

# **Ecologically diverse and distinct neighbourhoods trigger persistent phenotypic consequences, and amine metabolic profiling detects them**

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***Running headline.*** Neighbourhoods persistently change metabolites

## **Summary**

- Global change triggers rapid alterations in the composition and diversity of plant communities which may change ecosystem functioning. Do changes in community diversity also change traits persistently, i.e. does coexistence with numerous or functionally or phylogenetically distinct species trigger, in a given focal species, trait shifts that persist?
- We studied the grass *Dactylis glomerata*. *Dactylis* was grown in experimental plots with different species compositions for five years, sampled, cloned and grown in a common garden. We studied amines, regulators integrating growth responses of organisms to their environment.
- We found that the mean levels and variances of most amines depended on the diversity of the source community, notably the species richness and the phylogenetic and functional distinctness from *Dactylis*, unbiased by species identity or biomass shifts.
- *Synthesis*. Our results suggest that different levels of ambient diversity can, within a few years, select for different genotypes which have different compositions of growth regulators. Our study also suggests that a plant species can evolve in response to the diversity or distinctness of the surrounding plant community. Evolutionary changes of plant phenotypes might mediate an impact of past biological diversity on present ecosystem functioning.

**Key-words.** Community phylogeny; common garden experiment; determinants of plant community diversity and structure; Jena experiment; metabolome; microevolution; phenotypic response; species richness; trait shift.

## Introduction

Global change currently triggers rapid alterations in the composition and diversity of plant communities worldwide. Community composition is a selective environment and evolutionary change in response to particular coexisting species has been demonstrated repeatedly (Lack 1942; Thompson 2005). In particular in plants, it is increasingly acknowledged that changes in plant community composition can trigger changes in phenotypes of incumbent plant species with possible consequences on ecosystem functioning (Bolker *et al.* 2003). Up to now, studies have focused mostly on the idiosyncratic effects of one given plant species on the phenotype of another species (Aarssen & Turkington 1985) or on the relationship between species diversity in a community and genotypic diversity in a focal species (Vellend & Geber 2005). However, there is little evidence as to whether plant community composition and diversity *per se* (rather than a particular neighbouring species) affect the phenotypes (rather than the genetic diversity) of a given focal species. Plant community structure encompasses the diversity of species neighbouring a target species and

their distinctness from the target species. Phylogenetic distinctness of neighbours, for instance, might decrease competition and the impact of shared pathogens (e.g. Yguel *et al.* 2011) but might in particular situations also provide less facilitative support (Burns & Strauss 2011). Also, neighbouring species belonging to the same functional group as a focal species may increase competition and enemy pressure while many species from another, complementary group may decrease these pressures (MacArthur & Levins 1967), but see Yan *et al.* (2012). Finally, the combination of multiple species and multiple functional groups may have non-additive effects on phenotypes (Vellend & Geber 2005). Thus the distinctness and diversity of neighbours may impose multiple selection pressures on the individuals of a given species, whilst the above negative or positive interactions may trigger persistent growth responses in particular plants (Bolker *et al.* 2003).

Community composition may not only change mean phenotypes of a given species but also their variation. In particular, diverse or distinct neighbourhoods may increase variation by increasing the diversity of possible interactions (Navarro, Ayensa & Guitian 2007). However, an increasing number of possible interactions might also result in a diffuse and simultaneous interaction of a focal species with many others so that the only solution is particularly average phenotypes in all individuals, i.e. low persistent intra-population phenotypic variability, permitting each individual of the focal species to interact with all species (Navarro, Ayensa & Guitian 2007).

The few existing studies on the effect of neighbourhood diversity on plant phenotypes, to our knowledge, sampled plants *in situ* (e.g. Scherling *et al.* 2010; Walter *et al.* 2012). Characterizations of phenotypes may hence strongly reflect instantaneous plastic and reversible responses of the plants to neighbourhoods. As far as we are aware there have been no tests of whether the diversity and distinctness of the neighbourhood implies that there will be persistent, evolutionary responses in a focal plant species. We pragmatically define evolutionary responses of a focal plant species as a change in characters that is maintained even after plants are reduced to clones of standard size, acclimatized for several years in a standard common-garden environment and then traits are measured on newly-grown ramets. The acclimatization in the common garden excludes reversible plastic responses and permits the identification of persistent responses, i.e. irreversible and often heritable plastic changes of individuals (e.g. Nussey *et al.* 2005) or selection among individuals based on their epigenetic or genetic characters. Such persistent, evolutionary responses would be of major importance as they translate the diversity or distinctness of a neighbourhood irreversibly into characters of a plant population. Even after change of a community neighbourhood (as occurs frequently in nature) a plant population might still carry the signature of the initial neighbourhood.

