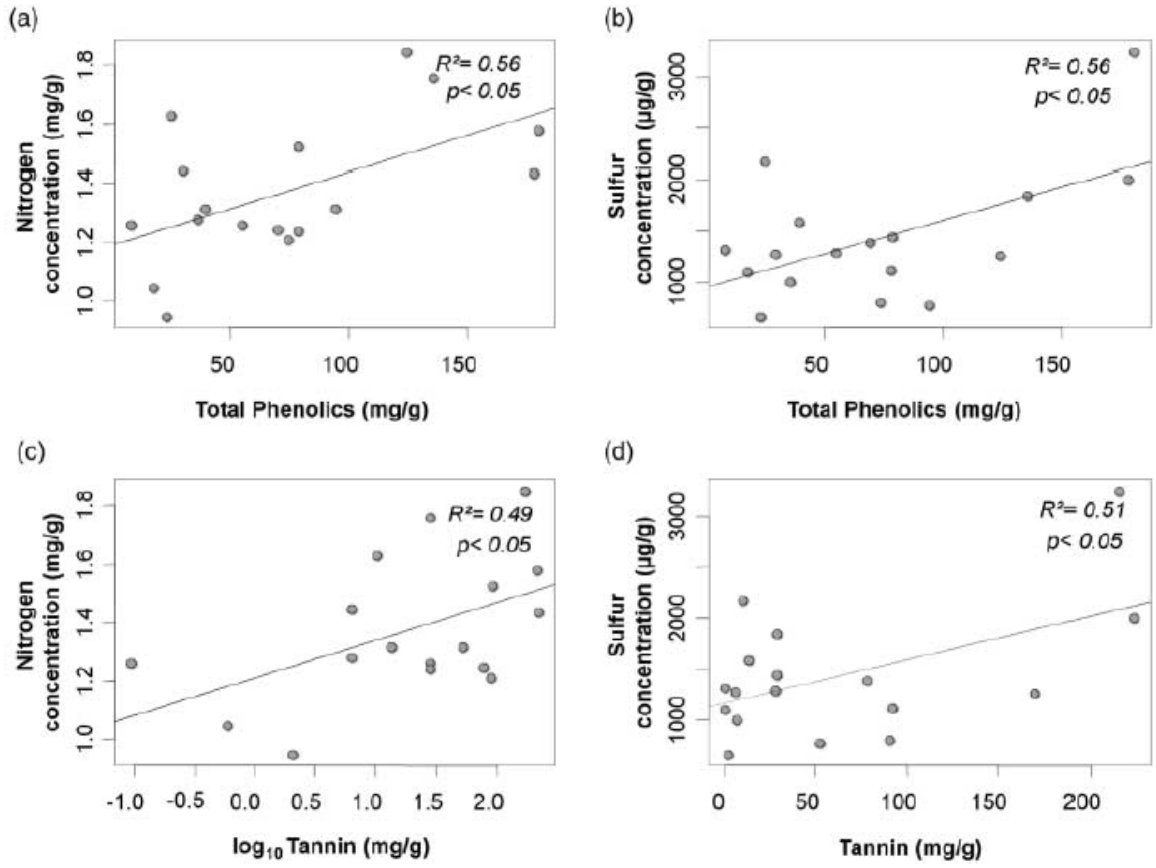

Figure 4-5: Univariate correlations between total phenolics as well as tannin concentrations of leaves with a) and c) leaf nitrogen concentration (LNC) and b) and d) leaf sulphur concentration (LSC); note that panel c) shows log10 transformed values of tannin concentrations

4.2.4. Discussion

As also reported in several other studies (e.g. Hagerman 1988, Salminen 2003) we detected noticeable changes in the polyphenol concentrations between the two sample sets for some species. Tannin concentrations were found to be less robust to differences in sample treatment than non-tannin phenolics. In general, we found higher concentrations in set I (possibly evoked by thermal or light-induced heterolysis phenolic substances bound to the cell wall). Our major finding, however, was that the amount of variance explained by the different sampling and storage conditions, was small as compared to the variance explained by the taxonomic levels *family* and *genus*. This result is further supported by the finding that, according to Pagel's λ, both secondary metabolites show highly significant trait conservatism along the investigated phylogeny. The finding of high explanatory power of *family* and *genus* is in line with the observations of Villar *et al.* (2006), who calculated the chemical composition and construction costs of 16 different plant species, taking into account

secondary compounds. They also found that *family* affiliation explained the largest proportion of variance (up to 80%). The authors also stated that the chemical composition in their studied species 'contain a large variation within genera', but gave no quantitative specification on explained variances. Depending on the taxonomic range investigated we found that considerable amounts as large as 46% of variance were explained by genus affiliation. Yet, as presented in other publications, such as Hagerman (1988) and Salminen (2003), the amount of hydrolysable tannins can be substantially altered by differences in sample drying and storage conditions. Moreover, several investigations demonstrate the strong effects of sampling leaves in different times of the year, due to seasonal variability in tannin concentration (Ammar *et al.* 2004; Salminen *et al.* 2004). Additionally, Julkunen-Tiitto and Sorsa (2001) demonstrated substantial changes in the structural composition of phenolics when applying heat-based drying methods as well as in freeze-drying, when samples were pre-frozen in liquid nitrogen. Our results do not necessarily contradict these findings, but only put them into perspective as we could observe shifts in the redox activity of polyphenols and the protein precipitation capacity of tannins found in the two sample sets. However, based on our results we cannot decide whether these changes were brought about by the differences in sampling protocols or by other environmental factors. Changes caused by storage conditions affected concentrations of tannins and non-tannin phenolics much less than the taxonomic levels included in the analysis. The strong impact of phylogeny described in the present study does also not seem to match the observation of Hattas *et al.* (2011), who found more similar polyphenolic compounds in phylogenetically more distant species than in closely related species. While this may ultimately be the case for the particular chemical structure of polyphenols, this does not render our results on total contents invalid. Many studies show that synthesis pathways of secondary metabolites are phylogenetically conserved (e.g. Peregrin-Alvarez *et al.* 2009). However, our data do not allow conclusions on the synthesis pathway, but our findings of increasing impact of differences in sample handling with decreasing taxonomic range also points to phylogenetic conservatism. Still it might be possible that the observed increase in the amount of variance explained by *sample set* was caused by changes in structure and/or activity of particular macromolecular tannin compounds as well as the reductive potential in the Prussian blue method.

Despite these caveats, the first hypothesis of non-transferability of results from studies with different handling of samples during collection and storage can be rejected in the case of total phenolic concentrations. However, although this applies for most of species, there were some notable exceptions. In some species, e.g. *Elaeocarpus chinensis*, differences in total phenolics

were remarkable. In contrast, tannin concentrations were found to be significantly different between the two sample sets, thus not allowing a full rejection of the first hypothesis. Thus, differences in sample handling may be important in cases when studying e.g. species-specific plant-herbivore interactions, where the concentration of specific polyphenol macromolecules have to be taken into account, especially when total tannin concentrations are considered. Specifically, we would like to make clear that we do not claim that sampling, storage and drying conditions are unimportant. However, we do state that the negative effects of suboptimal sample collection, storage and processing was found to be overridden by the variance brought about by including taxonomically widespread species.

In our second analysis we found that, compared to other traits in the leaf economics spectrum (*sensu* Wright *et al.* 2004), the two secondary metabolites were the most phylogenetically conserved ones. Trait conservatism was even more pronounced for total phenolics than for tannin concentrations. In the case of other leaf traits, such as LDMC and SLA, the within-species variation was much higher, probably as adaptations to different light regimes and indicating a high phenotypic plasticity of these traits (Givnish 1988). Similarly, the leaf nutrient concentration displayed a less pronounced phylogenetic conservatism, which can be seen in a high amount of within-species variation, probably brought about by differences in abiotic growth conditions such as nutrient availability and soil moisture conditions (Chapin III *et al.* 1987). Thus, we have to reject our second hypothesis, as total phenolics and tannins concentration showed a more pronounced phylogenetic conservatism, with a clearly hierarchical structure along the taxonomic gradient, compared to other leaf traits. In particular, for total phenolics and tannins this finding emphasizes the need for phylogenetic correction in inter-species comparisons of trait analyses as suggested by Westoby *et al.* (1995).

To our knowledge, the present study is the first one investigating the robustness of ordination techniques to differences in sample treatment of polyphenolic secondary compounds in plant leaf material. We showed that the two ordinations were highly significantly correlated. We found that the highest amount of variation could be traced back to single species such as *Meliosma flexuosa* and *Quercus serrata*. This finding is in accordance with that of Ammar *et al.* (2004), who reported that changes in polyphenolic concentration of leaves from different stages of maturity were dependent on species identity. Despite the impact of such sources of variation, we found no impact on the conclusions drawn from ordination techniques when analyzing, e.g. trait inter-relationships.

In conclusion we state that, under the prerequisite that a large dataset with a wide taxonomic spectrum is investigated, the well-documented effects of differences in sampling season, sample treatment and storage conditions are overridden by the taxonomic identity of the respective species. We suggest that future studies, using the sampling, drying and preservation procedures presented in this paper, should compare samples of different sampling times and environments to identify those factors that exert the strongest influence on the concentration of phenolic compounds. Conversely, in experiments where only few congeneric or closely related species are investigated, care has to be taken that specimens are sampled from plants growing in similar ecological conditions and are optimally preserved.

4.2.5. Acknowledgements

We are grateful to M. Böhnke and A. Hallensleben for their relentless field and lab work on the investigated species traits and thank the whole BEF-China team for the inspiring working atmosphere. O. Purschke provided the species phylogeny and assistance with the phylogenetic analyses.

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4.3. Shifts in community leaf functional traits relate to litter decomposition during secondary forest succession

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Abstract

Aims: We investigated shifts in community weighted mean traits (CWM) of 14 leaf functional traits along a secondary successional series in a laurophyllous rain forest in subtropical China. Most of the investigated traits have been reported to affect litter decomposition in previous studies. We asked whether these shifts followed a linear, logarithmic or quadratic pattern and reflected shifts indicate a change in resource use strategy along the successional gradient. Using community decomposition rates (k-rates) for all investigated communities derived from annual litter production and standing litter biomass we asked whether changes in litter k-rates along the successional series were caused by changes in leaf functional traits.

Methods: Twenty-seven plots were examined for shifts in leaf CWM traits as well as k-rates along the secondary succession in a subtropical rainforest established in the framework of the BEF-China project. We analyzed both physical traits (such as leaf toughness, C to N ratio) and chemical traits (such as polyphenols and tannins) that might affect leaf litter decomposition. Three alternative linear models were used to reveal the general patterns of shifts in CWM trait values. Multiple regression analysis was applied to investigate causal relationships between leaf functional traits and decomposition rates to assess which traits had the highest impact on community litter decomposition rates.

Important findings: Shifts in CWM functional traits generally followed logarithmic patterns whereas community k-rates remained stable along the successional gradient. In summary, the shifts indicate a change in resource use from high nutrient acquisition to nutrient retention with ongoing succession. We successfully linked litter decomposition rates to changes in leaf traits along the successional sere. CWM traits that are related to nutritional quality (such as leaf phosphorous concentrations) promoted community k-rates whereas physical and chemical defense traits (such as polyphenols and leaf toughness) decreased litter decomposition. Thus, our findings show that community leaf decomposition is dependent on

more than one trait and is a result from interactions of different traits that affect decomposition in opposing directions.

Keywords: secondary forest succession; BEF-China; nutrient cycling; plant polyphenols; multiple regression analysis

4.3.1. Introduction:

Secondary succession is defined as a directional changes in community composition after disturbance (Horn 1974; Finegan 1984). While early studies focused on the successional changes in plant species composition (e.g. Odum 1960; 1974; Vitousek and Reiners 1975; Guariguata and Ostertag 2001; Peña-Claros 2003), more recent studies have also taken functional traits into account. The investigated plant characteristics often comprised functional leaf traits such as specific leaf area (SLA) and leaf nitrogen content (LNC), which often exhibited a decrease in the course of succession, and leaf dry matter content (LDMC), which mostly displayed an increase. In a global analysis, Wright *et al.* (2004) demonstrated that SLA, LNC and LDMC among other traits determine the leaf economics spectrum (LES), defined by a trade-off between productivity and resource conservation. In general, high plant investments into structural compounds, as e.g. reflected in high values in LDMC, were found to be related to low productivity. In the course of succession, frequently a shift has been described from communities consisting of fast growing, highly productive species with short-lived leaves to communities consisting of slowly growing, lowly productive species with long-lived leaves (e.g. Garnier *et al.* 2004; Caccianiga *et al.* 2006; Vile *et al.* 2006; Raevel *et al.* 2012). However, as demonstrated by Mason *et al.* (2011), the shifts towards nutrient conservation during secondary succession does not hold true for every ecosystem. Studying a gradient of secondary succession in a New Zealandian rainforest, the authors observed an increase in community mean leaf nutritional value during forest maturation. With ongoing succession they also detected an increase in SLA and a decrease in leaf secondary metabolites (e.g. polyphenolics). The authors concluded that these shifts indicate an increase in leaf palatability as well as in leaf decomposability. However, as stated above, the majority of successional studies report the opposite trend. Thus, the New Zealandian rainforest seems to be exceptional.

Irrespective of the directional shifts in leaf traits along succession, a corresponding change in leaf litter decomposability has not explicitly been shown yet, although decomposition can be considered as one of the most important processes in terrestrial ecosystems (Aber *et al.* 1991).

The physical, biological and chemical breakdown of organic matter releases biologically bound nutrients and makes them available for plant growth (Swift *et al.* 1979; Berg and McClaugherty 2008). In accordance with the rationale of Mason *et al.* (2011), several studies have demonstrated that decomposition dynamics of leaf litter strongly depend on leaf functional traits (e.g. Cornwell *et al.* 2008; Freschet *et al.* 2012; Freschet *et al.* 2013).

Traits that were frequently found to promote litter decomposability were connected to high plant productivity, photosynthetic capacity and leaf nutritional value (Aerts 1997b; Aerts 1997a; Aerts and de Caluwe 1997; Cornelissen and Thompson 1997). In contrast, leaf chemical defense traits such as e.g. polyphenol contents were found to negatively affect decomposability (Hättenschwiler and Vitoussek, 2000). Similarly, leaf traits referring to physical resistance such as leaf toughness, LDMC and leaf carbon content have been reported to have negative effects on litter decomposability (e.g. Hättenschwiler *et al.* 2005). As stated above, most of these traits, in turn, were also reported to change in communities undergoing secondary succession; thus litter decomposability can be expected to change accordingly.

Besides functional and abiotic characteristics as well as stand age, leaf-litter species richness might affect decomposition rates. In natural ecosystems, leaf litter is unlikely to consist of single species debris but is merely a mixture of litter types from all species growing in the respective community. Recent studies have shown that decomposition rates of leaf litter mixtures cannot be predicted from those expected from the k-values of involved single-species leaf litter (see e.g. Hoorens *et al.* 2003; Wardle *et al.* 2003; Gartner and Cardon 2004). Non-additive effects, resulting from an impact of one litter type on the decomposition of another may either accelerate (synergistic effects) or reduce mixed-litter decomposition (antagonistic effects). Such litter mixing effects on decomposition rates may be substantial. For example, Hector *et al.* (2000) found an up to 37% greater mass loss in mixed grass litter than expected from mass losses of monospecific decomposition. Whether the effect of species mixture is synergistic or antagonistic was found to be highly dependent on which litter types were combined during the investigation of litter decomposition (see e.g. Wardle *et al.* 2003).

There are various ways in which litter mixtures may affect decomposability of species-specific litter. Nutrient transfer has been described from one litter type to another through leaching (Fyles and Fyles 1993; Briones and Ineson 1996) or via microorganisms such as fungi (e.g. McTiernan *et al.* 1997). Changes in the microclimatic conditions within the litter layer as well as changes in the decomposer abundance and activity have also been reported (see reviews of Facelli and Pickett 1991 and Gartner and Cardon 2004 for details).

As stated by the mass-ratio hypothesis (Grime 1998), it may be anticipated that the most abundant species in a community exerts the highest impact on ecosystem properties (see also Garnier *et al.* 2004, Díaz *et al.* 2007). Following this argumentation line, it can be expected that functional leaf traits of the most dominant species should have the highest influence on ecosystem functions such as decomposition. As demonstrated in a study on changes in community leaf trait composition along a successional sere investigated by Garnier *et al.* (2004), community weighted mean (hereafter CWM) trait values offer a convenient metric to describe shifts in functional trait values and may be used to relate functional characteristic of plants to ecosystem properties. In most cases, CWMs are aggregated species mean trait values, using the same species trait value across the different communities that are compared (but see Auger and Shipley 2013). However, such a procedure is only valid under the assumption that the trait variability within a certain species is low in comparison to the variability among species (see Albert *et al.* 2010; Albert *et al.* 2011), which is true for some traits such as phenolics and tannin as well as leaf toughness (Eichenberg *et al.* 2014a, b) but might not be for other traits. CMWs calculated in this way for diverse plant communities have successfully been used to predict ecosystem-specific above-ground net primary production along a gradient of secondary succession (Vile *et al.* 2006a). Thus, changes in CWM traits along secondary succession, which have been postulated to be indicative for community resource use strategy (e.g. Mason *et al.* 2011; Raevel *et al.* 2012) might also be used to investigate the relationships between leaf functional traits and community litter decomposition rates.

In the present study we analyzed shifts in CWM traits along a gradient of secondary succession established in the BEF-China project. We examined 27 communities along a gradient covering approx. 120 years of secondary forest succession. For a total of 143 species we assessed mean values of 14 traits which have been reported to relate to litter decomposability. In addition we measured community leaf litter decomposition along the gradient by calculating community decomposition rates (k-rates). We hypothesized that 1) along secondary succession, there is a shift from communities dominated by species with high productivity, high leaf nutrient contents and short-lived leaves with low physical or chemical resistance to communities consisting of species with low productivity, low leaf nutrient contents and more persistent leaves (i.e. higher investment into structural compounds and chemical defense). We expected that these shifts remain significant after accounting for species richness. We also tested whether these shifts followed a linear, logarithmic or quadratic pattern (see Mason *et al.* 2011). We further hypothesized that 2) community litter

decomposition rates decline with successional age, irrespective of species richness, and that 3) the changes in litter decomposition along the successional series can be explained by the observed shifts in CWM traits. More precisely, we predict that traits relating to high nutrient content and high productivity increase litter decomposability, whereas traits related to physical and chemical resistance decrease litter decomposition rates. To our knowledge this is the first study that relates shifts in trait composition to leaf litter decomposition in the course of a subtropical secondary forest succession.