Metabolites are known to being central in the functional responses of plants to the environment. Their levels are viewed as “the ultimate response of biological systems

to genetic or environmental change” (Fiehn 2002). Therefore, metabolites should play a prime role in the responses of populations to community diversity and distinctness. However, the impacts of community composition on the metabolite composition of a target species have so far been little addressed (but see Scherling *et al.* 2010; Mraja *et al.* 2011; Walter *et al.* 2012). These studies did not aim to identify persistent responses to community neighbourhood but sampled plants *in situ* which might show a particularly strong signal of reversible plasticity (which is very high in metabolites – e.g. Schweitzer *et al.* 2008). Among metabolites, amines are particularly interesting as these compounds are set at an interface between stress and developmental response in plants e.g. Hummel *et al.* (2004). Therefore, amines are well suited to integrate growth and stress responses to different neighbourhoods. Amine compounds are conveniently divided into aliphatic di- and polyamines and aromatic amines (Table 1). Polyamines (PAs) are small polycationic molecules bearing amino groups. Diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm) are the most common PAs found in higher organisms. The diamine agmatine is an intermediate in Put synthesis *via* arginine-decarboxylase (Tiburcio, Kaur-Sawhney & Galston 1990). Triamine (Spd) and tetraamine (Spm) are synthesized by transfer of aminopropyl residues to respectively Put and Spd (Tiburcio *et al.* 2014). Less widespread than Put, the diamine cadaverine is synthesized through the decarboxylation of lysine (Bouchereau *et al.* 1999). Finally, the diamine 1,3-diaminopropane is a product of catabolic oxidation of Spd and Spm (Martin-Tanguy 2001). Our study also included

acetylated polyamines. There is some evidence for the occurrence of the polyamine acetylation pathway in plants (Hennion, Frenot & Martin-Tanguy 2006) and references therein). Through this pathway in animals and bacteria, Put, Spd and Spm are acetylated by acetyltransferases to produce *N*-acetylputrescine (NAcPut), *N*<sup>1</sup>- and *N*<sup>8</sup>-acetylspermidine (*N*<sup>1</sup>AcSpd, *N*<sup>8</sup>AcSpd) and *N*<sup>1</sup>- and *N*<sup>8</sup>-acetylspermine, respectively. Of special interest is the “interconversion pathway” that converts acetylated Spd and Spm to Put and thus plays a major role in polyamine regulation in animals and bacteria (Seiler 1987). We suggested that this process may also be present in plants given the high flexibility of acetylated compounds in relation with environmental conditions (Hennion, Frenot & Martin-Tanguy 2006). Finally, aromatic amines are monoamines with either an arylalkyl or an indol radical, corresponding respectively to phenylalkylamines ie “*phenethylamines*”, or indolalkylamines ie “*tryptamines*”, both widespread in plants (Table 1). Phenethylamine *sensu stricto* is formed by decarboxylation of phenylalanine and tyramine by decarboxylation of tyrosine (Smith 1980; Facchini, Huber-Allanach & Tari 2000). Dopamine is synthesized *via* two equally active routes in plants (Table 1) (Kulma & Szopa 2007). Octopamine has been found in a number of plants (Smith 1977; Hennion & Martin-Tanguy 2000; Szopa *et al.* 2001; Gicquiaud, Hennion & Esnault 2002; Lee *et al.* 2009) where it is formed by hydroxylation of tyramine (Smith 1980; Matsuda *et al.* 2005). For indol amines, tryptamine is formed by decarboxylation of tryptophan and is widespread in plants (Smith 1980; De Luca 2000). Serotonin is formed *via* the decarboxylation of tryptophan followed by hydroxylation of tryptamine (De Luca

2000) and is also widespread in plants.

The aliphatic amines agmatine, putrescine, spermidine and spermine are called “growth regulators” because they are implicated in many developmental processes in plants (Tiburcio *et al.* 2014). Among these, putrescine, spermidine and spermine have been shown to be essential to cell viability (Tiburcio *et al.* 2014). Free polyamines and possibly also their acetyl conjugates are involved in plant response to abiotic stresses and some polyamines have demonstrated roles in plant tolerance to many abiotic stresses (Table 1; Hennion & Martin-Tanguy 2000; Hennion, Frenot & Martin-Tanguy 2006; Shevyakova *et al.* 2013; Minocha, Majumdar & Minocha 2014). Furthermore, putrescine, spermidine, spermine and cadaverine are involved in plant defense against aphids (Sempruch *et al.* 2012). Di- and polyamines are also precursors for alkaloid defense compounds against predators (Martin-Tanguy 2001), although these compounds are present only at low levels in *Dactylis glomerata* (Villalba *et al.* 2011). Hydroxycinnamoyl conjugates of agmatine are antifungal in several Poaceae (Batchu *et al.* 2006). The physiological roles of aromatic amines remain less well understood in plants, however some play roles in plant development and growth (Christou & Barton 1989; Kulma & Szopa 2007; Pelagio-Flores *et al.* 2011). Furthermore, many aromatic amines were found to respond to abiotic stresses (Dufeu, Martin-Tanguy & Hennion 2003; Hennion *et al.* 2012; Mukherjee *et al.* 2014). Finally, phenethylamines and tryptamines are precursors of hydroxycinnamoyl conjugates involved in plant defense against wounding and



pathogens (Facchini, Yu & Penzes-Yost 1999; Facchini, Hagel & Zulak 2002; Matsuda *et al.* 2005; Kulma & Szopa 2007; Ishihara *et al.* 2008) as well as allelopathy (Cutillo *et al.* 2003; Thi *et al.* 2014) and both phenethylamines and tryptamines are precursors for alkaloid defense compounds (Smith 1980; Facchini, Huber-Allanach & Tari 2000). Intraspecific differences in amine (polyamine and aromatic) composition were found between plant populations subject to different environments in nature (Gicquiaud, Hennion & Esnault 2002; Hennion, Frenot & Martin-Tanguy 2006; Hennion *et al.* 2012) and were shown to be heritable (Gicquiaud, Hennion & Esnault 2002). Overall, amine composition may be suitable to detect the evolutionary, i.e. persistent response of plant populations to other, biotic conditions such as for instance distinctness and diversity of the neighbouring community.

We tested the evolutionary responses of the amine composition, taken as metabolite phenotype, of a grass species (*Dactylis glomerata*) to diversity and distinctness of their community neighbourhood. We used an experimental approach exposing the same cultivars of *D. glomerata* for five years to neighbourhoods (“source communities”), varying in phylogenetic and functional distinctness from *D. glomerata* and in diversity of species, grasses and legumes. Individuals from these source neighbourhoods were sampled, cloned and grown in a common garden for three years and biochemically phenotyped. Means and variances for each source community were then regressed against the distinctness and diversity of the source communities. Overall we find robust evidence that the functional and evolutionary

structure of a community changes the amine metabolome of a plant species strongly, in multiple ways and that persist in the long term after cloning and cultivation in a common garden.

## **Materials and methods**

### STUDY SPECIES AND OVERVIEW OF STUDY DESIGN

We studied the grass *Dactylis glomerata* (cock's-foot or orchard grass), a perennial C<sub>3</sub> of high forage quality, cross-pollinated, and presenting a broad genetic variance (Casler *et al.* 2000; Xie *et al.* 2014). *D. glomerata* grows continuously throughout the year. Therefore, well-performing genotypes can rapidly replace less well-performing genotypes within months or a few years. Thus, fitter genotypes should represent a rapidly increasing share of a population even without sexual reproduction, only through vegetative growth.

Testing the evolutionary responses of the amine composition of *D. glomerata* to diversity and distinctness of their community neighbourhood is a regression approach: variation among species combinations representing different levels of distinctness or diversity is compared to variation among species combinations representing the same levels of distinctness or diversity. Replicates in the analysis are hence the different species combinations and not the different plots or plants from a given species combination. We used an experimental approach exposing the

same cultivars of *D. glomerata* to 25 different neighbourhoods (“source communities”), varying in phylogenetic and functional distinctness from *D. glomerata* and in diversity (species richness, richness within the most similar and the most different functional groups, grasses and legumes respectively). After five years we randomly sampled *D. glomerata* individuals in the plots, cloned them and grew the clones in a common garden for three years to ensure their physiological acclimatization to that common environment. We biochemically phenotyped each individual, calculated means and variances for each source community and related these metabolite profiles to the composition (distinctness and diversity) of the source communities. *D. glomerata* may respond to a given interspecific neighbourhood not only by individuals changing their metabolome but also by populations increasing in biomass, which in turn may increase intraspecific competition - and thereby again metabolomic profiles. To account for such confounding population-level effects we included responses of population biomass to community neighbourhood in our analyses. We also accounted for possible effects of the identity of species in the source community. We finally repeated our analyses for a classical phenotypic parameter in chemical analyses of plants, C/N ratios of the foliage.

## EXPERIMENTAL DESIGN

We used the so-called “dominance experiment” within the Jena Diversity

Experiment described in Roscher et al. (2004). This experimental design was especially designed to display the effects of species richness on phenotypic changes of a focus species, and to manipulate species richness and species identity fully independently. The initial composition of community has been controlled and the plot conditions known over the time. The plots (3.5 m × 3.5 m) were sown from 11–16 May 2002. Eight species, besides *Dactylis*, were sown: the herbs *Anthriscus sylvestris* and *Geranium pratense*, the legumes *Trifolium pratense* and *T. repens*, and the grasses *Alopecurus pratensis*, *Arrhenatherum elatius*, *Phleum pratense* and *Poa trivialis*. Numbers of species neighbouring *Dactylis* varied from zero to eight. Numbers of seeds were identical among species, so that increased richness of a group corresponds also to increased abundance. Numbers of Poaceae species ranged from zero to four; numbers of leguminous species from zero to two. Numbers of functional groups in addition to Poaceae ranged from zero to two. Mean phylogenetic distance of *Dactylis* from its respective neighbours ranged between 0 - 191 million years ( $2 \times$  age of the common ancestor of two species, averaged across all pairs of species, inferred from a recent, highly resolved phylogeny from Bartish in Hermant *et al.* (2012), see Appendix S2). In total 25 species combinations were studied (Appendix S1). All species mixtures were replicated once.