4.3.2. Materials and Methods:

Research location and species inventory

The Gutianshan National Nature Reserve is located in the Zhejiang Province in southeast China. It comprises more than 80 km² of laurophyllous rainforests undergoing secondary succession. With a mean annual temperature of 15.1°C and mean annual precipitation accumulating to 1964mm, the climate is characterized as typically subtropical, exhibiting a wet season from May to June (see Geißler *et al.* 2010 as well as Hu and Yu 2008 for further information) and exhibiting a bedrock formed by a granite intrusion. A total of 27 plots of 30 m by 30 m ('Comparative Study Plots', in short CSPs) have been established along a gradient of secondary succession. This sere covers communities with a range of duration in secondary succession from approx. 20 to 120 years (67.4 ± 26 yrs, mean ± SD) after periodic logging by the local population. Thus, the secondary succession series presents a secondary succession on previously forested terrain. In all 27 CSPs a census was carried out in 2008 and 2009 and all individuals exceeding 1 m in height were counted and identified to the species level. A total of 148 woody species were recorded across all 27 CSPs. Age determination was accomplished by counting tree rings from core drillings (see Bruelheide *et al.* 2011 for further details). Plant species names follow the nomenclature of the "Flora of China" (www.efloras.com).

Leaf traits assessment

In the present investigation, we focused on the shifts of leaf CWM traits from tree species along the successional gradient, for practical reasons. The importance of herbaceous species in the herb layer and their impact on forest-ecosystem functioning is well-known (e.g. Tchouto *et al.* 2006). However, with respect to the successional gradient established within the BEF-China project, Both *et al.* 2012) found that, in contrast to recruits of woody species, herbaceous species played a minor role in species composition of the 27 CSPs.

We compared the shifts in CWM values for 14 leaf functional traits (see Table 1), which have been related to litter decomposability in other studies. Nine of these leaf functional traits were compiled from Kröber *et al.* (2012) for 143 (out of the total 148) tree species. From all 143 species found in the CSPs, information on leaf polyphenolic content could be determined for 101 species. Fifty milligrams of dried leaf powder were extracted in 20ml of 50% aqueous acetone, according to the procedures described in Torti *et al.* (1995). Leaf total phenolics concentrations were determined using the prussian blue method (Price and Butler 1977) as modified by Graham (1992). Total tannin concentrations were determined using the radial diffusion method for increased sensitivity as described by Hagerman 2002). Both secondary metabolites were quantified by standardization against tannic acid (Roth, Germany, Charge Nr. 250153788) and expressed as milligrams per gram dry weight of tannic acid equivalents (for further details see Eichenberg *et al.* 2014a). In total, 381 specimens were analyzed for total phenolics as well as tannin content.

Leaf toughness was assessed as leaf tensile strength for a total of 91 species according to the methods described by Hendry and Grime (1993). Briefly, leaf fragments of 1-5 mm width were cut from the central part of the leaves (avoiding the midrib and major veins) along the longitudinal axis. These fragments were fixed between two clamps in the tearing apparatus and slowly pulled apart. The force needed to tear apart the leaf fragment was measured with a spring balance (Cornelissen *et al.* 2003). In total, 1725 single leaves were analyzed.

Information on life form (evergreen or deciduous) and leaf type (pinnate or entire) for 143 out of the 148 species was compiled from field observations and the Flora of China.

In general, we investigated traits that can be assigned to four different functional regimes (see Table 4-5): Firstly, we investigated traits relating to the productivity and photosynthetic efficiency of plants. Among the traits analyzed regarding productivity we included specific leaf area (SLA), leaf calcium content (LCaC) and stomatal density (StoD). While LCaC was found to be significantly correlated with the maximum rate of photosynthesis (Reich *et al.* 1995), StoD has been related to the efficiency of a plant's adaptation to the water vapor deficit and thus to the efficiency of photosynthesis (Carpenter and Smith, 1975; Kröber *et al*., 2012). Secondly, we aimed at assessing leaf traits concerning the nutritional value of the plant tissue by including information on leaf nutrient concentrations of nitrogen (LNC), phosphorus (LPC) and sulfur (LSC). A third group of traits were those related to chemical leaf defense. To this end, we included leaf total phenolics and tannin concentrations. These secondary metabolites have frequently been related to nutrient turnover dynamics in terrestrial

ecosystems and litter decomposability (see e.g. Hättenschwiler *et al.* 2005). The last group can be surmised as indicators of physical resistance due to investment into structural components: we included leaf toughness (LT), leaf carbon content (LCC), leaf dry matter content (LDMC) as well as the carbon-to-nitrogen ratio (C/N). As most of these traits might affect multiple functions simultaneously, the assignment to these three groups and the specific functions of each trait in Table 4-5 can only be taken as a rough guide. However, in order to test our hypotheses we decided to interpret the chosen traits under the light of the functional regimes presented above as their predominant roles.

Table 4-5: List of traits investigated in this study and a selection of their ecological functions. C/N: carbon-to-nitrogen ratio; eve: evergreen leaf habit; LCaC: Leaf calcium concentration; LCC: leaf carbon concentration; LDMC: leaf dry matter content; LNC: leaf nitrogen concentration; LPC: leaf phosphorus concentration; LSC: leaf sulphur concentration; LT; leaf toughness; Phenolics: leaf total phenolics concentration; pinnate: leaf pinnation; SLA: specific leaf area; StoD: stomatal density; Tannin: leaf tannin concentration

Trait	**Functional regime**	**Other ecological functions**
SLA [mm².g-1]	Productivity	adaption to light regime; leaf life span, structural defense, relative growth rate, photosynthetic rate, productivity [1,2,3,4]
LCaC [µgg-1]	Productivity	Leaf transpiration, leaf life span, nutritional quality, productivity, photosynthetic rate[12,13]
StoD [1.µm-²]	Productivity	Adaption to vapor pressure deficit, photosynthetic rate [11,12.13]
LNC [%]	Nutritional value	Nutritional quality, photosynthetic rate, productivity [1,9]
LPC [µg.g-1]	Nutritional value	Nutritional quality, photosynthetic rate, productivity [1,9]
LSC [µgg-1]	Nutritional value	Chloroplast construction, rate of photosynthesis, nutritional quality, productivity [9,10]
Phenolics [mg.g-1]	Chemical defense	Chemical defense, decomposability [13.15]
Tannin [mg.g-1]	Chemical defense	Chemical defense, decomposability [13,15]
C/N [%]	Physical resistance	Nutritional state of plant, physical resistance, disturbance regime, nutritive value, leaf longevity, decomposability, productivity [5.6]
LCC [%]	Physical resistance	Structural components, physical defense, relative growth rate [1,2]
LDMC [mg.g-1]	Physical resistance	Physical defense, leaf life span, relative growth rate [1,7,8]
LT [N.mm-1]	Physical resistance	Physical defense, decomposability [14,15,16]
Evergreen	--	Leaf longevity [17.18]
Pinnate	--	Adaption to light regime [17]
k rate	--	Decomposition [19]

References: [1] Cornelissen *et al.* (2003), [2] Wright *et al.* (2004), [3] Poorter and Garnier (1999), [4] Reich *et al.* (1999), [5] Matsuki and Koike (2006), [6] Tateno and Chapin III (1997), [7] Poorter and Garnier (1999), [8] Grime *et al.* (1997),[9] Aerts and Chapin III (1999), [10] Terry (1976), [11] Kröber *et al.* (2012), [12] Gindel (1969), [13] Carpenter and Smith (1975), [14] Eichhorn *et al.* (2007), [15] Hättenschwiler and Vitousek (2000), [16] Perez-Harguindeguy *et al.* (2000), [17] Roloff (2004), [18] Graca *et al.* (2005), [19] Cornwell *et al.* (2008)

Assessment of community decomposition rates.

For each of the 27 CSPs, the community decomposition constant k was estimated according to Olson 1963) as presented in equation 1:

$$k = \frac{\text{Annual leaf litter production } [\text{Mg} * \text{ha}^{-1}]}{\text{Forest floor leaf litter mass } [\text{Mg} * \text{ha}^{-1}]}$$

Leaf litter production (Mg ha^{-1}, oven-dried at 80 °C until constant weight) was assessed using five litter traps evenly spread across each plot. To account for inter-annual variation in monthly collected litterfall, we calculated annual mean leaf litter production based on data obtained in two subsequent years (2010 and 2011). Mean forest floor litter mass (Mg ha^{-1}) was seasonally determined (spring 2009, summer 2009, autumn 2009 and winter 2010) by sample cores taken in undisturbed litter patches in each CSP, excluding twigs with diameter >0.6 cm. In parallel we measured litter thickness at 12 additional points per CSP to correct for spatial variation of forest floor litter mass. The obtained k-rates do not only integrate over decomposition dynamics influenced by species abundance and composition at the community level, but also account for potential non-additive litter mixture effects that have been observed during decomposition (Gartner and Cardon 2004; Hättenschwiler *et al.* 2005).

Functional trait data

Mean trait values and weighted community aggregated mean trait

For each of the 12 continuous trait variables (see Table 4-5) species mean values were calculated. Dichotomous categorical variables (evergreen/deciduous, pinnate/entire) were 1/0 coded and treated as numeric variables to obtain community weighted relative proportions of evergreen and pinnate species within the respective communities. Community mean traits (CWM) were computed on the basis of species mean trait values species and abundances in the plot according to equation 2.

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i a_i}{\sum_{i=1}^{n} a_i}$$

with $\bar{x}$ = community abundance-weighted aggregated mean trait; x_i = mean trait value of species *i,* a_i = abundance of species *i* and n= species richness of the community.

We used the FD-package (Laliberté & Shipley, 2011) in R for CWM calculation. All statistical computations were performed using the statistical program R (Version 3.0.1; R Development Core Team 2011). In Table S1 (Supplementary Data) we provide information on the plot-specific CWMs as well as on plot age and species richness.

Tests for shifts in CWM trait values along the successional gradient

According to the original design of BEF-China, the 27 CSPs were established along two crossed gradients, species diversity and stand age (see Bruelheide *et al.* 2011). To assess whether shifts in CWM trait values may be related to changes in species diversity in addition to successional age, we conducted regression analyses including plot species richness in addition to plot age. Species richness ranged from 25 to 69 species/plot and was independent of plot age. To assess the general patterns of trait shifts along secondary succession we ran three alternative types of linear models. These models were used to determine whether shifts in CWM for each single trait were best reflected by i) linear, ii) logarithmic or iii) quadratic patterns along the successional gradient. In each model, the respective trait was used as response, whereas the successional stand age as well as species richness were used as predictor variables. The most informative model was selected according to the AIC criterion.

Relating CWM shifts to decomposition along the successional gradient

To test for correlations between the changes in CWM traits and litter decomposition rates along the successional gradient we ran multiple regression analysis. According to hypothesis 3 we only included continuous traits from the four functional regimes presented above. Thus, the variables 'evergreen' and 'pinnate' were excluded from the analysis. We used principal components analyses (PCA) to assess multidimensional trait interrelationships prior to multiple regression analyses.

Model selection of the most informative model was based on stepwise backwards selection applying the stepAIC routine available in the MASS-package (Venables and Ripley, 2002) for R. Potential model inconsistencies due to multicollinearity were assessed using variance inflation analysis available in the R package faraway (Faraway, 2011). To assess the relative strength of the functional traits affecting community litter decomposability we used standardized trait values (mean=0, sd=1) in multiple regression analyses.

4.3.3. Results

Shifts in CWM traits and litter decomposition along the successional gradient

In Table 4-6 we present the pattern of changes as well as the direction of CWM shifts determined along secondary succession. According to the AIC criterion, species richness was not included as an informative predictor affecting shifts in any final trait model along secondary succession. All functional traits changed according to logarithmic patterns (Figure 4-6) whereas community k-rates followed a quadratic pattern with ongoing secondary succession.

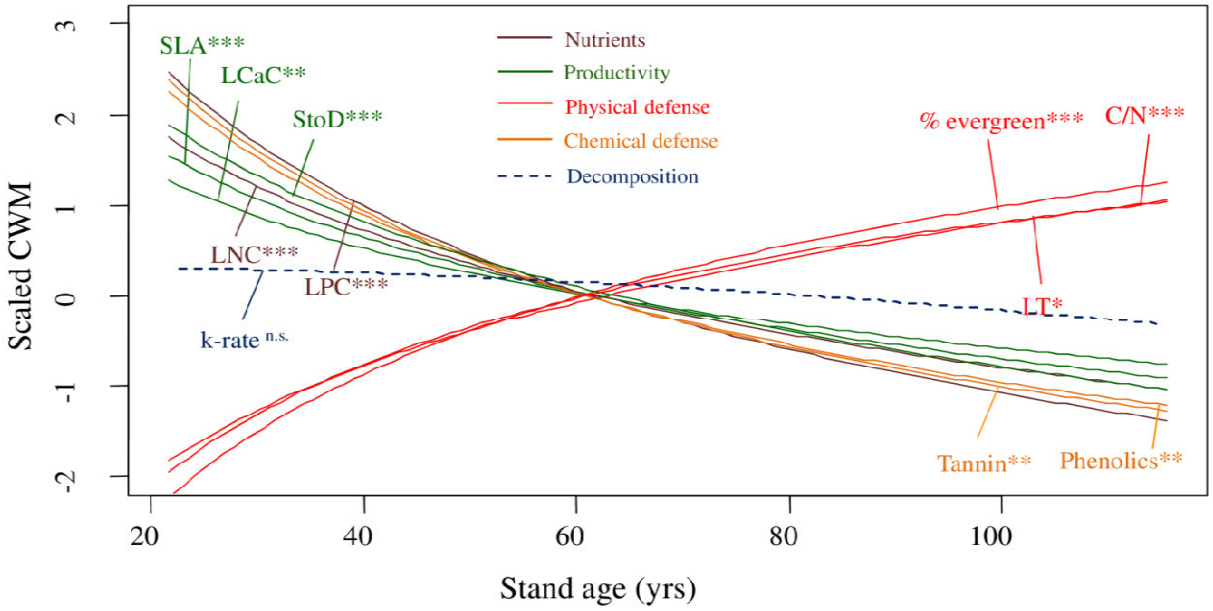

Figure 4-6: Shifts of standardized community leaf functional traits and decomposition rates along secondary succession. Only significant trait changes are displayed. Colors indicate functional regime affiliation (see Table 4-5). *: p< 0.05; **: p<0.01; ***: p< 0.001; n.s.: not significant.

In general, traits related to high community productivity and to nutritional quality decreased along the successional gradient, whereas in the trait group related to physical resistance, C/N and LT as well as the proportion of evergreen species increased. Surprisingly we detected a decrease of total phenolics and tannin concentrations with increasing stand age. The changes in litter carbon and sulfur concentrations, LDMC and the percentage of pinnate individuals with ongoing succession were non-significant. Moreover, community litter decomposition rates did not change significantly along the investigated successional gradient.

Table 4-6: Shifts in community abundance-weighted aggregated mean traits (CWM) along the successional gradient. The favoured model was selected according to the AIC criterion;. plot species richness was not included in any final model.

Trait	Favre model	Stand age	Direction
SLA	logarithmic	***	↓
LCaC	logarithmic	**	↓
StoD	logarithmic	***	↓
LNC	logarithmic	***	↓
LPC	logarithmic	***	↓
LSC	logarithmic	n.s.	↓
Phenolics	logarithmic	**	↓
Tannin	logarithmic	**	↓
C/N	logarithmic	***	↑
LCC	logarithmic	n.s.	↑
LDMC	logarithmic	n.s.	↑
LT	logarithmic	*	↑
Evergreen	logarithmic	***	↑
Pinnate	logarithmic	n.s.	↓
k-rate	quadratic	n.s.	↓

***: p< 0.001; **: p< 0.01 *: p< 0.05; n.s.: not significant

Relationships between CWM traits and decomposition rates along succession

Trait interrelationships

In Figure 4-7 we present the results from the principal components analysis including all 13 leaf functional traits used in multiple regression analysis. In sum, the first two axes represented approx. 70% of the over-all variance in the multivariate dataset. Along the first axis, communities were arranged from those indicating high productivity with leafs of high nutritional content and chemical defense on the left-hand side and those with leaves of high carbon content and leaf toughness on the right-hand side. The traits included in the productivity and nutritional value regime (with the exception of LSC) as well as in the chemical defense regime show a high degree of collinearity. The second axis was predominantly related to differences in community LDMC, which was the most uncorrelated traits to the main trait gradient.

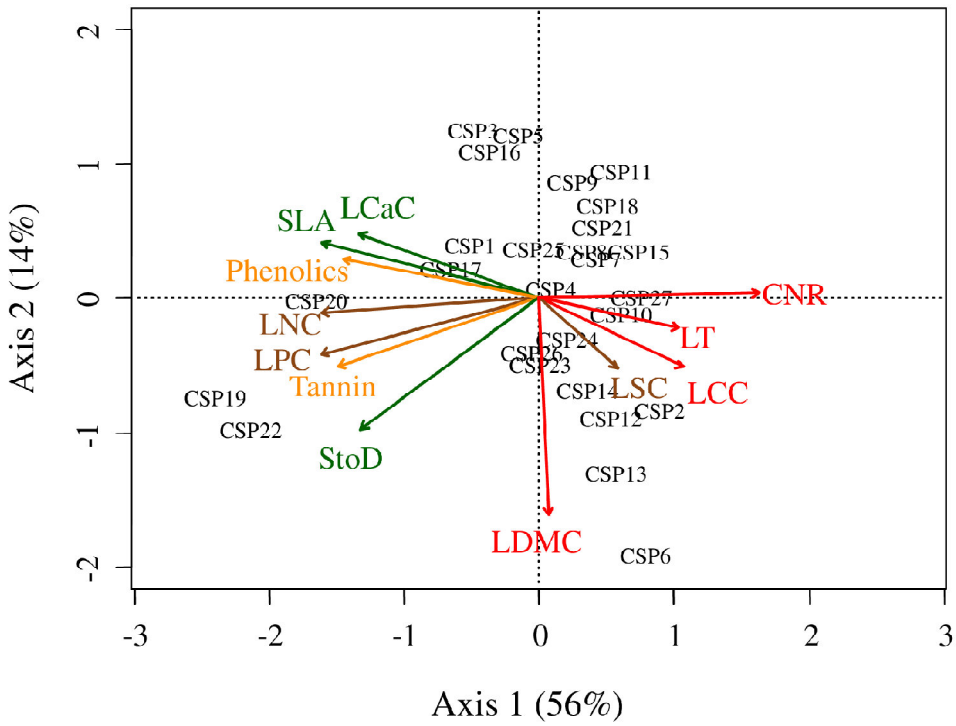

Figure 4-7: Principal components of functional traits included in the study. Colors indicate traits corresponding to the functional regimes indicated in Figure 4-6. Numbers in parentheses indicate the proportion of variance accounted for by the respective axis.

Multiple regression analysis

The most informative multiple regression model included the predictor variables SLA, LCC, LCaC, LPC, LSC, total phenolics and tannin concentrations as well as LDMC and leaf toughness. However, variance inflation factor analysis indicated multicollinearity with the highest variance inflations in tannin and leaf phosphorous concentrations (see also Figure 4-7). Tannin concentrations were strongly correlated to phenolic concentrations ($r=0.72$, $p< 0.001$) and LPC was strongly correlated to LCaC ($r=0.65$; $p< 0.001$). We thus used linear models to residualize tannin vs. total phenolics concentrations and LPC vs. LCaC, respectively. Repeated multiple regression analyses based on the residualized values of Tannin and LPC resulted in a more informative and accurate model (lower AIC values) than when computed from the original values ($R^2=0.35$, $p<0.05$ and $R^2=0.21$, $p=0.12$, respectively). As indicated by the regression coefficients, LPC_{resid} had the strongest positive, whereas $Tannin_{resid}$ had the most negative effect on community litter decomposability (see Table 4-7). LCC had a strong positive effect, whereas LDMC and leaf toughness decreased litter decomposition. LSC was also included in the final model although this variable affected litter decomposition rates with marginal significance. However, removal of LSC decreased R^2 and increased overall p values in the reduced model. Moreover, variance inflation factor

analysis did not indicate severe multicollinearity between the remaining predictors (data not shown).

Table 4-7: Leaf functional traits that affect community leaf decomposition rates. The final model according to AIC is presented. Regression coefficients were calculated from standardized trait values. Thus, the absolute value of the coefficients shows the importance of this predictor on leaf decomposition. Removal of LSC increased AIC values, indicating less appropriate models. Note: LPC_{resid} and $Tannin_{resid}$ depict the residuals from linear regression analyses against LCaC and total phenolics concentrations, respectively.

Trait	Coefficient	Significance
LPC_{resid}	0.97	*
LCC	0.81	**
LSC	0.34	'
$Tannin_{resid}$	-0.75	*
LDMC	-0.62	*
LT	-0.61	**

4.3.4. Discussion

In the present investigation we detected shifts in CWM trait values along a gradient of secondary succession. None of these shifts were related to differences in plot species richness. Traits belonging to the functional regimes of productivity, nutrient quality and chemical defense regimes decreased along succession. In contrast, C/N and LDMC, belonging to the physical defense regime, increased with ongoing succession, whereas community litter decomposition rates did not change significantly. Multiple regression analysis revealed causal relationship between shifts in CWM traits and litter decomposition rates. These results are in full accordance with findings of Kröber *et al.* (2012) who investigated the importance of abiotic vs. biotic factors in trait shifts along the secondary successional gradient of the BEF-China experiment. In addition to the investigation of Kröber *et al.* (2012), in the present study we analyzed changes in total phenolics and tannin concentrations as well as leaf toughness and litter decomposition rates. We found that LT increased in parallel to C/N with increasing stand age. Other traits related to investments into structural compounds (LCC and LDMC) did not change along the successional gradient. Surprisingly, CWM traits related to chemical defense such as total phenolics and tannin contents, decreased with ongoing succession. Moreover, as indicated by the increase in the proportion of evergreen leaf habits, species in late successional communities tended to have more persistent leaves. In young communities, highly productive species with leaves of high nutritional value were more dominant; in older

communities, species exhibited lower productivity and lower nutritional quality. We thus conclude that there is a change in the resource use strategy from high nutrient acquisition to nutrient retention, and thus, can confirm our first hypothesis. In contrast, our second hypothesis, predicting a decrease in community litter decomposition rates could not be confirmed. In contrast to our results, Garnier *et al.* (2004) detected a decrease in litter decomposition along a Mediterranean old-field succession. However, in accordance with Garnier *et al.* (2004) we present evidence that the inference of species functional traits to community based aggregation of traits can be successfully used to predict effects of trait shifts on ecosystem processes such as litter decomposition. Using multiple regression analysis we demonstrated that along the successional gradient encountered in the GNNR, litter decomposition rates were positively related to traits of community leaf nutritional quality such as LPC and negatively to leaf physical resistance and chemical defense traits (i.e. leaf toughness and leaf tannin concentrations). Surprisingly, in contrast to our expectations, LCC had a strong positive effect on litter decomposability. In contrast to these findings, Perez-Harguindeguy *et al.* (2000) reported a negative effect of litter carbon content on litter decomposability. In addition to leaf carbon, the authors also reported a negative effect of C/N and leaf toughness on litter decomposition rates for a wide variety of species. However, in contrast to the present study, Perez-Harguindeguy *et al.* (2000) used separate linear models to relate each single trait to litter decomposability. In multiple regression, LCC positively affected litter decomposition whereas LT and LDMC slowed down this process. We initially assigned both former traits to the group of leaf traits related to physical defense. Although the collinear arrangement of LT and LCC in the principal components analysis indicated that LT was affected by the same leaf structural compounds as LCC, both traits had opposing effects on leaf litter decomposition. As variance inflation factor analysis of the most informative multiple regression model did not indicate detrimental influences of multicollinearity between LT and LCC, the positive effect of LCC might rather be related to more labile carbon-fraction than to structural carbon compounds. Such labile compounds might be readily used by the decomposer community, thus resulting in higher leaf litter decomposition rates.

Wardle *et al.* (2003) also used multiple regression analysis to relate functional traits to decomposition rates. The authors used a wide variety of leaf functional traits, including tissue nitrogen content, lignin, cellulose, fibre and leaf polyphenol content in their analyses and reported a positive effect of leaf nitrogen concentrations on litter decomposition. Moreover, tissue tanning capacity was reported to be positively related to decomposition rates. The authors do not discuss the positive influence of tannins although this finding is interesting as

it contradicts other decomposition studies (e.g. Salamanca *et al.* 1998; Hoorens *et al.* 2003; Hättenschwiler *et al.* 2005). Among many other studies, Perez-Harguindeguy *et al.* (2000) highlighted the negative effect of tannins and total phenolics content on decomposition when investigating decomposability of 52 Argentinean and 15 species from England. Moreover, Coq *et al.* (2010) analyzed a wide variety of different condensed tannins and provided evidence, that these have an important influence on litter decomposition and nutrient dynamics. This is in line with our findings that tannins decrease rates of litter decomposition. Hättenschwiler *et al.* (2005) provide information on the mode of action of tannins on litter decomposition such as the inhibition of nitrification or on the complexation of nutrients, rendering these unavailable for decomposers (see also Kraus *et al.* 2003a). We therefore conclude that decomposition dynamics along the successional gradient under study can be explained by CWMs of leaf functional traits (our hypothesis 3). However, in contrast to our expectations, differences in community productivity (as indicated by the traits related to productivity) did not affect community decomposition rates. Nevertheless, this might be due to strong multicollinearities among the traits related to productivity and those related to the nutritional value, as depicted in the principle components analysis.

We are not aware of any other study investigating shifts in leaf functional traits in subtropical forest communities along secondary succession, directly linking these shifts to community litter decomposition rates. In summary, we conclude that along secondary succession in the species-rich subtropical forest ecosystem under study, leaf traits that promote litter decomposition decreased in importance, whereas traits that reduce the community k-rates did not consistently increase in importance along the succession. While leaf physical resistance increased, chemical defense traits decreased in older forest communities. In consequence, litter decomposability remained stable along secondary succession. The adverse effects of polyphenolic substances on litter decomposition are most prominent in young successional stages, whereas in the late successional stages, structural components dominated decomposition. We suggest that future studies that aim at relating shifts in leaf functional traits to ecosystem processes such as litter decomposition along secondary succession should use multiple regression analyses rather than separate linear models to analyze causal relationships.

4.3.5. Supplementary Data

Table 4-S1: Site-specific information on each of the 27 CSPs. This table includes CMW values for all investigated traits as well as the community-specific decomposition rates. CWM traits were computed according to equation 1 whereas community k-rates were calculated following equation 2. In addition we present important information on plot age and species richness.

CSP	SLA	CNR	LNC	LCC	LCaC	LPC	LSC	Phenolics	Tannin	% evergreen	% pinnate	StoD	LT	LDMC	k-rate	Age	Species richness
1	12.79	38.24	1.30	46.32	7187.73	506.05	1354.72	80.58	51.66	0.82	0.04	32.48	0.65	402.87	0.44	71.71	44
2	11.28	40.13	1.25	46.76	4581.30	472.65	1432.42	57.48	36.21	0.87	0.04	32.38	1.05	412.29	0.39	73.60	69
3	13.84	36.11	1.43	46.40	7086.54	486.21	1385.23	72.74	51.69	0.58	0.08	26.90	0.60	389.01	0.49	54.24	49
4	11.93	39.11	1.28	46.25	4527.87	488.75	1473.90	64.00	51.99	0.97	0.02	30.53	0.65	405.80	0.56	87.57	44
5	13.03	36.81	1.33	46.34	7471.25	459.28	1504.22	73.23	41.27	0.78	0.03	29.01	0.76	388.53	0.55	85.20	25
6	10.24	40.05	1.24	47.10	3895.31	447.78	1848.82	47.60	38.08	0.90	0.01	35.65	0.86	434.63	0.33	55.78	39
7	11.07	40.29	1.24	46.32	4410.96	432.02	1491.22	70.69	39.31	0.83	0.02	28.73	0.67	403.28	0.46	77.99	46
8	11.85	38.33	1.29	46.31	5294.61	477.39	1700.08	61.89	36.86	0.90	0.05	30.48	1.06	389.76	0.47	73.44	53
9	12.48	38.18	1.30	46.41	5439.61	465.51	1489.24	55.50	35.61	0.92	0.02	32.38	0.85	381.27	0.53	83.56	55
10	10.73	42.46	1.16	46.31	5436.06	476.50	1688.99	59.63	41.72	0.88	0.01	29.99	0.79	400.16	0.40	85.96	41
11	10.35	41.64	1.16	44.92	3117.12	441.62	1487.39	65.67	32.84	0.90	0.02	25.77	0.67	392.27	0.38	61.86	35
12	9.97	43.69	1.14	46.92	4835.46	471.50	1361.02	72.73	56.44	0.94	0.00	31.15	0.79	421.95	0.49	106.20	29
13	10.38	41.60	1.19	46.85	4823.14	461.11	1792.63	66.47	52.93	0.96	0.00	32.22	0.79	421.53	0.43	58.68	32
14	10.73	40.40	1.22	46.57	4012.81	507.49	1453.31	63.44	52.26	0.92	0.02	29.24	0.76	414.32	0.34	115.54	38
15	10.20	43.33	1.15	46.74	5523.86	451.72	1274.24	72.78	52.65	0.95	0.02	27.59	0.72	404.58	0.41	81.76	39
16	13.31	38.01	1.25	45.80	8060.86	494.92	1721.84	74.76	47.75	0.82	0.06	30.85	0.64	387.52	0.56	51.58	37
17	12.75	38.31	1.26	46.21	8089.31	554.17	1414.39	80.67	50.13	0.88	0.01	34.67	0.60	409.39	0.62	44.98	39
18	10.96	40.92	1.21	46.22	5148.15	435.68	1368.82	63.83	39.90	0.87	0.01	26.38	0.73	399.13	0.45	101.30	52
19	15.29	28.20	1.68	45.24	7513.57	630.63	1362.81	81.59	106.90	0.08	0.08	41.33	0.44	410.47	0.44	25.00	30
20	14.71	33.06	1.48	45.26	8743.65	608.05	1318.71	89.79	78.93	0.48	0.07	36.98	0.85	400.82	0.31	36.31	38
21	11.19	41.67	1.22	46.81	6245.96	466.39	1412.47	61.68	41.66	0.87	0.03	27.98	0.77	393.64	0.40	105.77	56

22	14.41	31.48	1.54	45.41	8550.49	633.39	1409.25	90.56	92.58	0.42	0.02	43.37	0.52	413.07	0.36	21.72	33
23	11.58	37.78	1.32	47.00	4813.04	506.01	1525.12	71.11	48.75	0.91	0.06	34.02	0.78	409.57	0.59	34.00	55
24	11.69	40.87	1.22	47.22	6721.95	505.25	1274.38	62.18	31.99	0.93	0.01	33.29	0.69	414.17	0.48	48.40	34
25	12.34	37.87	1.28	47.00	8123.41	496.98	1316.63	68.10	31.38	0.89	0.05	32.65	0.86	397.29	0.39	45.49	27
26	11.32	37.61	1.33	46.83	5061.60	531.40	1446.88	67.19	45.36	0.91	0.04	32.91	0.69	416.96	0.71	38.98	44
27	9.91	43.16	1.15	46.41	4442.21	469.17	1414.75	61.81	50.31	0.92	0.02	27.22	0.89	401.55	0.38	93.28	46

4.3.6. Acknowledgements

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4.4.Trade-offs between physical and chemical carbon-based leaf defense: disentangling the relative importance of intraspecific variation and trait evolution

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Summary

1. Current research and Rationale: Despite recent advances in studies of trade-offs between plant defense traits, little is known to which extent trade-offs are due to evolutionary or allocation constraints at the individual level. Here, we quantified the levels of covariation between physical and chemical carbon-based leaf defense traits across different taxonomic levels and across the phylogeny. In addition to using species trait means, we analyzed the importance of intraspecific trait variability (ITV) in trade-off analyses.

2. Methods: We assessed **l**eaf toughness, leaf total phenolics and tannin concentrations for 51 subtropical tree species. ITV was quantified by variance components analysis based on taxonomic information. Species trait means, sample-specific values and phylogenetic independent contrasts were used in regression analyses. Phylogenetic signals and trait evolution were assessed across the entire phylogenetic tree and along single nodes.

3. Main results: While ITV was negligible, significant trade-offs were encountered in cross-species analyses, where however, ITV significantly decreased the strength of the covariations. The defense aspects showed strong phylogenetic signals, but differed in degree of divergence and conservatism along the phylogeny. Strong trade-offs between physical and chemical defense were detected when phylogenetic non-independence was accounted for.

4. Conclusion: Our analyses indicate that the trade-offs between differential defense mechanisms were due to evolutionary and not resource allocation constraints.

Keywords: leaf toughness, plant polyphenols, plant defense, tannins, trade-off, trait evolution

4.4.1. Introduction:

Plants have evolved a multitude of defense mechanisms in order to protect their leaves from herbivory or pathogen attack. These defenses are either based on physical structures such as trichomes or sclerophylly, or on chemical composition, such as toxic or deterrent compounds (Koricheva *et al.* 2004; Moles *et al.* 2013). During the last decades, a range of different theories on plant defenses has been proposed (reviewed by Stamp 2003). Generally, the basic assumption underlying all these theories is that defense is costly and will only be cost-effective if investments into defense pay off damage costs (e.g. caused by herbivores). In this sense, the optimal defense theory by Rhoades and Cates (1976) predicts that those organs most valuable to plants will be defended best while less resources will be invested to defend other organs. Plant leaves rank high in importance as they assure carbon supply by photosynthesis, and thus, in most species can be expected to be well defended. Based on the assumption of allocation constraints, several studies have addressed the idea of trade-offs in defense traits by investigating trait interrelationships between different aspects of leaf defenses. These trade-offs comprised polyphenolic substances vs. leaf toughness (Cornelissen *et al.* 2009; Read *et al.* 2009) or analyses within single categories of defense such as between different chemical compounds (e.g. Agrawal *et al.* 2009; Koricheva *et al.* 2004). However, results from these analyses are inconclusive. For example, in studying 34 subarctic species Cornelissen *et al.* (2009) encountered either species showing high phenolic concentrations in combination with low fibre (a proxy for leaf toughness), species with high fibre and low phenolics concentration, or species exhibiting low quantities of both aspects. In contrast, Read *et al.* (2009) detected either none or positive correlations between leaf toughness and leaf polyphenols. This indicates that these trade-offs may not be a universal feature.

Overcoming the idea of plain bivariate relationships, trade-offs are increasingly being investigated from a multivariate perspective in recent literature (e.g. Koricheva *et al.* 2004; Cornelissen *et al.* 2009; Moles *et al.* 2013. Results from such studies led scientists to formulate the concept of defense syndromes (Kursar and Coley 2003; Agrawal and Fishbein (2006) where suites of covarying defense traits may result in distinct defense strategies. As in bivariate trade-offs, evidence for defense syndromes also is equivocal. While some studies detected discrete defense syndromes, e.g. in 24 species of *Asclepias (*Agrawal & Fishbein, 2006), others like Kursar and Coley (2003) and Moles *et al.* (2013) suggested that different trait combinations might vary continuously.

Most of these analyses, however, were conducted using species mean trait values, thus neglecting the potential importance of intraspecific trait variability (ITV), which may be non-negligible in species' trait variability (Albert *et al.* 2010; Hulshof and Swenson 2010). If intraspecific variability is high, species mean values may be less informative, as they may only poorly reflect a plant's true *in-situ* trait value (Kattge *et al.* 2011).

In general, ITV may be assigned to two major components (e.g. Albert *et al.* 2011): adaptation (i.e. the variation among individual genotypes of a given species) and acclimation (i.e. phenotypic variation of the same genotype of a given species evoked by *in-situ* habitat conditions), the latter point potentially being of special importance in trade-off analyses. For example, He *et al.* (2009) could show that differences in habitat conditions significantly altered the intensity of trade-offs in leaf productivity vs. persistence. Thus, for studies investigating trade-offs, quantification of ITV may be of importance e.g. to assess the representativeness of species mean values. In addition, ITV may gain significance when analyses are conducted along short gradients (Auger and Shipley 2013), whereas ITV may be the less important, the larger the scale, e.g. when studying relationships on a global scale (Swenson and Enquist 2007; Wright *et al.* 2004). However, cross-species studies mostly ignore ITV and focus on among-species variation with different lineages potentially having evolved different defense strategies at different times (e.g. Baldwin and Schultz 1988). From an evolutionary perspective, closely related species may not be taken as independent replicates, thus requiring the use of phylogenetic corrections (Felsenstein 1985; Garland *et al.* 1992. Ignoring similarities between species brought about by evolutionary constraints may lead to situations where a) trait relationships (e.g. trade-offs) are spurious, i.e. based on relatively few independent evolutionary events or b) strong trait correlations within groups of closely related species may be masked if there are uncorrelated shifts in trait mean early in the phylogeny (see Ackerly 1999; Orme *et al.* 2013). Phylogenetically independent contrasts (PICs) allow the detection of trait relationships unbiased by species' phylogenetic non-independence, i.e. the tendency for traits of closely related species to resemble each other (Blomberg *et al.* 2003). However, as stated by Agrawal (2007), blindly correcting for phylogeny may not be appropriate in every case and it is important to evaluate the significance of the phylogenetic signal including information on intraspecific trait variation (Kembel *et al.* 2010).

Although statistics to detect phylogenetic signals offer a convenient tool to detect the level of phylogenetic trait-conservatism in cross-species studies, these tests usually integrate over the

complete phylogeny and thus do not allow for the possibility that phylogenetic signal may change throughout evolutionary history (see Moles *et al.* 2005, Webb *et al.* 2008, Revell 2013b).