Seeds for all species were obtained from a commercial supplier (Rieger-Hofmann GmbH) using nearest possible origins. The plots were sown in May 2002 with 1000 viable seeds per square meter in a replacement design. Eight weeks after sowing all

plots were mown to a height of 10 cm and the biomass was removed in order to support the establishment of the sown species and to remove annual weeds. A second mowing was carried out mid-September. In the following years plots were hand weeded for non-sown species in April and June and mown twice, in early June and September. No fertilizer was applied to the field. Aboveground community biomass was harvested twice a year shortly before mowing on all experimental plots. The first harvest was at peak biomass in late May and the second in late August. In each plot two rectangles of 0.2 x 0.5 m were clipped at 3 cm above ground level. The position of the rectangles was chosen by random selection of coordinates in the 3 x 3 m core area of the plot. The harvested biomass was sorted into species sown at the particular plot and dried at 70 °C for at least 48 h before weighing. For further analyses we used the mean biomass of the two samples taken per plot.

#### SAMPLING AND CLONING

Plots were sampled in October 2006. Given our hypothesis, replicates correspond to different species compositions: variance is compared between species compositions of increasing diversity/distinctness to species compositions of the same diversity/distinctness. We hence sampled 25 different species compositions (Appendix S1). For most species compositions we sampled 4 plants (mean 3.6), i.e. 2 plants randomly sampled from each of 48 plots (Appendix S1). Multiple plants within each of the replicate species-compositions permitted to calculate for each species

composition a mean and a standard deviation. Means more precisely represent a given species composition than a single plant, and more precise data points permit more powerful analyses. Our analyses yielded numerous significant relationships indicating that it does not lack power and the number of plants used to characterise a given data point was sufficiently large. In order to be sure to sample different plant individuals (not tillers from the same individual), we sampled the tillers at a distance of 0.4 meter. *D. glomerata* has a caespitose, tuft-like growth form and in our experiment the radius of an individual did not exceed a few centimeters. In total, 91 genotypes were sampled and transplanted in sand in a greenhouse at INRA in Lusignan, SW France. Each of these 91 individuals was cloned in March 2007 and each clone (replication) was planted in a common garden (field trials and spaced plants) at INRA in Lusignan in May 2007.

#### PLANT COLLECTION TO STUDY AMINE COMPOSITION

Plants were collected on the 27<sup>th</sup> and 28<sup>th</sup> April 2009 from the common garden in Lusignan. The entire sampling was performed during a short, two-day period so as to avoid variation in amine contents due to seasonal differences (F. Hennion unpublished data). Plants were sampled between 10.30 am and 17.00 pm to avoid bias from daily variation in amine metabolism (Tiburcio, Kaur-Sawhney & Galston 1990; Fujihara & Yoneyama 2001). Because amine composition may vary also within a given plant at a given moment between leaves of different developmental stages

(Foster & Walters 1991; Fujihara & Yoneyama 2001), we sampled an appropriate and constant leaf developmental stage, i.e., young developing, fully photosynthetic leaves (Hennion, Frenot & Martin-Tanguy 2006; Hennion *et al.* 2012). Two to four subapical leaves were hence sampled from each individual plant. The samples were frozen in liquid nitrogen and stored at -20°C then lyophilized and ground to powder.

#### DETERMINATION OF FREE AMINES AND ACETYLATED POLYAMINES

In this study, we considered only soluble, i.e., free and acetyl-conjugated amines, and not insoluble amines, i.e., either conjugated to phenolic acids or covalently bound to macromolecules. Because the synthesis of conjugated or bound amines involves free amine compounds (Martin-Tanguy 1997), we considered the free amine pool to be a good signature of the entire amine metabolism. Thirty mg of powdered samples were thoroughly mixed with 400  $\mu$ L of 1 mmol/L HCl supplemented with 10  $\mu$ mol/L heptanediamine, as an internal standard, on a magnetic stirring plate (2000 rpm) for 1 h at 4°C. The homogenates were then centrifuged for 15 min at 10 000 *g* at 4°C, and the supernatants collected. The pellets were further extracted twice with 300  $\mu$ L of 1 mmol/L HCL and 10  $\mu$ mol/L heptanediamine. After short stirring, the homogenates were centrifuged for 15 min at 10 000 *g* at 4°C. The combined supernatants were used as the crude extracts for characterization and determination of free and acetylated amines and polyamines and stored frozen at -20°C before chromatographic analyses. HPLC and fluorescence

spectrophotometry were used to separate and quantify amines (in  $\mu\text{mol.g}^{-1}$  dry weight) prepared as their dansyl derivatives as described in Hennion et al. (2012).

#### DETERMINATION OF C/N RATIOS

C and N contents were analyzed on dried material (80°C, 48h) of leaves showing no signs of senescence, using Perkin Elmer CHN PE 2400 (Perkin Elmer, Massachusetts) according to standard protocols (Cornelissen *et al.* 2003).