In the present study we analyzed trade-offs in carbon-based chemical and physical leaf defense along secondary forest succession in a cross-species study at different hierarchical levels of taxonomy: a) within species, b) across species with and without incorporation of ITV and c) across species including phylogenetic information. An ubiquitous aspect of carbon-based chemical defenses are polyphenolic substances such as tannins and non-tannin phenolics (Harborne 1980). These metabolites are considered to partake in plant herbivore defense, amelioration of light stress and pathogen resistance (Hättenschwiler and Vitousek 2000; Ingersoll *et al.* 2010). In contrast, leaf toughness (LT) as a key physical leaf trait is also considered an effective defense trait against herbivore damage (e.g. Kursar and Coley 2003). LT is mainly attributed to leaf morphology and anatomy such as sclerophylly, tissue density and density of leaf venation (e.g. Westbrook *et al.* 2011). As for polyphenols, most of these aspects are based on carbon-allocation to structural compounds (Kitajima *et al.* 2012). Thus, as both facets of leaf defense, polyphenolic compounds as well as LT, rely on carbon, these may be considered to trade-off one another. To investigate trade-offs between these chemical and physical defense traits, we assessed LT from 1205 single leaves from 51 woody species out of 24 families. First, we investigated trait relationships between leaf polyphenol (total phenolics and tannin) concentrations and LT. Analyses of trait variability on the level of individuals within species allowed us to assess the proportion of ITV in comparison to interspecific trait variation by conducting variance components analyses. Second, we used linear models based on the species specific mean trait values, and linear and mixed effects models (considering taxonomic as well as habitat information). Third, we assessed trait evolution, i.e. the phylogenetic signal, both at the level of the whole phylogeny as well as for single nodes. Where necessary, we corrected for phylogenetic non-independence which potentially could obscure trade-offs between traits. We hypothesized that, i) intraspecific trade-offs between polyphenolic substances and leaf toughness are consistent in all investigated species. We further hypothesized that ii) across all species trade-offs will be encountered when calculated on species trait means and iii) intraspecific trade-offs based on trait values including ITV are matched by interspecific trade-offs based on species trait means. In other words, incorporation of ITV will not significantly alter the results in trade-off analyses in comparison to the results based on species mean trait values. We further analyzed the role of evolutionary constraints in the interrelationships between the two aspects of

defense. We hypothesized that iv) across all species, the investigated traits show a strong phylogenetic signal and therefore cannot be considered independent when subjecting them to cross-species analyses. Moreover, we hypothesized that v) chemical and physical defense traits show similar phylogenetic signals, indicating that the two traits have experienced similar selective pressure along evolution. A consequence of this hypothesis would be that correcting for phylogenetic non-independence does not affect the strength of the trade-offs detected in the cross-species analysis. Our hypotheses iii) and v) account for the possibility that both, inconsistent ITV in trade-offs based on resource allocation and different patterns of trait evolution can obscure potential trade-offs expected at the level of species mean trait values. To our knowledge, our study is the first to address the two sources of variation independently from each other and the first to study the phylogeny of physical and chemical defense traits along a forest succession series.

4.4.2. Methods:

Site description:

Leaves were sampled across the BEF-China project, comprising 27 plots (the so-called comparative study plots, CSPs) in a subtropical evergreen mixed forest undergoing secondary succession and two experimental sites (Bruelheide *et al.* 2011, 2014). The successional gradient covered by the CSPs spans from approx. 20 up to more than 120 years. Within the CSPs all individual trees above 1m stem height were recorded during 2008 and 2009 and identified to the species level. A total of 148 woody species were recorded in the CSPs of BEF-China. In the experimental sites, 62 different tree and shrub species from the regional species pool were planted on two sites (Site A and Site B) approx. 30 km west of the CSPs (for detailed information see Yang *et al.* 2013 and Bruelheide *et al.* 2014).

Trait assessment

Leaf toughness

Leaf toughness (LT) was assessed for 1725 leaves from 91 species collected in the GNNR as well as on the experimental sites of the BEF-China experiment. Each species was sampled with one to seven individuals, with each individual being sampled with five to ten single leaves. In the case of pinnate leaves, leaf toughness was measured on one single leaflet each from seven to ten different leaves. Leaf toughness was recorded as leaf tensile strength, following the approach of Hendry and Grime (1993). Small leaf fragments of 1-5mm width

(avoiding the midrib as well as major leaf veins) were cut along the longitudinal axis from every single leaf and fixed between two clamps of a tearing apparatus. The force needed to rupture the leaf (in N mm^{-1}) was recorded with a spring balance (see Cornelissen *et al.* 2003). Samples were processed within one day after collection. Subsequent to fragment excision, the remaining leaves were stored in a freezer at -20°C until lyophilization for 24h at ambient temperature and 1Pa pressure for further analysis in the lab. A complete list of the investigated species is presented in Table 4-S2 (supporting information).

Collection of Near-infrared (NIR) Spectra

We used NIR Spectrometry (MPA, Bruker Optics, Germany) to determine the concentrations of total phenolics and tannins, respectively. NIR-spectra were collected from all 1725 leaves used in the LT assay. The spectra were recorded from powdered leaf material, stored in glass vials (ø7mm). Spectral information was compiled using OPUS (V. 7.2, Bruker Germany) for every sample in three replicates. Powdered samples were packed in equal densities by gently tapping the vials on a table in order to minimize light scattering due to inhomogeneous packing. Between each replicate measurement the powder was thoroughly shaken in order to minimize spectral measurement error. Spectral reflectance was collected over the maximal screening interval (9403 to 3880 wave numbers, 1 wave number per step).

Calibration sets for NIR spectrometry and reference data collection

We aimed at determining total phenolics and tannin concentrations (hereafter phenolics and tannins) for all 1725 leaves with known LT. In order to obtain reliable trait information for every single leaf, NIR spectrometry needs careful calibration with a sufficiently large set of reference samples with known trait values. The reference data was compiled from previous investigations within the BEF-China experiment (see Bruelheide *et al.* 2011; Kröber *et al.* 2012; Eichenberg *et al.* 2014a,b). All reference samples were collected between 2008 and 2012. Most samples were from lyophilized powdered leaf material (approx. 70% of all samples) whereas the rest was dried at 80°C in a drying oven (c.f. Cornelissen *et al.* 2003). We used this heterogeneous sample set to cover the entire range of spectral variation typically found in large sample-sets.

For calibration of NIRS against the secondary metabolites, we recorded 543 spectra from samples with known phenolics and tannin concentrations, different from those used in the LT assay. Within the reference samples we covered a wide range of taxonomy (126 species from 45 families). These samples were analyzed for phenolics concentrations by the Prussian blue

method Price and Butler 1977 as modified by Graham (1992), using 50 mg of dried plant powder extracted in 50% aqueous acetone as described in Torti *et al.* (1995). Tannin concentrations were quantified using the radial diffusion method for increased sensitivity Hagerman (2002). Concentrations of the secondary metabolites were standardized against tannic acid (Roth, Germany, Charge Nr. 250153788) and expressed as mg g_{dw}^{-1} tannic acid equivalents (TAE). For further details see Eichenberg *et al.* (2014a).

NIRS model development

We used the Quant 2 software (OPUS V 7.2, Bruker, Germany) in the optimization procedure. Spectral pretreatment was set to the first derivation of the complete spectrum and standard vector normalization. The optimization procedure was applied separately to the different traits to determine the ideal spectral intervals for the calibration for each trait separately. Partial least squares regression (PLS) was used to relate the concentrations to NIR spectra. Detecting the local minimum of the error of validation, the maximum number of factors to be included in the PLS model was determined. Subsequent removal of spectral outliers and model validation was conducted until no further improvement could be achieved. Regression coefficients (R^2), root mean square errors of cross validation (RMSECV) as well as the residual prediction deviation (RDP, i.e. the ratio of SD of the calibration set and the RMSECV) were considered to evaluate the quality of calibrations. RPD values higher than 2.5 indicate a sufficient validity of calibration; RPD values between 1.5 and 2 allow to qualitatively distinguish between high and low values; RPD values below 1.5 are uninformative and must not be used for calibration purposes (Hildrum 1992; Ozaki *et al.* 2006; Parsons *et al.* 2011).

Trait prediction for samples with unknown trait values

We used all 1725 spectra of leaves with known LT to predict phenolics and tannin concentrations. As reported by Eichenberg *et al.* (2014a), up to 85% of the variability in phenolics and tannins were explained by the taxonomic levels 'family' and 'genus', whereas species affiliation only accounted for minor proportions of variability. Thus, from all 1725 samples with known leaf toughness, we only included spectra of samples belonging to families that also were present in the calibration set. This resulted in 1531 samples. After prediction of the trait values, we excluded all samples with predicted values outside the ranges of concentrations included in the calibration and then excluded species that were represented with less than three individuals in the remaining dataset. This resulted in 1205

single leaves with known values for leaf toughness, total phenolics concentration and total tannin concentration from 186 individuals from 51 species from 24 families.

Trade-off analyses

Assessment of intraspecific trait variability vs. interspecific trait variability

To assess the relative importance of ITV in comparison to the interspecific variability we computed variance components analysis based on linear mixed effects models for each of the three defense traits separately. The models were specified without fixed factors as predictors but included all taxonomic levels (family, genus, species, individual) as hierarchically nested factors in the random term. As indicated by Lambrecht and Dawson (2007), in-situ habitat differences may also influence intraspecific trait variability and thus, we added information on the habitat of the respective individual as a crossed random factor. The factor 'habitat' combined information on whether the sample was collected in the CSPs or in the experimental sites (Site A or B) and the respective plots. It gives unequivocal information on where the samples were collected. Although we did not specifically measure *in-situ* conditions (e.g. soil fertility, soil moisture, light regime) including the factor 'habitat' allowed for correcting for environmental differences between individual growth locations.

In summary, ITC comprised the trait variability within and among individuals as well as among habitats. This allows disentangling the structure of ITV in terms of contribution of the different random factors to intraspecific trait variability. Variance components were calculated as the percentage of estimated variances explained by each random factor in relation to the overall variance Crawley (2007).

Assessment of trade-offs on the species-level

To achieve an even spread in trait values, LT values were square-root transformed prior to analyses. To allow for a direct comparison between models corrected for taxonomy and habitat as well as models based on species mean trait values, all traits were scaled to mean= 0 and SD= 1 in regression analyses. Different functional traits may be differently affected by habitat-specific conditions, such as nutrient availability or light regime (e.g. Lambrecht and Dawson, 2007). Thus, we included the factor 'habitat' as a crossed factor in the linear mixed effects model. To test our first hypothesis (the intraspecific trade-offs in chemical vs. physical defense are consistent in all species), we conducted separate regression analyses for all 51

species. We used linear mixed effects models incorporating 'individual' nested within 'habitat' in the random structure.

Cross-species analyses

To assess whether trait covariations between the two aspects of carbon-based defense were evident across all species (our second hypothesis) we applied two different types of linear models. Firstly, we conducted regression analyses based on species mean trait values. Secondly, we specified linear mixed effects models using a random structure analogous to that used to determine ITV (i.e. individuals nested in species, species nested in genera and genera nested in family; in addition, habitat was used as a crossed factor). To test our third hypothesis (intraspecific and interspecific trade-offs match), we compared the slopes of the two different models. The slopes were considered to significantly differ from each other if estimates and standard errors for the slopes in the models did not overlap.

All linear mixed effects models were conducted using the routines available in the lmerTest package (Kuznetsova *et al.* 2013).

Phylogenetic analyses

To assess to which extent the investigated traits were phylogenetically conserved at the level of the whole phylogeny (i.e. trait conservatism, our hypothesis iv), we calculated Blomberg's K statistic (Blomberg *et al*., 2003) for the species-level mean value of each trait. Phylogenetic information was obtained from Michalski and Durka (2013). K-values less than one indicate a lower phylogenetic signal than expected from Brownian motion model of trait evolution (low levels of phylogenetic trait conservatism). K values close to, or higher than one indicate a strong phylogenetic signal. Significance of the phylogenetic signal was determined by shuffling species' trait values (999 times) across the tips of the phylogenetic tree and comparing the resulting K-values to those computed from the original trait data.

Because using species mean trait values can underestimate the phylogenetic signal if there is a high intraspecific trait variability (Ives *et al.* 2007), K-values were also calculated incorporating standard errors from averaging over single sample values, to obtain more accurate estimates of phylogenetic signal.

Trait interrelations analyses using phylogenetically independent contrasts

If significant phylogenetic autocorrelation was detected we calculated trait-specific phylogenetic independent contrasts (PICs; Felsenstein 1985; Harvey *et al.* 1995; Westoby *et al.* 1995) to account for phylogenetic non-independence in the relationships between defense traits. If negative associations between physical and chemical defense were detected and these relationships persisted (or even become more pronounced) when phylogenetic non-independence was accounted for, we interpreted this as a strong evolutionary based trade-off between chemical and physical leaf defense. To assess evolutionary shifts in trait value transitions between the different traits (hypothesis v), we calculated the mean trait values and the trait variance for each single node. We visualized the phylogenetic signal (i.e. the divergence size) at different depths in the phylogenetic tree according to the approach proposed by Moles *et al.* (2005) (see also Purschke *et al.* 2013): (1) trait values were reshuffled across the tips of the phylogenetic tree; (2) the standard deviation (divergence size) of the trait values across the descendent terminal taxa was calculated for each node; (3) divergence size values were recalculated after permuting (999 times) trait values across the tips of the phylogenetic tree to generate a random distribution of divergence size values; (4) for each trait, the rank of the observed divergence size within the null distribution was plotted against node age to assess whether phylogenetic signal was higher (low ranks) or lower (high ranks) than expected by chance at different depths in the phylogeny. To aid interpretation of the results from the phylogenetically corrected regression analyses, we visualized the evolution of each trait along the phylogeny by mapping ancestral trait estimates on the phylogenetic tree using Method 2 in Revell (2013b).

All phylogenetic analyses were carried out using the R packages 'caper' (V 0.5, Orme *et al.* 2013 and 'phytools' (Revell 2013a) as well as Phylocom (V 4.2, Webb *et al.* 2008).

4.4.3. Results

NIRS calibration and trait assessment

Table 4-8 lists the configurations and quality measures for the NIRS calibration against phenolics and tannins. The calibration for phenolics yielded an R^2 of approx. 0.87 and an RPD of approx. 2.8, whereas the calibration for tannins performed slightly less reliable ($R^2 \approx 0.72$, RPD$\approx$ 1.9).

Table 4-8: Quality measures in NIRS calibration models to determine secondary metabolites concentrations.

Trait	unit	Spectral region (wave-numbers)	n ranks	R^2	RMSECV[1] [%]	RPD[2]
Phenolics	mg/g_{dw}	6086.6 - 5361.5 ; 5037.5 - 4104.1	6	0.87	7.97	2.77
Tannin	mg/g_{dw}	8894.7 - 8424.1; 7513.8-5677.8; 4767.5 -4296.9	6	0.72	8.84	1.9

[1]: root mean square error of cross-validation; [2]: residual predictive derivation

Minimum phenolics concentrations were found in *Ilex elmerrilliana* with 4.9 mg g_{dw}^{-1}. A maximum of 194.5mg $g_{dw}^{-1}$was detected in *Alniphyllum fortunei*. *Vaccinium bracteatum* showed the lowest concentration of tannins (2.7 mg g_{dw}^{-1}), whereas in *Liquidambar formosana* we detected highest tannin concentrations (92.8 mg g_{dw}^{-1}). Minimum values of LT ranged from 0.05 N mm^{-1} in *Sapindus saponaria*, whereas the toughest leaves were found in *Castanopsis eyrei* with 1.59 N mm^{-1}. A complete list of species' ranges for the three traits is provided in Table 4-S2 (Supporting Information).

Variance components analysis

Variance components analysis revealed that the taxonomic levels of 'family' and 'genus' together accounted for approx. 75% of the total variation in phenolics as well as in tannins (Fig. 4-9). The 'among species' level accounted for approx. 10% and 13% for total phenolics and tannin, respectively. The variability in 'individuals among habitats' accounted for only minor proportions of the over-all variance for both secondary metabolites. Approximately equal amounts of variance in the concentration of secondary metabolites were explained by the 'individuals among habitats' level. Another total of approx. 3.5% of variation was explained by 'within-individual' variability for both secondary metabolites. In consequence, ITV accumulated to approx. 14% and 13% of the overall variability for total phenolics and tannin, respectively.

In the case of LT, 'family' and 'genus' affiliation together accounted for approx. 40% of total variance in the dataset. Further 40% of variance were explained by the 'among-species' level. The variation 'individuals among habitats' and 'among individuals within species' together explained about 14 %. The 'within-individuals' variability accounted for approx. 7 % of over-all variability in the data. Thus, ITV for leaf toughness accumulated to approx. 21% of over-all variance.

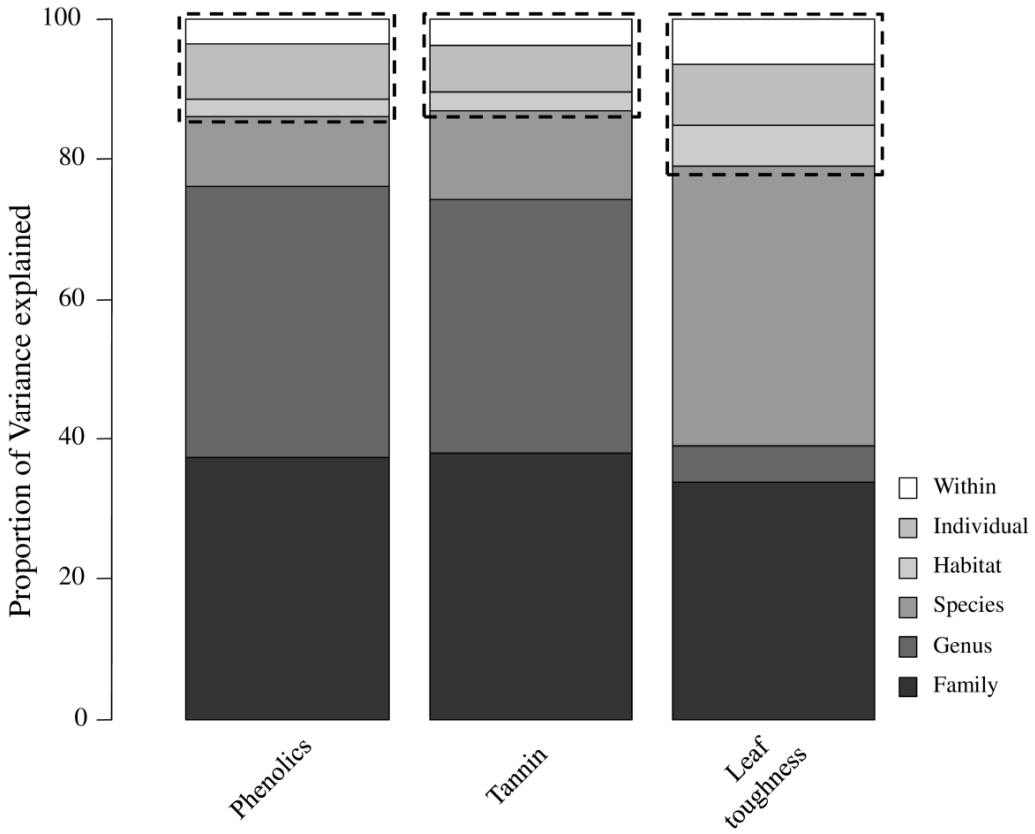

Figure 4-9: Variance components analysis of phenolics and tannin concentrations as well as leaf toughness. Variance components were computed based on the full sample set containing 1205 single leaves. Dashed boxes: variance components summed up to intraspecific variability (ITV). Within: variability 'within-individuals'; Individual: variability 'among individuals within species'; Habitat: variability 'among habitats', which was crossed with individuals, species, family and genus. Species: variability 'among species'; Genus: variability 'among genera'; Family: variability 'among families'.

Compared to the proportion of variability explained by differences among species (i.e. all variance components above 'among individuals within species'), the proportions of ITV were small. In the case of polyphenolics, interspecific variability accounted for more than six-fold higher proportions of variability than ITV. In leaf toughness, interspecific variability was four-fold higher, as compared to ITV.

Trade-off analyses

Intraspecific analyses

In Table 4-10 we present the results of the 51 species-specific linear mixed effects models of phenolics and tannins vs. LT, respectively. Of all 51 species, 13 showed a significant covariation between phenolics concentrations and LT; of these significant estimates, nine

were negative. Moreover, we detected significant covariation between tannins and LT in 11 species. Again, nine of the significant estimates were negative. Interestingly, in those genera in which covariation was detected, the direction was consistent in that genus (i.e. within one genus we never detected positive and negative covariation simultaneously). Similarly, in those cases where significant covariation in phenolics vs. LT coincided with significant covariation between tannins and LT, both were in the same direction. Although we detected a higher proportion of negative than positive estimates for the slopes between the traits related to chemical defense vs. physical defense, most species showed no significant correlation between these two different components of carbon-based defense.

Table 4-10: Species-specific regression analyses of total phonics and tannin concentrations v.s. leaf toughness, respectively. Regression analyses were carried on the basis of species-specific mixed-effects models; numbers indicate slopes.

Species	Family	total n leaves	LT vs.	
			total phenolics	tannin
Acer davidii	Aceraceae	21	0.383	0.043
Viburnum setigerum	Adoxaceae	21	-0.189	-0.658**
Choerospondias axillaris	Anacardiaceae	21	-0.216	-0.236
Rhus chinensis	Anacardiaceae	17	0.105	0.807
Rhododendron simsii	Apiaceae	20	-0.211	-0.192
Ilex elmerilliana	Aquifoliaceae	9	-0.205	-0.372
Daphniphyllum oldhamii	Daphniphyllaceae	31	-0.110	-0.179
Diospyros glaucifolia	Ebenaceae	21	-0.536**	-0.462*
Elaeocarpus chinensis	Elaeocarpaceae	38	-0.234	-0.170
Elaeocarpus glabripetalus	Elaeocarpaceae	35	0.118	0.096
Elaeocarpus japonicus	Elaeocarpaceae	21	0.112	0.185
Vaccinium bracteatum	Ericaceae	30	0.851***	0.452
Vaccinium mandarinorum	Ericaceae	28	-0.159	-0.126
Phyllanthus glaucus	Euphorbiaceae	14	-0.142	-0.140
Triadica cochichinensis	Euphorbiaceae	19	-0.042	-0.217
Triadica sebifera	Euphorbiaceae	15	-0.129	-0.196
Castanea henryi	Fagaceae	20	-0.289*	-0.521**
Castanopsis eyrei	Fagaceae	59	0.011	-0.071
Castanopsis fargesii	Fagaceae	28	-0.073	0.031
Castanopsis sclerophylla	Fagaceae	20	-0.553*	-0.581**
Cyclobalanopsis glauca	Fagaceae	32	-0.625***	-0.579**
Cyclobalanopsis myrsinifolia	Fagaceae	21	-0.367	-0.318

Table 4-10 (continued)

Lithocarpus glaber	Fagaceae	48	-0.203	-0.306*
Quercus acutissima	Fagaceae	21	-0.068	-0.081
Quercus fabri	Fagaceae	21	0.244	0.251
Quercus phillyreoides	Fagaceae	35	0.148	0.129
Quercus serrata	Fagaceae	18	-0.126	-0.137
Distylium buxifolium	Hamamelidaceae	21	0.205	0.206
Distylium myricoides	Hamamelidaceae	21	-0.221	0.047
Liquidambar formosana	Hamamelidaceae	18	-0.645***	-0.691**
Loropetalum chinense	Hamamelidaceae	16	0.804	0.222
Cinnamomum camphora	Lauraceae	27	0.086	0.080
Machilus leptophylla	Lauraceae	21	-0.226	0.130
Machilus thunbergii	Lauraceae	21	-0.035	-0.152
Neolitsea aurata	Lauraceae	23	-0.449*	-0.397*
Phoebe bournei	Lauraceae	21	0.037	0.026
Manglietia yuyuanensis	Magnoliaceae	35	-0.230	-0.043
Syzygium buxifolium	Myrtaceae	15	0.422*	0.470*
Nyssa sinensis	Nyssaceae	21	-0.171	-0.040
Photinia hirsuta	Rosaceae	21	-0.278	-0.360
Gardenia jasminoides	Rubiaceae	20	-0.061	-0.407
Meliosma flexuosa	Sabiaceae	21	-0.026	0.278
Koelreuteria bipinnata	Sapindaceae	21	-0.479**	-0.472**
Sapindus saponaria	Sapindaceae	15	-0.338*	0.098
Itea chinensis	Saxifragaceae	19	0.529	0.001
Alniphyllum fortunei	Styracaceae	20	-0.503*	-0.303
Adinandra millettii	Theaceae	20	0.488*	-0.002
Camellia chekiangoleaosa	Theaceae	20	-0.092	-0.043
Eurya muricata	Theaceae	23	-0.054	-0.068
Schima superba	Theaceae	42	0.252*	0.225*
Celtis biondii	Ulmaceae	19	0.219	0.111

*: $p<0.05$; **: $p< 0.01$; ***: $p< 0.001$

Interspecific analyses based on species means

We detected significant negative correlations in the regression analyses based on species mean trait values (slope= -0.349, SE= ±0.134, p< 0.05) as well as in the linear mixed effects model between phenolics and LT (slope= -0.064, SE= ±0.031, p< 0.05; see Fig. 4-10a and b, respectively). Similarly, LT was found to be negatively correlated with tannins in the linear model using species trait means (slope= -0.459, SE= ±0.127, p< 0.001) as well as in the linear mixed effects model (slope= -0.071, SE= ±0.029, p< 0.05; see Fig. 4-10d and e, respectively). The slopes in the models without considering ITV were significantly steeper than the slopes in the models including ITV for both, total phenolics vs. LT and tannin vs. LT (i.e. means ± SE did not overlap).

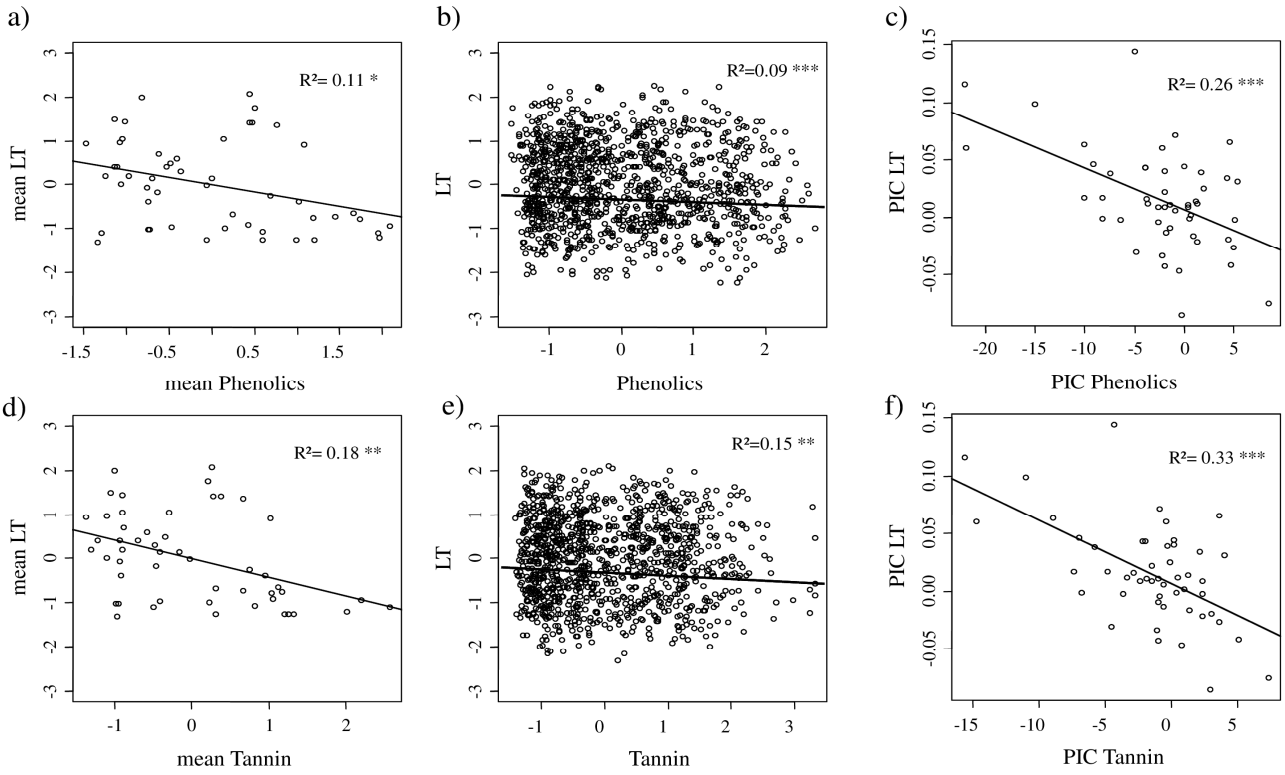

Figure 4-10: Regression analyses of leaf toughness against total phenolics concentrations (panels a-c) and tannin concentrations (panels d-f). Panels a and d: regression analyses based on mixed effects models, using family, genus, species, individual and habitat as random factors; Panels b and e: regression analyses based on linear models with species mean trait values; Panels c and f: regression analyses based on phylogenetically independent contrasts. Note: trait values were scaled to mean= 0 and SD= 1

Phylogenetic analysis and regression analyses based on PICs

Based on Blomberg's *K,* we detected significant phylogenetic signals in all three investigated traits (Table 4-11). Incorporation of the intraspecific measurement error substantially increased the phylogenetic signal in all traits and had the highest effect on LT, whereas it was less pronounced for phenolics and tannins. To account for the high levels of phylogenetic non-independence in the traits in the trade-off analysis, we ran linear models on PICs for all three traits. We found that phenolics were negatively correlated with LT (slope= -0.511, $p< 0.001$; Fig. 4-10c) and detected the same for tannins and LT (slope= -0.571, $p< 0.001$; Fig. 4-10f). In both cases the encountered trade-offs were much stronger (indicated by the absolute value of the slopes) than when analyzed without accounting for phylogenetic autocorrelation.

Table 4-11: Blomberg's *K*–values for leaf toughness, total phenolics and tannin concentrations, calculated either solely from species mean trait values or by taking into account intra-specific variability (standard error). p-values were calculated from a permutation test as the ratio of predicted to observed values based on 999 permutations.

Trait	**Without intraspecific variation**		**With intraspecific variation**	
	Blomberg's K	**p-value**	**Blomberg's K**	**p-value**
Leaf toughness	0.664	0.027	0.939	0.002
Phenolics	0.85	0.001	0.923	0.001
Tannin	0.774	0.003	0.866	0.003

Both physical and chemical defense traits increased in mean trait values with evolutionary time. However, this increase was earlier in tannin concentration than in leaf toughness, while phenolics concentrations increased more recently (Fig. 4-11a). Because tannins and phenolics showed similar variances (i.e. rates of transition) along the phylogeny (Figs. 4-11b), mapping trait evolution of tannin concentrations onto the phylogeny, revealed patterns in the evolution of tannin concentrations that closely resembled those of the phenolics concentrations. We therefore only present evolution in trait values of total phenolics (Fig. 4-12a) vs. leaf toughness (Fig. 4-12b). A corresponding figure for tannin concentrations vs. LT is given in Figure 4-S1 (Supporting Information).

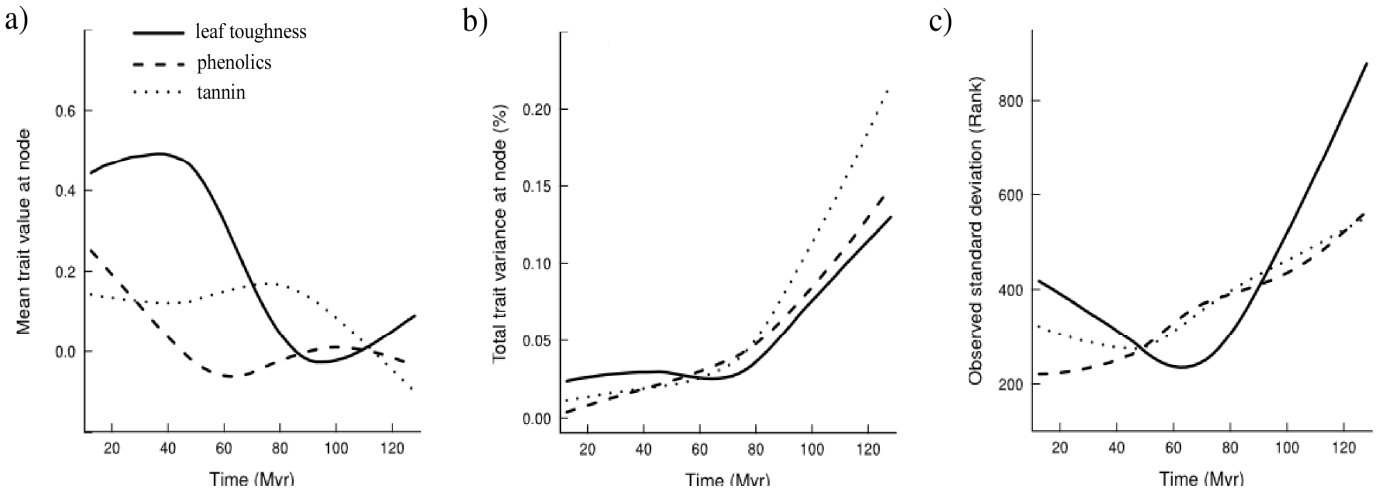

Figure 4-11: a) Node-level mean trait values, b) percentage of total trait variance contributed by species descending at that node, and c) ranks of the observed divergence size (standard deviation) within a null distribution of expected divergence size values (see Material and Methods). Lines represent, for each trait, fitted curves from local polynomial regressions (loess; smoothing span = 0.66, polynomial degree = 1) of node age (x-axis) against node-level values in trait means, variances and divergence size ranks, respectively (y-axis).

Reconstructions of trait values suggest that phenolics and LT differed in their rates of transitions between trait states in different branches of the phylogenetic tree. For example, while phenolics remained moderately high throughout Fagaceae evolution (*Quercus fabri* to *Q. serrata* in Fig. 4-12), LT evolved into very contrasting values within this clade. The high levels of trait divergence in LT within the Fagaceae (Fig. 4-12b) line up with our finding that, compared to phenolics content, LT showed low phylogenetic signal (Figs. 4-11c) close to the tips in the phylogeny. Even for closely related species, such as *Q. acutissima* and *Q. phillyreoides*, LT strongly diverged, while both species consistently showed intermediate values of total phenolics.

Assessment of divergence sizes (i.e. phylogenetic signal) at different depths in the phylogenetic tree revealed that phenolics and tannins generally showed a stronger phylogenetic signal across the phylogeny than leaf toughness (Fig. 4-11c). Basally as well as terminally, the two secondary metabolites showed a higher degree of trait conservatism than leaf toughness, with phenolics and tannins exhibiting increasingly less conservatism over time.

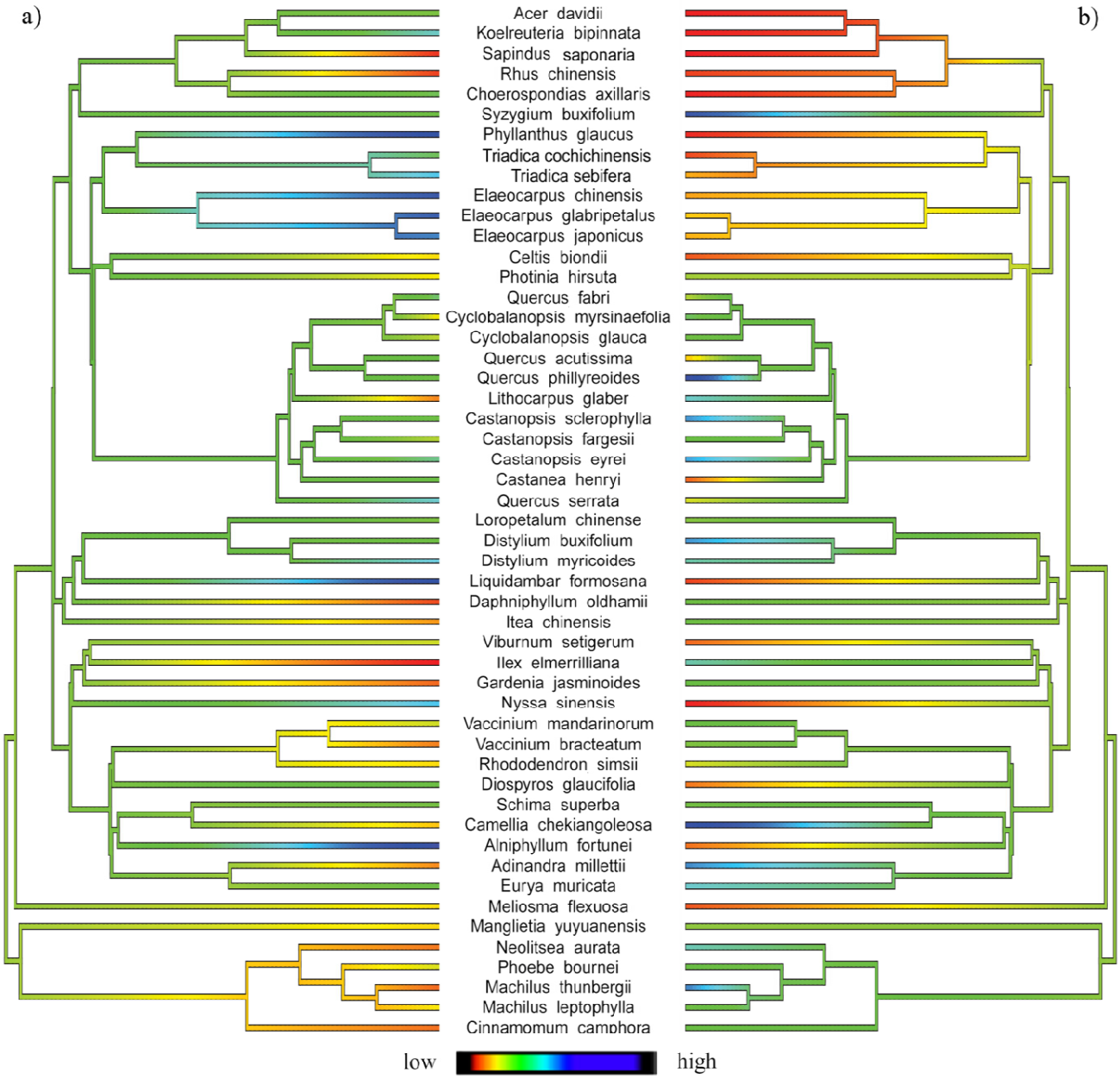

Figure 4-12:Trait evolution of a) total phenolics concentrations and b) leaf toughness mapped onto the phylogeny. Color ranges indicate high (blue) to low (red) trait values.