#### DATA ANALYSIS

We calculated means and standard deviations across the plants sampled from a given species combination. Each species combination was hence a single data point, representing one level of neighbourhood diversity or distinctness; different species combinations are true replicates of neighbourhood diversities or distinctnesses (repetitions of the same species combination would be pseudoreplicates and are hence averaged into a single value). We then used multiple regression analysis to explain the mean or the variance of the level of a given amine (or of C/N ratio) by the source neighbourhood: its functional and phylogenetic distinctness, species richness and richness in legumes and grasses. Such a parallel consideration of all variables in the same model is important to account for the partial redundancy among these variables (mean pairwise relationship  $R^2$  0.33) and permitted to distinguish their



respective effects. (We considered using legume/grass ratios instead of species richnesses of each group. However this implies opposing effects of legumes and of grasses, but we found that often only either of the variables has an effect, or their effects go into the same direction.) We then used best-subset selection to identify the best performing combination of independent variables, and avoid significance estimation for a given variable being obstructed by the presence of other less important but colinear variables in the same model. As a criterion for subset choice we used adjusted  $R^2$ , belonging to the same family as Akaike or Mallows criterion, maximizing the explained variance while accounting for the numbers of independent variables rather than minimizing the number of independent variables. We used residual plots to verify normality and homogeneity of variance and if necessary excluded (up to 2, on average 0.5) outliers that strongly biased the respective analysis. (We cannot say why these respective points were statistical outliers. However, experience shows that our measurements are stable so metabolomic data are robust. An outlying point might hence reflect a particularity of the clones arising from a given species composition, not a measurement error.) We rerun these analyses including the proportional biomass change of the *D. glomerata* source population in the respective neighbourhood. Proportional biomass change was calculated as the natural logarithm of the ratio of final to initial biomass of *D. glomerata*. An increase in biomass of *D. glomerata* populations in a given community neighbourhood might increase intraspecific competition and the observed biochemical response might rather reflect intraspecific competition than favourable

interspecific neighbourhood (e.g. Petermann *et al.* 2008). Increase of intraspecific competition can hence be partly accounted for by including proportional biomass change as a covariable, and the remaining variables would then better capture the true effect of interspecific neighbourhood. We moreover related each of the dependent variables (means and SDs of individual amines, total amines and C/N) to the presence and absence of each of the neighbouring species in univariate ANOVA. Neighbouring species that had a significant effect were included as additional covariables in the above analyses. Such effects of particular neighbour species on phenotypes of a focal species have long been described (Introduction) and should be filtered out when identifying the general effect of diversity or distinctness of neighbour species. All analyses were done on Statsoft 10 (2011).

## Results

### FREE AMINE PROFILES OF *DACTYLIS GLOMERATA*

Fifteen different compounds were detected and measured. They belonged to three biochemical categories: aliphatic amines, and their acetyl conjugates; arylalkylamines; and indolalkylamines. Amine and polyamine levels in *D. glomerata* were in the expected order of magnitude of levels of these compounds in Poaceae (Gicquiaud, Hennion & Esnault 2002; Hennion *et al.* 2012). For aliphatic amines agmatine, 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine were widely represented in the plants. Among acetyl conjugates, *N*<sup>1</sup>-acetylspermine

and, rarely, *N*-acetylputrescine were present. The arylalkylamines octopamine, 3-methoxy-4-hydroxy phenylethylamine and tyramine were always present, whereas phenylethylamine and dopamine were more variously present. The indolalkylamine serotonin was present in nearly all samples whereas tryptamine was more infrequent.

#### EFFECTS OF NEIGHBOURHOOD DIVERSITY AND DISTINCTNESS ON AMINE COMPOSITION IN *DACTYLIS GLOMERATA*

##### Mean amine levels

Overall, the diversity and distinctness of the initial neighbourhood persistently controls amine, and C/N compositions even after transfer to a common garden. Mean values of seven out of the 15 amines showed significant relationships to neighbourhood diversity and distinctness, and three further amines showed marginally significant relationships (Appendix S3: Tyr, Agm, NAcPut, Put, Oct, Spm, and Phe, Ser, Spd). All of these relationships were confirmed after including biomass ratios to account for population-level responses to neighbourhood diversity and distinctness (Appendix S3). Among the 15 amines studied only five amines (as well as C/N) corresponded, in simple ANOVA, to the presence or absence of particular species in the neighbourhood. Including these species into the corresponding multiple regression analyses on the effect of diversity and distinctness of the neighbourhood changed six of the results (two relationships disappeared, 3

appeared and two reversed sign) but did not affect six other relationships (Appendix S3). We will focus on these most complete analyses that account for biomass ratios and identity of neighbouring species (Table 2a). A given diversity or distinctness was often related positively to the levels of some amines and negatively to others, emphasizing the power of studying the changes in levels of each amine and not restricting to the total level of amines. Increased richness of functional groups other than Poaceae and increased numbers of Poaceae species corresponded to decreases of levels for all amines. Total amine levels declined with increasing functional group richness. Total amine levels also declined with legume-species richness but increased with total numbers of species. In Fig. 1 we illustrate the relationship between diversity and distinctness of the neighbourhood and the amine most strongly affected by neighbourhood diversity or distinctness, i.e. Agm. We note that neighbourhood diversities or distinctnesses also were related to foliage C/N ratios. Notably C/N ratios decreased with species richness of the neighbourhood. However, adjusted  $R^2$  values were lower than those for many of the amines (Table 2a).