4.4.4. Discussion

Variance components analysis showed that intraspecific trait variability only accounted for minor proportions of over-all variance when compared to differences across species. Intraspecific regression analyses revealed inconsistent patterns, whereas interspecific regressions based on species mean traits indicated negative trait covariation (i.e. trade-offs) between chemical and physical carbon-based defense. Including ITV into regression analyses significantly decreased the strength of these trade-offs. A strong phylogenetic autocorrelation

was detected in all investigated traits. Cross-species analysis based on phylogenetic independent contrast revealed a strong evolutionary trade-off between the plant polyphenolic substances and LT.

Trade-off analyses

Intraspecific trade-off analyses: a lack of clear trends

Considering species-specific trade-off analyses we encountered positive, negative or no correlations between leaf toughness and leaf polyphenols. This is in line with the findings of Cornelissen *et al.* (2009) and Read *et al.* (2009). The former authors reported positive as well as negative or absent correlations between polyphenols and leaf toughness, the latter reported either positive or absent species-specific trait interrelations. Following the terminology of Cornelissen *et al.* (2009) we encountered tripartite relationships between physical and chemical aspects of carbon based defense: we detected high concentrations of polyphenols coinciding with high leaf toughness (e.g. *Distylium myricoides*), high values of leaf polyphenols coinciding with low values of leaf toughness (e.g. *Liquidambar formosana*) as well as low values of polyphenols coinciding with low values of LT (e.g. *Rhus chinensis*). The finding that the majority of (the few) significant relationships between the two defense traits showed a negative slope points at allocation constraints in some of the species. However, for most of the species no significant trait interrelationships were detected and no clear pattern emerged from the species-specific trade-off analyses. In consequence, we found that trade-offs between the two investigated aspects of carbon-based defense were not an ubiquitous phenomenon. Thus, our first hypothesis has to be rejected.

Interspecific analyses: intraspecific variability and trade-off assessment

Cross-species analyses based on species mean trait values revealed significant trade-off between chemical and physical leaf carbon based defense, thus supporting our second hypothesis. When partitioning the trait variability based on the taxonomic affiliation across all species we found that over-all trait variability generally followed a hierarchical pattern in all three investigated traits with a decreasing contribution in the following sequence: families, genera, species, among individuals and within individuals. Trait variation attributable to habitats only accounted for a minor component of over-all variability. This indicates that the investigated traits did not strongly respond to differences in habitat conditions. The same hierarchical trends were also encountered by Hulshof & Swenson (2010) who assessed the relative importance of ITV in a set of relevant leaf traits from the Costa-Rican tree species. In

addition, Reich *et al.* (1999) reported this hierarchical pattern for LDMC and leaf thickness in 769 species from the British flora. However, the latter authors reported that SLA did not follow this pattern but showed higher intraspecific variability compared to the other traits. This indicates that the hierarchical patterns encountered in our study may not hold for all functional traits. In none of the above mentioned studies the relative importance of ITV in polyphenolic substances has been investigated. However, in a recent study Eichenberg *et al.* (2014a) found that similar proportions of trait variability in the concentrations of the two polyphenolic substances could be assigned to the family and genus affiliation. The latter study included 20 subtropical tree species but did not assess trait variability below the species level.

Other studies, such as those of Albert *et al.* (2010), reported much higher proportions of ITV in SLA, LDMC, maximum height and leaf nitrogen and carbon contents for 13 species collected in the French Alps . They reported that ITV may account for up to 30% of over-all trait variability in these traits. However Albert *et al.* (2010) did not partition trait variability between species across higher levels of taxonomy (i.e. genera and families). In the present study we found remarkable differences in the explanatory power of taxonomy across different hierarchical levels. For polyphenolic substances the major part of over-all trait variability was accounted for by 'genus' and 'family' affiliation. In contrast, for LT the major part of trait variability was explained by the 'species' level. This hints at the high relevance of phylogeny in over-all trait variability.

In a global analysis comprising approx. 260 species, Moles *et al.* (2013) did not find significant trade-offs between physical and chemical aspects of plant defense. However, in the present study we analyzed interrelationships between leaf traits that were determined from the same tissues whereas Moles *et al.* (2013) used mean trait values compiled from different sources. Our approach allowed for the accurate determination in of ITV across 51 species and thus allows for more universal conclusions that analyses carried out within single families (e.g. Agrawal *et al.* 2009).

We detected a weaker trade-off between chemical and physical leaf carbon based defense across all species when ITV was incorporated in comparison to cross-species analyses based solely on species mean trait values. As this was the effect of absent or inconsistent trait correlations within different species we have to reject our third hypothesis of a match between trades-off based on ITV and species mean values.

Phylogenetic analysis: strong evolutionary trade-offs between traits

In the present study, significant phylogenetic signals were detected in all three investigated traits, confirming our fourth hypothesis. High levels of phylogenetic autocorrelation in comparative studies may cause increased Type-I-error rates in the estimation of model coefficients and hence introduce bias in the quantification of trade-offs (Ackerly 1999). The fact that regression analyses based on phylogenetically independent contrasts (PICs) revealed even stronger negative relationships between chemical and physical defense trait mean values than did analyses that did not account for phylogenetic non-independence indicates that, in the cross-species approach phylogenetic autocorrelation masked trade-offs between physical and chemical carbon-based defense at least to some extent. In a previous study Ackerly (1999) has pointed out that the strength of correlations between traits may be underestimated if there are uncorrelated shifts in the values of traits deep in the phylogeny. Our results showed that such contrasting trait shifts have also obscured the detection of a strong evolutionary trade-off between physical and chemical plant defense. Trait reconstruction along the phylogeny indicates that there were strong evolutionary shifts in mean LT values along the phylogenetic tree, whereas phenolics and tannins showed a relatively constant increase in mean trait values and low levels of trait variation with evolutionary time. Although the tree-wide phylogenetic signal assessed using Blomberg's *K* was highly significant for all three traits, node-wise trait reconstruction suggested that high divergences in the strength of conservatism in the two aspects of defense have occurred at different periods during trait evolution. In comparison to phenolics and tannins, LT was found to be the least conserved trait, with a period of maximum conservatism between 55 and 80 Mya. In consequence, we reject our fifth hypothesis that physical and chemical leaf traits followed the same patterns of conservation during evolutionary history. Being aware that node ages only represent rough estimates, Swenson and Enquist (2007) advocate that caution should be used when interpreting times based on node-wise trait reconstruction. However, the depicted evolutionary differences between the traits under consideration still allow to interpret relative differences in the intensity of trait conservatism. From the root of the phylogeny up to 70 Mya we detected an increase in the phylogenetic signal in LT. During that period the angiosperms gained dominance over the gymnosperms in many ecosystems (Soltis *et al.* 2008). Our results indicate that at that time there was a high selective pressure on LT. However, given the limited number of species included in our phylogeny, this interpretation is, of course, highly speculative.

In contrast to LT, phenolics and tannin concentrations seem to have been subjected to higher conservatism throughout trait evolution. This is possibly evoked by conservatism in their specific synthetic pathway. In this respect, Agrawal *et al.* (2009) reported evidence for an escalation of defense in the complexity of phenolic structures in 35 species of milkweeds. The authors interpret this escalation as a co-evolution between herbivores and their plant resources. They argue that the differences in the complexity of the phenolic structures are closely linked to the synthetic pathways within the respective species and that these pathways are very similar among the investigated species. As a result of differential trait conservatism between the two investigated aspects of carbon-based defense, and thus based on their independent evolution, even closely related species may feature different combinations of these two defense aspects. Integrating these results into the findings of the present study, we conclude that the trade-offs between the concentrations of polyphenolic substances and leaf toughness have a strong evolutionary basis.

Performance of NIRS calibration models

Both calibration models showed RPD values above 1.5, indicating a moderate to good model performance (Hildrum 1992; Ozaki *et al.* 2006). The phenolics calibration model comprised spectral regions with high resonance in the first and second overtones of C-H and C-H bonds in aromatic ring systems (Conzen 2006) which are typically found in polyphenolic molecules. R^2 values around 0.9 and an RPD around 2.8 indicate a high model accuracy. For the optimal model for tannin concentrations, the RPD value of 1.9 is within a range between 1.5 and 2, allowing to differentiate between high and low tannin concentrations (e.g. Saeys *et al.* 2005). For tannins the spectral regions selected in the optimal model comprise a wide range of wave-numbers which are typically associated with the absorbance of the second overtone region of C-H bonds in aromatic ring systems, the first overtones and combination oscillations of O-H bonds aromatic ring systems and the combined oscillations of C-C bonds and C-O-H bonds (Conzen 2006). Tannin molecules are highly variable and many different types of molecules of this group can be found within single plant species (Waterman and Mole 1994; Salminen *et al.* 2001; Hagerman 2002; Salminen 2003; Salminen *et al.* 2004). This complicates the detection of distinct spectral regions of tannin molecules for a multitude of species. Given the broad spectrum of families incorporated in this study, the tannin concentrations predicted by NIRS are sufficiently accurate to allow interpretations of trait-interrelationships on a semi-quantitative basis.

Conclusion

In the present analysis we demonstrated significant trade-offs between plant polyphenolic substances and leaf toughness when analyzed across all species. While incorporation of ITV significantly lowered the intensity of this trade-off, including phylogenetically independent contrasts made the trade-offs more pronounced. The low contribution of ITV in these relationships shows that the trade-offs are not mainly constrained by carbon allocation but rather are dominated by evolutionary constraints. We conclude that detailed phylogenetic analyses may greatly contribute to our understanding in trait covariances.

4.4.5. Supporting information

Table 4-S2: Species-specific trait ranges in the investigated species. Total number of individuals, number of leaves per species, trait mean values as well as minima and maxima are reported. Species names and given authorities follow the nomenclature of the Flora of China (http://flora.huh.harvard.edu/china).

Species	Author	number of individuals	n leaves (total)	leaf toughness			total phenolics concentration			tannin concentration		
				min	max	mean	min	max	mean	min	max	mean
Acer davidii	Franchet	3	21	0.07	0.16	0.11	55.24	95.10	70.98	45.09	69.13	59.17
Adinandra millettii	(Hooker & Arnott) Bentham & J. D. Hooker ex Hance	3	20	0.64	1.15	0.90	12.32	45.20	29.74	18.24	27.18	22.17
Alniphyllum fortunei	(Hemsley) Makino	3	20	0.16	0.27	0.20	139.57	194.50	163.90	107.21	132.84	117.07
Camellia chekiangoleosa	Hu	3	20	0.61	1.56	1.07	29.25	60.65	38.05	13.92	28.29	18.91
Castanea henryi	(Skan) Rehder & E. H. Wilson	3	20	0.12	0.25	0.19	61.92	97.96	80.04	41.47	74.86	56.39
Castanopsis eyrei	(Champion ex Bentham) Tutcher	8	59	0.49	1.60	0.88	24.42	160.00	106.43	19.01	107.92	70.04
Castanopsis fargesii	Franchet	4	28	0.38	0.86	0.57	46.80	71.01	57.55	26.53	46.41	34.96
Castanopsis sclerophylla	(Lindley & Paxton) Schottky	3	20	0.69	1.05	0.90	53.93	140.34	93.02	35.54	92.57	61.37
Celtis biondii	Pampanini	3	19	0.14	0.22	0.18	24.26	60.71	41.41	10.89	39.14	20.61
Choerospondias axillaris	(Roxburgh) B. L. Burtt & A. W. Hill	3	21	0.07	0.16	0.11	78.45	113.72	99.19	72.70	101.35	90.05
Cinnamomum camphora	(Linnaeus) J. Presl	4	27	0.46	0.74	0.60	13.43	43.98	25.63	13.76	28.29	20.84
Cyclobalanopsis glauca	(Thunberg) Oersted	4	32	0.47	0.90	0.65	22.59	95.60	56.13	10.63	57.63	31.90
Cyclobalanopsis myrsinifolia	(Blume) Oersted	4	21	0.57	0.82	0.68	32.30	66.96	46.37	13.07	35.67	22.36
Daphniphyllum oldhamii	(Hemsley) K. Rosenthal	6	31	0.39	0.70	0.54	11.12	25.86	19.86	6.07	13.16	9.70

Diospyros glaucifolia	Metcalf	3	21	0.16	0.24	0.21	67.71	111.54	92.23	63.07	100.42	81.83
Distylium buxifolium	(Hance) Merrill	3	21	0.61	1.27	0.90	78.07	122.11	93.80	41.82	82.74	57.94
Distylium myricoides	Hemsley	3	21	0.55	0.93	0.75	98.92	144.74	120.56	59.57	101.26	80.75
Elaeocarpus chinensis	(Gardner & Champion) J. D. Hooker ex Bentham	5	38	0.18	0.45	0.25	88.25	186.41	148.37	48.69	112.38	81.60
Elaeocarpus glabripetalus	Merrill	5	35	0.20	0.41	0.28	115.08	180.11	145.32	64.95	111.41	84.14
Elaeocarpus japonicus	Siebold & Zuccarini	3	21	0.20	0.37	0.26	116.99	158.25	136.28	54.58	87.41	69.98
Eurya muricata	Dunn	4	23	0.27	1.14	0.79	66.82	94.53	79.37	32.63	51.33	40.50
Gardenia jasminoides	J. Ellis	3	20	0.41	0.73	0.60	15.10	32.76	24.32	7.34	18.91	11.95
Ilex elmerrilliana	S. Y. Hu	3	9	0.51	0.88	0.75	4.88	14.61	9.51	4.65	9.15	7.21
Itea chinensis	Hooker & Arnott	3	19	0.47	0.66	0.54	9.53	55.34	31.56	10.10	33.06	21.73
Koelreuteria bipinnata	Franchet	3	21	0.08	0.15	0.11	74.60	144.38	116.45	62.73	100.40	86.74
Liquidambar formosana	Hance	3	18	0.09	0.29	0.15	132.83	175.74	157.60	92.81	152.02	128.49
Lithocarpus glaber	(Thunberg) Nakai	6	48	0.51	1.06	0.79	14.99	63.86	28.39	12.34	55.07	22.01
Loropetalum chinense	(R. Brown) Oliver	3	16	0.35	0.59	0.47	42.32	136.05	70.73	31.49	94.51	48.73
Machilus leptophylla	Handel-Mazzetti	3	21	0.33	0.92	0.52	24.45	60.01	43.23	20.49	54.22	36.78
Machilus thunbergii	Siebold & Zuccarini	3	21	0.69	1.19	0.92	15.19	33.58	24.59	10.56	26.21	17.33
Manglietia yuyuanensis	Oliver	5	35	0.25	0.65	0.46	29.84	54.91	41.03	13.97	25.74	20.65
Meliosma flexuosa	Pampanini	3	21	0.12	0.25	0.18	30.07	51.86	42.09	13.29	26.83	19.10
Neolitsea aurata	(Hayata) Koidzumi	4	23	0.51	0.97	0.76	12.89	40.21	26.99	10.63	26.19	15.90
Nyssa sinensis	Oliver	3	21	0.08	0.15	0.11	99.94	157.98	125.94	67.04	112.67	88.20
Phoebe bournei	(Hemsley) Yen C. Yang	3	21	0.43	0.82	0.60	36.24	66.67	50.91	22.14	38.49	28.06
Photinia hirsuta	Handel-Mazzetti	3	21	0.31	0.55	0.43	37.63	54.66	45.78	30.70	42.49	35.50
Phyllanthus glaucus	Wallich ex Müller	3	14	0.09	0.16	0.12	136.74	190.18	158.53	97.83	129.65	111.22
Quercus acutissima	Carruthers	3	21	0.12	0.41	0.28	59.64	106.72	84.38	39.47	78.79	59.28
Quercus fabri	Hance	3	21	0.33	0.49	0.41	85.56	119.98	103.18	60.34	89.33	72.65

Quercus phillyreoides	A. Gray	5	35	0.67	1.45	1.09	65.02	127.90	92.47	37.16	85.10	57.34
Quercus serrata	Murray	3	18	0.27	0.47	0.37	92.33	151.86	117.44	61.82	103.36	78.88
Rhododendron simsii	Planchon	4	20	0.26	0.47	0.37	29.61	74.18	41.31	8.37	55.77	21.34
Rhus chinensis	Miller	3	17	0.11	0.21	0.15	8.07	24.07	17.99	25.16	39.82	34.45
Sapindus saponaria	Linnaeus	3	15	0.05	0.18	0.10	6.01	22.49	15.58	12.50	28.02	19.61
Triadica cochinchinensis	Loureiro	3	19	0.14	0.20	0.16	70.71	124.78	99.37	61.97	90.70	74.65
Triadica sebifera	(Linnaeus) Small	3	15	0.18	0.48	0.26	94.40	148.71	125.29	60.33	107.78	85.51
Schima superba	Gardner & Champion	6	42	0.22	0.88	0.52	39.66	119.09	73.82	25.21	78.47	44.64
Syzygium buxifolium	Hooker & Arnott	3	15	0.82	1.26	1.00	79.49	140.88	95.09	46.25	86.52	56.13
Vaccinium bracteatum	Thunberg	6	30	0.32	0.75	0.49	10.88	58.76	27.80	2.69	38.30	15.60
Vaccinium mandarinorum	Diels	4	28	0.25	0.82	0.62	43.18	66.12	52.89	27.69	47.80	39.19
Viburnum setigerum	Hance	3	21	0.14	0.25	0.19	41.77	58.57	53.08	29.44	43.32	37.09

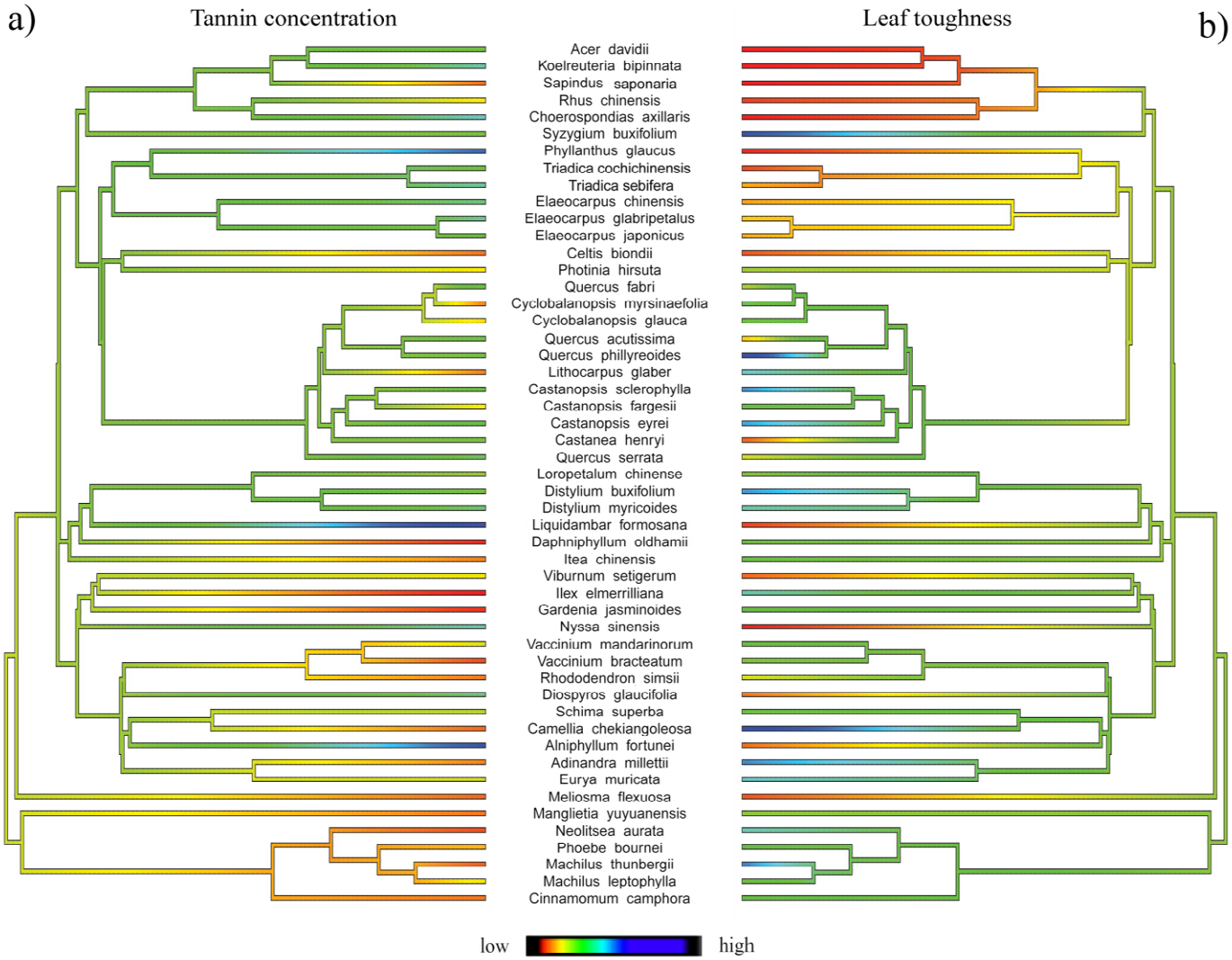

Figure 4-S0-1: Trait evolution of a) total phenolics concentrations and b) leaf toughness mapped onto the phylogeny. Color ranges indicate high (blue) to low (red) trait values.