#### Variability of amine levels

The standard deviations of amines and C/N were persistently controlled by the diversity and distinctness of the initial neighbourhood even after transfer to a common garden. Standard deviations of six out of the 15 amines showed significant relationships to neighbourhood diversity and distinctness and two further showed

marginally significant relationships (Table 2b, Agm, Phe, DAP, Cad, Oct, Dop, and N<sup>1</sup>AcSpm, Spm). All of these relationships were confirmed after including biomass ratios to account for population-level responses to neighbourhood (Appendix S3). Among the 15 amines studied variances of only five corresponded, in simple ANOVA, to the presence or absence of particular species in the neighbourhood. Including these species into the corresponding multiple regression analyses changed five of the relationships to diversity and distinctness of the neighbourhood (two disappeared, two appeared and one reversed sign) but did not affect one other relationship (Appendix S3, Table 2b). The overall conclusion that the diversity and distinctness of the initial neighbourhood controls persistently variance in amine and C/N compositions even after transfer to a common garden remains unchanged. Shifts in variances were independent of shifts in means, given that the latter had been accounted for as co-variables. Note that most of the compounds without significant relationships to initial neighbourhood had very low levels (below an average of 0.05  $\mu\text{mol. g}^{-1}$  dry weight), strongly limiting the power to detect effects on variability due to many zero values. We also observed no effect of neighbourhood diversity and distinctness on the variance of total amines, which is not surprising given the limited effects of neighbourhood diversity and distinctness on variance of most of the major amines. In Fig. 2 we illustrate the relationship between diversity and distinctness of the neighbourhood and the amine whose standard deviations were most strongly affected by neighbourhood diversity or distinctness, i.e. Phe. While relationships of neighbourhood to amine variance were not always significant,

they still yielded a higher adjusted  $R^2$  stronger than the relationships to C/N variation for ten of the amines (Table 2b, including biomass ratios as co-variables).

## **Discussion**

In the present study we tested for persistent changes of plant phenotypes to the distinctness and diversity of its neighbourhood, i.e. changes that persisted during the production of standard-sized clones, the acclimatization for years in a common garden, and the establishment of new tillers. To our knowledge this question has not been asked before. To answer the question we used amine screening, which to our knowledge has not been applied before to detect imprints of the community neighbourhood. Twelve out of the 15 amines persistently responded to neighbourhood in terms of either means (four), variances (two), or both (six). Amines responded to the overall richnesses of all species, of Poaceae, of legumes, to phylogenetic distance and to numbers of functional groups. Some of the amine compounds showed stronger responses than total amine levels or the widely-used C/N ratios, indicating the power of amine screening approach compared to the C/N trait frequently used to functionally characterize plants. These overall conclusions were robust to different models including different sets of variables.

The present study may have a number of shortcomings. First, as stated in Methods,

we only considered soluble, *i.e.* free and acetyl conjugated amines, and not insoluble amines, notably amines conjugated to hydroxycinnamic acids. As the synthesis of conjugated amines involves free amine compounds (Martin-Tanguy 1997), we considered the free amine pool to be a good indicator of the entire amine metabolism. Second, the study is based on only a single species, the grass *D. glomerata*. Other species might respond differently, notably dicot species (Cahill *et al.* 2008, see below). The precise results hence risk being specific to *D. glomerata* – but we suspect that the overall phenomenon of persistent evolutionary responses to diversity and distinctness of the neighbourhood may occur in many other species. Our study has developed powerful metabolomic tools to test for similar effects in other species in the future. Third, results on individual-level amine-responses may be biased by changes in population-level responses of total biomass: reduction in population size might trigger bottleneck effects (Nestmann *et al.* 2011) but also a release from intraspecific competition. However, we had at least partly controlled for such biases by including, as covariable, ratios of population biomasses. Fourth, results might be biased by a sampling effect: a particular species being more likely to be present in a species combination of highest species richnesses than under lower species richness. However, we found strong species-richness effects already between lowest and intermediate richnesses (Figs. 1 and 2). Moreover, we had controlled our analyses for the presence of species that affected a given amine. Finally, the limited number of plants sampled per species combination may have introduced noise (Methods). However noise can only explain absence of signal, while

we found significant and robust signals. We acknowledge however that separating the highly correlated explanatory variables may be to some degree contingent on the species pool considered and should be repeated with other species pools elsewhere.

In our study changes in neighbourhood diversity triggered either increases or decreases in amine levels, depending on the type of diversity considered. High species richness increased total amines whereas high richness of functional groups, of legumes or of Poaceae decreased total amines. Given the evidence listed in the Introduction that increases of amines often respond to increased abiotic stress we suggest that stressful neighbouring environments for *D. glomerata* were characterized by high total numbers of species, but this effect was reduced by richness of functional groups or of legumes and Poaceae. In fact it has been repeatedly demonstrated that presence of legumes and hence N<sub>2</sub>-fixation can decrease nutrient stress of non-legumes like *Dactylis* (Bedoussac & Justes 2010). Also, Cahill et al. (2008) suggest that monocots like *D. glomerata* are less stressed in the presence of other Poaceae species than in the presence of dicots. Finally, a high number of functional groups distinct from *D. glomerata* may increase complementarity between *D. glomerata* and its neighbours and hence decrease competition for resources (limiting similarity, e.g. Schwillk & Ackerly 2005). Conversely, an increase in species richness may, everything else being equal, result



in a more complete occupation of ambient niche space and hence a stronger limitation of resources for any given species.

Among polyamines, agmatine was the polyamine for which mean levels were most strongly affected by parental neighbourhood. The acetyl conjugate of putrescine *N*-acetylputrescine responded less but in an overall similar way. Agmatine is a growth regulator that is particularly involved in root formation and growth (Davis 1997; Hummel *et al.* 2002), and that correlates to increased cold tolerance (Paldi *et al.* 1998; Hennion & Martin-Tanguy 2000) and possibly to tolerance to Na<sup>+</sup> and Ca<sup>2+</sup> (Aziz, Martin-Tanguy & Larher 1999). Similarly, *N*-acetylputrescine accumulates under drought and salt stress (Hennion, Frenot & Martin-Tanguy 2006). These physiological functions are consistent with observed response to neighbourhoods: cold tolerance may help *D. glomerata* to advance its phenology which has been demonstrated to provide major competitive advantages to this species (Ross & Harper 1972). A neighbourhood with high species richness (for a given level of other neighbourhood variables) might impose such competition which would also result in higher competition for water. Therefore, both increases in agmatine (stimulating root growth) and in *N*-acetylputrescine (increasing under water stress) are consistent with our above hypotheses on the competition pressure imposed by a species-rich neighbourhood and the corresponding avoidance strategies. Interestingly, serotonin, the only other amine having demonstrated roles specifically in root growth (Pelagio-

Flores *et al.* 2011), shows a similar response to agmatine. Inversely, neighbourhoods composed of closely related species (i.e. other Poaceae), of *N*-fixers (i.e. legumes) or functionally different species might exert particularly little competition pressure (Cahill *et al.* 2008; Temperton *et al.* 2007; and Best, Caulk & Stachowicz 2013, respectively). Consistently, we find reduced levels of agmatine and of *N*-acetylputrescine.

Among amines, octopamine had a strong response to various neighbourhoods, and this response was exactly the same as for agmatine. Whereas polyamines have direct roles in growth, aromatic amines are more known as being directly involved in chemical interspecific interactions (Table 1). Octopamine belongs to the phenethylamines group within which particular hydroxycinnamoyl conjugates have been demonstrated to be allelopathic (Cutillo *et al.* 2003; Thi *et al.* 2014). Therefore, octopamine may help plants to defend themselves in a highly competitive neighbourhood, equivalent to agmatine which permitted to avoid competitors through early or deep growth. Furthermore, all phenethylamines, including octopamine, are precursors of hydroxycinnamoyl conjugates that are involved in plant defense against pathogens and of which synthesis is activated by infections, namely by fungi (Matsuda *et al.* 2005; Kulma & Szopa 2007). However, this antipathogen function appears particularly useful in neighbourhoods where octopamine levels are particularly low. Notably in a neighbourhood composed of

many grass species the grass *D. glomerata* might suffer strong attacks by fungal pathogens (Parker *et al.* 2015). A possible explanation for the low levels of free octopamine might be that the pool of these amines is depleted by intense biosynthesis of their conjugates (Burtin *et al.* 1990). Besides, attack by fungal pathogens may not only be favored by Poaceae-rich neighbours sharing the same pathogens, but also by highly competitive neighbourhoods, i.e. such poor in Poaceae or legumes or rich in species as explained above: shading by competitors on one hand, and early phenology in response to competitors on the other hand may expose *D. glomerata* to a moister microclimate where fungal pathogens are more abundant and powerful, rendering the antifungal properties useful where competition is high. These speculations are consistent with the observed increases of octopamine. Tyramine shows a similar response as octopamine to Poaceae possibly due to the same reasons as tyramine is also involved in plant defense against pathogens both as free form (Facchini, Yu & Penzes-Yost 1999) and conjugated forms (Matsuda *et al.* 2005; Kulma & Szopa 2007). Besides, the negative response of tyramine to phylogenetic distance may reflect a lower share of pathogens with distantly related species beyond Poaceae.

Even with constant mean levels, amines changed in variation in response to diversity and distinctness of the neighbourhood. Decreases of variation were observed in response to presence of many non-grass functional groups (phenethylamine,

cadaverine, dopamine, C/N ratios, and, in tendency, spermine and total amines) and to many Poaceae species (phenethylamine). Such a decline in variation of a given trait with diversity of functional groups may be due to a bottleneck after decline in population size, but we had roughly accounted for population decline by including biomass ratios. Decline in phenotypic variation may also be explained by niche preemption, i.e. available functional roles being occupied by different species instead of different genotypes and phenotypes within a given species (Vellend & Geber 2005; Silvertown, Biss & Freeland 2009). The number of functional roles occupied by species other than *D. glomerata* (rather than by different genotypes within *D. glomerata*) may indeed increase with the number of functional groups different from *D. glomerata*, and occasionally the number of species within its own group.

We also observed amine variation that increased with neighbourhood diversity and distinctness, notably with (i) total species number for phenethylamine, 1,3-diaminopropane, dopamine, spermine, cadaverine and octopamine, (ii) phylogenetic distance for cadaverine, octopamine, and C/N, and (iii) Poaceae richness for *N*<sup>1</sup>-acetylspermine, and C/N (marginally significant trends included). Increase in phenotypic variance may reflect increased interaction heterogeneity (models in Vellend & Geber 2005). First, within a focal species, a given phenotype may succeed best in the interaction with a given neighbouring species, and numerous

neighbouring species would select for numerous phenotypes (Vellend & Geber 2005, focusing on genetic diversity). Second, within the focal species the same phenotype may succeed in interacting with conspecifics and with closely related neighbouring species, but interaction with distantly related neighbours may require a distinct phenotype, increasing the phenotypic variation within the focal population.