4.4.6. Acknowledgements

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5. Synthesis

5.1. General discussion

The present thesis focused on the effects that plant polyphenolic substances exert on ecological processes in the context of BEF studies. However, prior to investigating specific questions on the effects of polyphenols on ecosystem processes, the consequences of methodological problems needed to be assessed. In many previous studies (e.g. Cork and Krockenberger 1991; Hagerman 1988; Salminen 2003), the detrimental effects of sub-optimal sample handling during sample collection, drying and the storage conditions on the structure and amount of extractable polyphenols have been demonstrated. Moreover, seasonal variability as well as variations between different stages of leaf maturation have been described as factors affecting species-specific compositions and concentrations of polyphenolic substances in plant tissue (Brunt *et al.* 2006; Salminen *et al.* 2001, 2004). In the present thesis the hypothesis was tested, that these methodological as well as biotic and ontogenetic sources of variability render data on leaf polyphenol concentrations determined according to different protocols as incomparable. Moreover it was tested whether information on leaf polyphenol concentrations based on sub-optimally handled specimen leads to erroneous ecological conclusions. It has been found that total phenolics concentrations are less sensitive to non-optimal sample treatment, whereas tannin concentrations were affected by sub-optimal sample treatment in some species. However, as plant polyphenolic substances were found to be highly phylogenetically conserved, we found that the detrimental effects of non-optimal sample handling were widely overridden by the variability in polyphenol concentrations brought about by the taxonomic extent. Decreasing the taxonomic extent in the analyses increased the relative effect of suboptimal sample handling. Most importantly, it has been shown that data from studies on polyphenol concentrations analyzed from plant material sampled under non-optimal conditions may be used to draw ecologically valuable conclusions with high accuracy. However, this is only true under the prerequisite that a wide range of species is examined in studies addressing the functional role of plant polyphenolic substances on the investigated ecosystem process.

In addition, this thesis assessed general patterns of shifts in traits relating to leaf productivity, nutrient quality and defense in forest communities studied along a gradient of secondary forest succession, established in the framework of the BEF-China project. These shifts were functionally linked to a shift in the community resource-use strategy, with high nutrient

acquisition rates in young forests and high nutrient retention in old forests. Moreover, the present thesis demonstrated that the dynamics of community leaf litter decomposition rates can be best explained when simultaneously considering a multitude of functional traits. The combined effect of traits related to leaf resistance (comprising chemical and physical resistance), productivity and nutritional quality together were found to affect litter decomposition rates. While litter mixtures that consisted of leafs with high nutritient quality exhibited higher rates of litter break-down, litter mixtures with leafs possessing higher trait values related leaf resistance had lower decomposition rates. Along the successional gradient, no significant changes in leaf litter decomposition rates were observed. The opposing directions of trait shifts detected along the successional gradient were found to ensure a constant nutrient release from leaf litter during succession.

Moreover, it has been found that the investigated leaf resistance traits did not evolve independently from each other during the phylogeny of the investigated species. While the three aspects of leaf resistance, total phenolic and tannin concentrations as well as leaf toughness, were found to be highly phylogenetically conserved, it has been demonstrated that the degree of phylogenetic conservatism varied independently throughout the trait evolution. Disentangling the evolutionary signals of the three investigated traits from species-specific variations resulted in the detection of an evolutionary based trade-off between chemical and physical carbon-based resistance traits. Interestingly, the finding of this evolutionary trade-offs may indicate that the width of the phylogenetic diversity in communities is less important for the dynamics of community litter decomposition: even closely related species may vary significantly in the combination of polyphenolic concentrations and leaf toughness and may thus exhibit a diversity in these traits similar to the diversity of communities comprising very distantly related species.

Furthermore, the protocols for the optimized sample collection, storage and quantification of total phenolics and tannins presented in the Chapters 4.1 and 4.2 allow for a straightforward and comparable assessment of information on plant polyphenolic concentrations in future BEF experiments. With a focus on the BEF-China project, the methods developed to accurately quantify the over-all concentrations of leaf polyphenolic substances by near infrared spectrometry constitute a breakthrough for further investigations on the role of plant polyphenols in ecosystem functioning. Near infrared spectrometry (NIRS) is increasingly used to determine leaf properties such as leaf nutrient contents and carbon-to-nitrogen rations; traits that are routinely assessed in BEF studies, and thus the use of the established NIRS

methods for the determination of total phenolics and tannin concentrations will give information on the concentrations of these two metabolites with no additional expenses. As total phenolics and tannin concentrations can now be reliably quantified in no time, these traits may be now considered as 'soft traits' (sensu Hodgson *et al.*, 1999) for the species planted in the experimental sites of BEF-China.

It has to be stated that the analyses presented this thesis did quantify the over-all concentrations of polyphenols in plant tissue, whereas the quantification of specific polyphenol molecules was beyond the scope of the present thesis. In other fields of biological research, such as in chemical ecology, the interplay of specific polyphenols with processes and functions in particular combinations may be important (e.g. Barbehenn 2005; Padmavati 1997). In early stages of BEF research on polyphenols, it may be more important to accurately determine the total amount of polyphenolic substances rather than to quantify specific polyphenols. For example in the case of nutrient turnover in ecosystems, the BEF approach presented in this thesis was to assess litter decomposition rates in given communities, without determining the exact composition of the decomposer community. Thus, in the context of nutrient turnover, the over-all concentrations of polyphenolic substances were considered to be of higher importance when assessing their effects on this ecosystem process. However, information on compound-specific polyphenol concentrations may significantly contribute to the understanding of the mechanisms underlying the general patterns detected e.g. in nutrient turnover dynamics. Future studies may address these mechanisms.

Summing up the insights provided by the investigations of this dissertation, it is becoming increasingly evident that the role of plant polyphenolics in ecosystem functioning cannot be addressed using these secondary metabolites as isolated variables. It has been demonstrated that the dynamics of leaf litter decomposition in the diverse forest communities of BEF-China are driven by complex interactions between different functional leaf traits. These include plant polyphenols, but not as the only drivers for certain ecosystem functions, such as leaf litter decomposition rates.

5.2. Outlook

The insight that the concerted link between different leaf functional traits determines ecosystem processes such as nutrient turnover through litter decomposition highlights the necessity to use approaches that involve multiple traits when analyzing the role of plant

polyphenols in ecosystem functioning. This may not only be the case for litter decomposition (as demonstrated in the present dissertation), but also may apply to other ecological functions of leaf polyphenols such as herbivore defense. Interestingly, Cornelissen *et al.* (2004) reported that the same factors that affected litter decomposability also affected litter digestibility in ruminants. Litter digestibility may also be linked to feeding preferences in insect herbivores via the tannin concentrations in the respective diet (Brehmer *et al.* 2002).

In the present thesis we did not explicitly investigate the relationships between leaf polyphenols and herbivory. However, the patterns of intensity and the causes of the herbivore damage experienced by plant species growing in the Gutianshan National Nature Reserve (GNNR) have been investigated by Schuldt *et al.* (2010, 2012, 2014). As a general result on the patterns of herbivore damage, the authors reported that the herbivore community is dominated by generalist herbivores. As stated by *Wittstock et al.* (2003), generalist herbivores are expected to be deterred by unspecific strategies of plant defense, while specialist herbivores have been reported to have evolved adaptations to certain strategies of plant defense. Specialist herbivores are restricted to one or few host species, whereas generalist herbivores may feed upon a wide spectrum of host plants.

Considering the high herbivore damage encountered on the majority of tree species in the GNNR, it may be assumed that leaves are mostly consumed by generalist herbivores (Schuldt *et al.* 2010). Thus, a high selective pressure to produce leaves that are well defended against generalist herbivore attack should exist. Plant polyphenolic substances as well as leaf toughness are generally considered as unspecific characteristics of leaf defense. Based on the results presented in Chapter 4.5, it may be inferred that most tree species in the GNNR exhibit combined defense strategies against generalist, and to a lesser extent against specialist herbivores. This hypothesis has not been tested yet. In this respect, information on the concentrations of specific chemical leaf defenses may become useful. Future studies can be designed to investigate the relative abundances of specific versus non-specific chemical defense compounds. The hypothesis to be tested in such a study could then be formulated as: 'For the species in the Gutianshan National Nature Reserve, the proportion of leaf defense traits against specialist herbivores is lower than the proportion of defense traits against generalist herbivores.'

Albeit the lack of information on the proportion of specialized versus generalized defense traits, Schuldt *et al.* (2012) did not find significant relationships between over-all herbivory and leaf total phenolics concentrations in the 27 CSPs established within the BEF-China

project. In contrast to an expected negative influence of chemical defense traits, the authors reported that geographical range characteristics such as species niche marginality were the best predictors for herbivory rates. In a later study, however, the authors reported a significant correlation between the community-level diversity of leaf chemical traits and total herbivore damage in the 27 communities (Schuldt *et al.* 2014). The chemical defense traits incorporated in this multivariate variable comprised the effect of total phenolics concentrations, leaf carbon content, leaf carbon-to-nitrogen ratio and leaf carbon-to-phosphorous ratio. In support to the general conclusions drawn from the investigations presented in this dissertation, leaf polyphenol concentrations as single traits did not significantly affect herbivory. However, Schuldt *et al.* (2014) did not include leaf toughness (LT) as a physical defense trait in their study due to the fact that this trait was only known for a low proportion of all investigated species. Based on the results presented in Chapter 4.5, the lack of a direct link between leaf polyphenolics concentrations and herbivore damage may also be interpreted from a different perspective: as a result of the evolutionary trade-off encountered for the investigated species, even closely related species were found to defend their leaves from herbivores not solely through chemical or solely through physical defense, but rather specific combinations of these defense traits. The combined effect of leaf toughness and polyphenolics is then masked by the lack of information on leaf toughness. Although the defense strategies may vary from species to species, depending on the specific contribution of chemical and physical leaf defense traits on over-all defense, it may be hypothesized that the leaves of the species investigated by Schuldt *et al.* (2010, 2012, 2014) are generally (equally) well defended against generalist herbivores. Consequently, generalist herbivores may not profit from avoiding highly defended host species and switching to other, less defended tree species. The lack of significant direct correlations between leaf polyphenols and herbivore damage reported by Schuldt *et al.* (2012, 2014) may thus be interpreted as a complicit effect of the of chemical and structural defense traits. In other words, this lack of significant correlations may be attributed to the lack of information on leaf toughness for the respective species. Simultaneously considering both aspects of leaf defense rates in multiple regression analyses may result in significant correlations between defense and herbivory, similar to the effects of the two aspects reported for litter decomposition. This hypothesis has also not been tested yet, but future investigations can be designed to do so. Therefore, methods to assess data on chemical and physical herbivory repellents across a wide range of ecological contexts and across a multitude of plant species is required. Such methods have been developed and tested in the present thesis.

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9. Appendix

Curriculum vitae

David Eichenberg

Date and place of birth:	20.10.1982, Lichtenfels
Nationality:	German
Address:	Hempelstraße 1, 04177 Leipzig, Germany

Education:

11.2010 - present	PhD student, Martin-Luther-University Halle-Wittenberg
	Research topic: 'Plant Polyphenols in the context of Biodiversity Ecosystem Functioning', supervisors: Prof. Helge Bruelheide, Prof. Christian Wirth
10.2003 - 09.2009	Studies in biology, University of Bayreuth
	Major subject: Plant Ecology
	Minor subjects: plant physiology, animal ecology, animal morphology, animal physiology
	Diploma thesis: 'Natürliche C- und N- Isotopenverhältnisse bei ausgewählten einheimischen Cyperaceen', supervisor: Prof. Dr. Gerhard Gebauer, Isotopenbiogeochemie, University of Bayreuth
09.1993 - 06.2003	Abitur: Meranier-Gymnasium Lichtenfels

List of Publications:

Eichenberg D, Ristok C, Kröber W, Bruelheide H (2014): Plant polyphenols- Implications of different sampling, storage and sample processing in Biodiversity Ecosystem Functioning experiments. Chemistry and Ecology; doi: 10.1080/02757540.2014.894987

Eichenberg D, Trogisch S, Huang Y, He JS, Bruelheide H (under review): Shifts in community leaf functional traits relate to litter decomposition during secondary forest succession. Journal of Plant Ecology

Eichenberg D, Purschke O, Bruelheide H (submitted): Trade-offs between physical and chemical carbon-based defense: Disentangling the relative importance of intraspecific variation vs. trait evolution. New Phytologist,

Other publications by the author

Schuldt A, Bruelheide H, Durka W, Eichenberg D, Fischer M, Kröber W, Härdtle W, Ma KP, Michalski SG, Palm WU, Schmid B, Welk E, Zhou HZ, Assmann T (2012): Plant traits affecting susceptibility to herbivory of tree recruits in highly diverse subtropical forests. Ecology Letter 15: 723-739. doi: 10.1111/j.1461-0248.2012.01792.x.

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Declaration of Originality

Eigenständigkeitserklärung

Hiermit erkläre ich, dass die vorliegende Arbeit mit dem Titel 'Plant Polyphenols in the context of Biodiversity-Ecosystem Functioning' bisher weder bei der Naturwissenschaftlichen Fakultät 1 Biowissenschaften noch bei einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion vorgelegt wurde.

Ferner erkläre ich hiermit, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst sowie keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die anderen Werken wörtlich oder inhaltlich entnommenen Stellen wurden von mir als solche kenntlich gemacht.

Ich erkläre weiterhin, dass ich mich bisher noch nie um einen Doktorgrad beworben habe.

Leipzig, den 31.03.2014

David Eichenberg

Appendix:

1. Optimized laboratory protocol

2. Sketch for the construction of vertical gel plates

3. Template for gel loading (30 wells)

A) Preparation of necessary solutions

Standard Solutions for Total Phenolics Assay:

Use great caution when preparing standard solutions!

Prepare a stock solution of Tannic acid in 50% AcOH with a concentration of 500mg/L. Therefore dissolve 50mg of Tannic acid in 100ml of AcOH(50%).

Standard solutions for the assays

V(Tannin solution from the stock) [ml]	V(Aceton 50%) [ml]	Concentration [mg/L]	
10	0	500	**Radial diffusion**
8	2	400	
6	4	300	
4	6	200	
2	8	100	**Total Phenolics**
1.5	8.5	75	
1	9	50	
0.4	9.6	20	
0.2	9.8	10	

Store the solutions in the refrigerator in light sealed and individually labelled vials.

It's best to take out small aliquots on the day of total phenolics analysis. These should be stored on ice.

Dye reagents for Prussian blue Total Phenolics Assay:

0,02 M $FeNH_4(SO_4)_2$ in 0,10 M HCl

Dilute 830µl HCl (conc.) in 100ml H_2O_{demin} to obtain 0,10M HCl.

Dissolve 324mg of $FeNH_4(SO_4)_2$ in 100ml 0,10M HCl. This will result in a pale yellow solution.

0,016M K3Fe(CN)6

Dissolve 526mg $K_3Fe(CN)_6$ in 100ml H_2O_{demin}. This will result in al yellow solution.

Stabilizer-Solution for Prussian Blue Assay:

General recipe:

H_2O_{demin}; 85% H3PO4; 1% Gummi arabicum (3:1:1)

Preparation of a stock Solution for approx. 200 Samples (stable 1 week in refrigerator):

1% gum arabic:

0,25g gum arabic + 20ml H_2O_{demin} -> boil for 25min. Vacuum filter solution through filter paper and put in the refrigerator to cool down the suspension.

Complete stabilizer solution mix (sufficient for approx. 200 samples):

60ml H_2O_{demin}; 20ml 85% H_3PO_4; 20ml 1% Gummi arabicum

Note: the stabilizer solution mixture should be prepared on the day of the assay. It is not stable for longer periods.

Dye reagents for Radial Diffusion Assay:

2 N NaOH:

Dissolve 8.0g of NaOH in 100ml H_2O_{dest}.

10 N NaOH:

Dissolve 40.0g of NaOH in 100ml H_2O_{dest}.

0.3 M NaCl:

Dissolve 17.6g of NaCl in 1L H_2O_{dest}. Prepate sufficient solution; 3l is convenient.

0.10 M HCl:

Dilute 9.910ml of 32% HCl in 1000ml H_2O_{demin} to obtain 0.10 M HCl.

0.1M $FeNH_4(SO_4)_2$ in 0.1 M HCl:

Dissolve 48.2g of $FeNH_4(SO_4)_2$ in 1000ml of 0.1 M HCl. This will result in a pale yellow/clear solution.

0.008 M $K_3Fe(CN)_6$:

Dissolve 2.63g of $K_3Fe(CN)_6$ in 1000ml H_2O_{dest}. This will result in a yellow solution.

Gel Buffer: – 0.05 M acetate containing 60µM ascorbic acid; pH 5.0

Dilute 2,85ml of glacial acetic acid in 800ml H_20_{dest}. Add 10.6mg of ascorbic acid. Adjust pH to 5.0 with 2 N NaOH and 10 N NaOH. Bring final volume to 1L with H_20_{dest}.

Note: the buffer solution should be prepared at the day of analysis.

B) Phenol extraction from dried leaf material:

Material:

- Falcon tube (10ml) for each specimen
- Falcon tube (50ml) for each extract
- Ice bath
- Analytical Scale
- Homogenizer
- Centrifuge (4°C)
- Storage Vial/Tube for each Specimen (we use 20ml UV-tight borosilicate glass)

Chemicals:

50% Acetone in H_2O_{demin} (stored in fridge and then put on ice to keep cool)

Note: Always make sure to close all tubes as quickly as possible to prevent evaporation of acetone

Procedure:

It is convenient to prepare as much extracts as can be centrifuged (we prepare 20-24 Samples per extraction day).

Label each Tube and each Storage vial unequivocally prior to the extraction procedure.

1. About 50 mg (record weight exactly!) of material is suspended in 5 ml 50% Acetone in the 10ml falcon tubes.
2. Homogenize Sample at maximum speed (30.000rpm) for 60 sec.
3. Store extract on ice until further processing.
4. Clear the homogenizer from plant material residue with 1ml of Acetone 50% between each sample to prevent cross-contamination.
5. After all prepared samples have been homogenized the tube is sealed (screwcap) and the specimen are centrifuged for 15 min. at 4°C at maximum rotation speed.
6. Transfer supernatant quantitatively to a UV-tight using a pipette and filled, labelled with the respective sample label.
7. Extract the remaining pellet three times according to points 2-5.

Note: Make sure to clean homogenizer between each additional specimen to prevent cross-contamination.

For each specimen, the liquid phases are combined. The pellet can be discarded after the final extraction step. Store the extract in the fridge at 4°C until further analysis (within 1 week).

C.) Total Phenolics Determination using the Prussian blue method as modified by Graham 1992:

General information: This essay uses a time dependent redox-reaction between the two Prussian blue reagents (see Chapter 'Preparation of necessary solutions'). It is therefore important to stick to constant time intervals during the determination of the total phenolics content. The time-dependent color formation can be stabilized (slowed down) by the addition of a 'Stabilizer solution'. Details are given below.

Material:

- Photospectrometer set at 710nm
- Pipettes (1000µl, 1x 100µl, 1x 20µl)
- Cuvettes (1/Sample) - ***Note: need to be acetone-resistant!***
- 2 reaction tubes/Sample (1x 2500µl; 1x 300µl)
- stop watch set to 15min

Chemicals (per Sample):

- 100µl 0,02 M $FeNH_4(SO_4)_2$ in 0,10 M HCl
- 10µl of Extract/Standard or Solvent
- 100µl 0,016M $K_3Fe(CN)_6$
- 500µl Stabilizer-Solution (see above)
- H_20_{demin}

Procedure:

Label each cuvette unequivocally. Include one cuvette for the ***blank*** and five for the ***standards***.

Note: Take care not to touch the absorption measurement area.

It is convenient to place the tubes in the order of measurement in an appropriate rack.

Label each reaction tube (2 per sample) unequivocally. Place them in the order of measurment into an appropriate rack.

Note: From point 3 on it is important to keep the same time intervals throughout the whole procedure (see below). 30sec is convenient.

1. 300µl reaction vials: prepare a 1:5 dilution* (Extract, **NOT Standard**: H_2O) for each specimen; vortex thoroughly.
2. 2500µl reaction vials: add 100µl of $FeNH_4(SO_4)_2$
   add 100µl of $K_3Fe(CN)_6$
   Add 290µl of H_2O_{demin}
3. add 10µl of **solvent** to the reaction vial ***'Blank'***. Start the clock. Vortex Eppi.
4. Subsequently add 10µl of ***pure Standard*** or ***diluted extract*** to the respective cuvette. Keep time intervals between cuvettes as constant as possible. ***Always vortex the extracts.***
5. Give exactly **15min**. reaction time.

** This is an empirical value for most of the species investigated in BEF-China; may differ, depending on species*

6. Add 500µl of stabilizer solution to each reaction vial. This stabilizes the colour development.
   Note: Keep same interval in adding stabilizer solution as with the addition of the extract.
7. Read baseline absorption (the ***Blank***) or set absorption of Blank to 0.
8. Read absorption of the Samples at ***710nm***.
9. Note the absorption.

Evaluation:

The 5 standard solutions with known concentrations are used to establish a standard curve. The R^2 of the standard curve should be from 0.98 to 0.99.

Sample concentrations can be calculated from the linear relationship between nabsorption and concentration established according to the calibration curve.

Note: samples with absorption > than the highest standards need to be re-measured with a higher dilution.

D) Radial Diffusion Method for Determining Tannins

General information:

Tannins precipitate proteins from the surrounding matrix. While the extract diffuses into the gel, a standardized protein (here: BSA fraction V) dispersed in the gel matrix, reacts with the tannins. After appropriate time the gel can be dyed with the Prussian blue reagents (see Chapter 'Preparation of necessary solutions'). Blue circles of precipitated protein will emerge. The diameter of a circle is in direct proportion to the tannin concentration. This procedure needs some experience. It is advisable to train gel preparation several times before the actual analysis.

Material:

- Vertical glass plates for agarose gels (distance between glass plates max. 1.6mm)
- Desk scanner (we use a DIN-A3 format scanner)
- 1 Glass flasks (250ml)/vertical glass plate
- 1 Glass pipette (100ml) and pilleus ball
- 1 Pipette (10 – 100µl)
- Water bath set to 45°C
- Well punch
- Thermometer
- Incubator set to 30°C
- 1 forceps
- 1 zip-lock bag/gel plate

Chemicals:

- Buffer for agarose gel (see Chapter 'Preparation of necessary solutions')
- Agarose
- Bovine Serum Albumin Fraction V
- Standard solutions (100mg/l tannic acid - 500 µg/l tannic acid)

Procedure:

Preparation of plates

0.8% agarose gel:

1. Add 1.0g agarose to 125ml of buffer (in a 250ml glass flask). Heat the solution with continuous stirring until the agarose completely dissolves.
2. Put the solution into the water bath set at **45°C**.
3. When the solution has reached 45°C (check with care), add **0.02g BSA**. Make sure the BSA is completely suspended.
4. Dispense 100ml of the gel solution into the upright oriented vertical gel plate, using the 100ml glass pipette. **Dispense the solution carefully to avoid development of bubbles and phases.**
5. Allow the gel solution to harden.
6. Bring the plate to horizontal position.
7. **Carefully** lift the top glass plate (if not gel may disrupt).
8. Allow the condensed water to evaporate at 40°C in the drying oven for a **maximum of 5 minutes**.

The layer of agarose in the plate should be of uniform thickness and free of bubbles or other imperfections.

Tannin Assay

1. Use well punch to punch wells in each plate. We normally prepare are 30 wells/plate (5 rows and 6 columns). Carefully remove punched Gel remains with a forceps.
2. Apply 20µl of each specimen (e.g. 1 blank [=solvent only], 5 standard, 24 extracts) to each well. Note which sample is in which well at which position. For convenience we added a 'Loading template' at the end of this description.
3. **If necessary** (i.e. if the tannin is very dilute) add larger volumes by dispensing repetitive 20µl samples. Don't allow the well to become completely dry between successive aliquots that are to be added. Note which wells received multiple volumes.
4. Cover the gels with the top glass plate and put in air-tight zip lock bags. Place the plates in a level incubator at **30°C**. Allow the rings to form for **96h**.

Staining

1. After incubation uncover the plates and place them into a large dish for staining.
2. Wash each gel with an adequate volume of 0.3 M NaCl saline for **20min** under continuous shaking.
3. Discard the saline and rinse the gel twice with 0.3 M NaCl. **Repeat the entire wash procedure three times**.
4. Stain each gel with **equal volumes** of 0.1 M $FeNH_4(SO_4)_2$ in 0.1 M HCl and 0.008 M $K_3Fe(CN)_6$. We use spray flasks to evenly cover the gel plates with the staning solutions.

Note: the staining solutions contain 0.1N HCl; it is therefore advisable to conduct the staning procedure under a fume hood.

5. Stain for **15 minutes**.
6. Pour off excess stain.
7. Rinse the gel with 0.1 N HCl.
8. Cover the gel with top glass plate
9. Scan the plate.
10. Save scanned image on hard drive or appropriate storing device.

Evaluation:

1. The scanned image is loaded into an image processing software
   (We use freeware: Image J, http://imagej.softonic.de/
   we additionally use the plug-in 'Chart-white balance' URL:
   http://imagejdocu.tudor.lu/doku.php?id=plugin:color:chart_white_balance:start).
2. Enhance contrast of the image (about 8% are convenient at this stage).
3. To sharpen the boundaries of the precipitation rings, we make use of the chart-white balance add-in: draw a straight line in the image **from dark to light** (see instructions for "Chart-White balance").
4. Run 'Chart-White balance' macro (we use QP 201 chart; see details of 'Chart-White-Balance').
5. Repeat point 4, if necessary.

   Note: Always cross-check original image and processed image to prevent loss of information (see Figure 1)

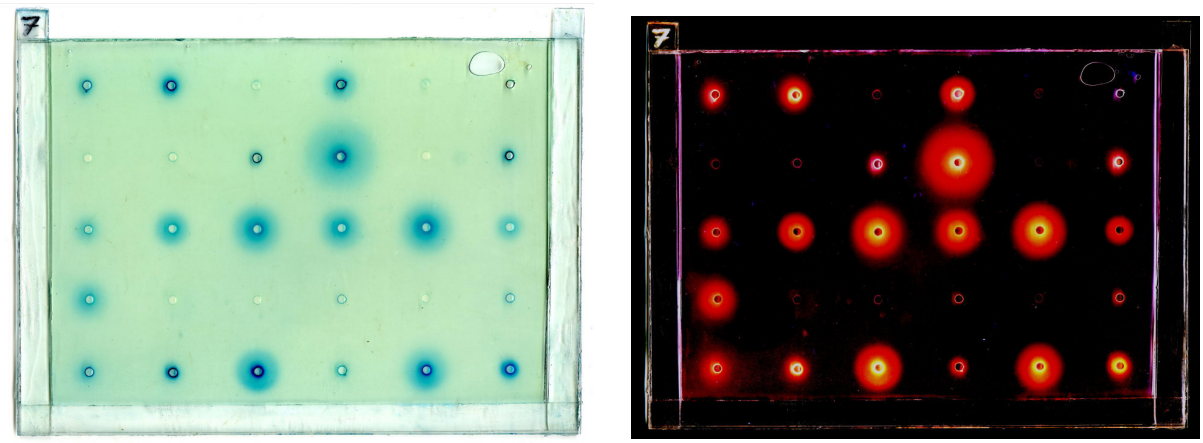
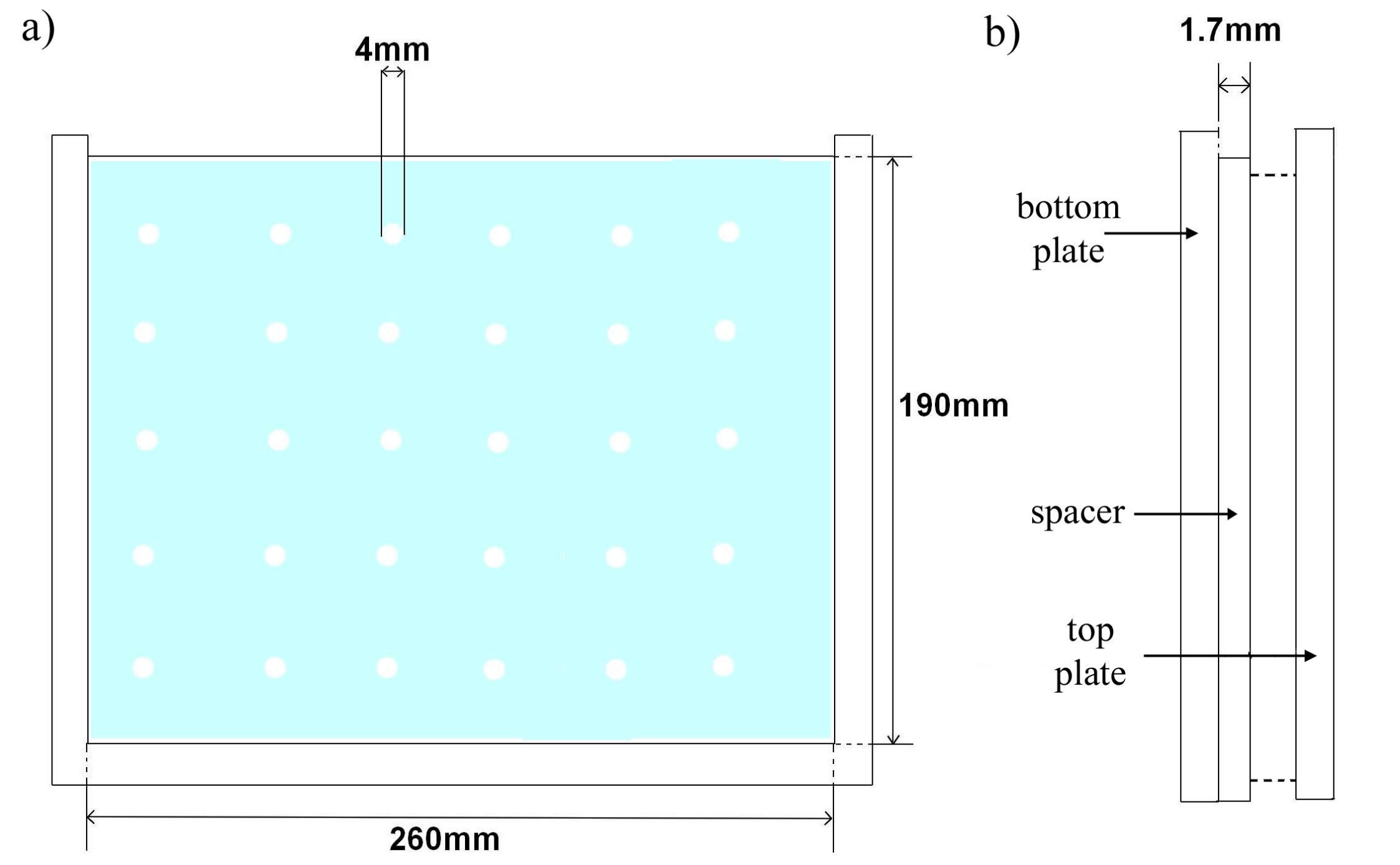
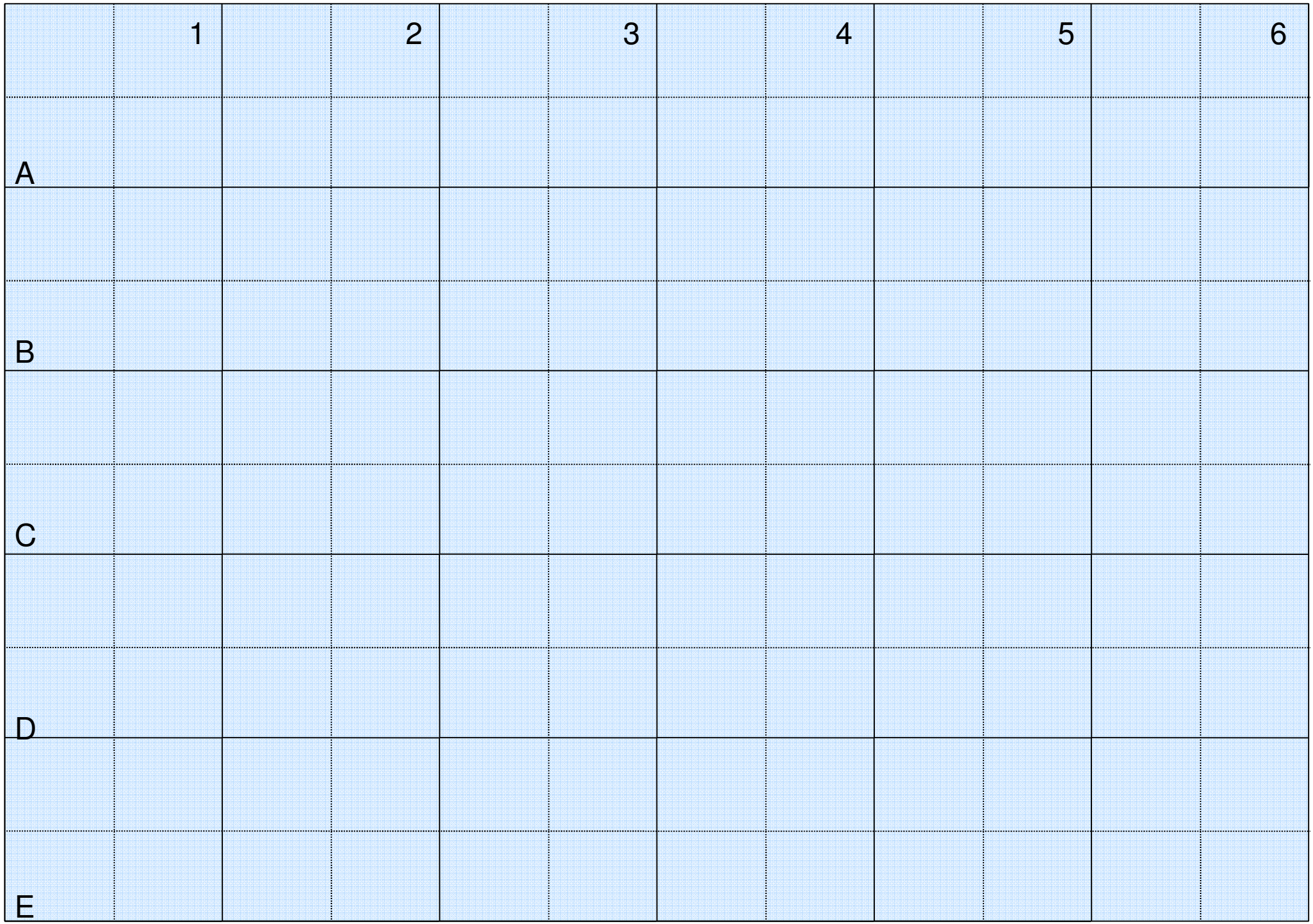


Figure 1: left: original scan of a gel; right: optimized scan of a gel using 'Chart-White balance'.

6. Use ‘Polygon selection’ Tool to determine the outlines of the precipitation ring.
7. Measure the area of the precipitation ring: ‘*Analyze -> Measure*’
8. Note precipitation ring area.
9. Calculate calibration curve from the precipitated area caused by the standard solutions. The linear relationship between concentration and area can be used to determine the concentration of tannins in the extracts. Correct the precipitation area for the ***Blank***, if necessary.


a)
4mm
190mm
260mm
b)
1.7mm
bottom plate
spacer
top plate


1
2
3
4
5
6
A
B
C
D
E