Responses of amines to the neighbourhood were highly persistent. They might therefore reflect either irreversible developmental plasticity of all individuals in a population, or natural selection among individuals on genetically or epigenetically fixed characters. However, even developmental plasticity is truly irreversible only within a plant module developed at a given moment. After cloning standard-sized offspring and growing them in a common garden for years, numerous new modules and even ramets have developed. Moreover, the traits we studied are not rigidly maintained like a growth form, but undergo hourly fluctuations (Tiburcio, Kaur-Sawhney & Galston 1990; Fujihara & Yoneyama 2001). Overall, developmental plasticity in the source environment might quickly fade out. This leaves natural selection among individuals on (epi) genetically fixed characters as a major explanation for the patterns of amine variation that we found. In fact, differential growth and death rates of plants in the source community have the potential to quickly change the presence and dominance of plants with such heritable characters in a population. To further evidence such changes, future studies should test

whether changes are also maintained through sexual reproduction, as seeds may retain irreversible developmental plasticity less than clones.

## **Conclusions**

We conclude that metabolomic characters of a focal species respond to the functional and phylogenetic distinctness and diversity of the surrounding community. The responses suggest that a functionally diverse and phylogenetically distinct ambient plant community results in a decrease in physiological stress, and increase in niche pre-emption and interaction diversity. Metabolomic responses persist far beyond the initial community and into new modules and individuals, suggesting a role for natural selection. Metabolomes, in turn, are known to control ecosystem functioning (Iason, Dicke & Hartley 2012). Our results therefore suggest that ecosystem functioning may not only depend on biodiversity of its present community. It may depend also on past diversity, which may be very different from the present one, with the effect of past diversity on present ecosystem functioning being mediated via an evolutionary change of plant metabolomes.

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### **Data accessibility**

Species compositions of source neighbourhoods: uploaded as Supporting Information. Methods for quantifying phylogenetic distances: uploaded as Supporting Information. Data on source neighbourhood and amine composition and contents: archived as an excel file “data for Hennion et al JEcology-2015-0385” on Dryad.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Species compositions considered.

**Appendix S2.** Quantifying phylogenetic distances.

**Appendix S3.** Full set of analyses conducted.

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**Table 1.** Amine compounds that were detected in our study: biochemical categories, metabolic origins, and known roles in plant physiology. The abbreviations used for the amines in the text are given. DOPA: dihydroxyphenylalanine.

<i>Biochemical category</i>	<i>Pathway</i>	<i>Amine</i>	
<i>Aliphatic di- and polyamines</i>	<b>Arginine decarboxylase</b>	Agmatine (Agm)	Growth regulator, root growth (Hu Accumulation or high levels involv Conjugates are antifungal in Poace
	<b>Arginine and/or ornithine decarboxylase</b>	Putrescine (Put)	Essential growth regulator (Tiburc Increases result in greater toleranc Possible antixenotic activity agains Precursor of alkaloids (Martin-Tan
	<b>Aminopropyltransferase s</b>	Spermidine (Spd)	Essential growth regulator (Tiburc Increases result in greater toleranc Possible antibiotic properties durin
		Spermine (Spm)	Essential growth regulator (Tiburc Increases result in greater toleranc Possibly involved in induction of lo

	<b>Lysine decarboxylase</b>	Cadaverine (Cad)	Growth regulator (Bouchereau <i>et al.</i> 2002) Accumulation in salt-stressed bean Possible antibiotic properties during senescence Mechanism against aphids (Sempere <i>et al.</i> 2003)
	<b>Spermidine or spermine oxidases</b>	1,3-diaminopropane (DAP)	Growth regulator (Bouchereau <i>et al.</i> 2002) Accumulates in plants subject to stress
<b>Acetylated conjugates of aliphatic di- and polyamines</b>	<b>Acetyltransferases</b>	<i>N</i> -acetylputrescine (NAcPut)	High levels in <i>P. antiscorbutica</i> under stress
		<i>N</i> <sup>1</sup> -acetylspermine (N <sup>1</sup> AcSpm), <i>N</i> <sup>8</sup> -acetylspermine	High levels in <i>P. antiscorbutica</i> under stress
<b>Aromatic amines</b>			
<b>Phenethylamines</b>	<b>Phenylalanine decarboxylase</b>	Phenylethylamine or Phenethylamine (Phe)	Precursor of hydroxycinnamoyl compounds (Facciola <i>et al.</i> 2002) and involved in allelopathy (Facciola <i>et al.</i> 2002)
	<b>Tyrosine decarboxylase</b>	Tyramine (Tyr)	Inhibits growth in plant cell culture small size and/or slow growth (Heilmann <i>et al.</i> 2003) Accumulates in plants subject to stress (Heilmann <i>et al.</i> 2003) Involved in plant defense against viruses Precursor of hydroxycinnamoyl conjugate (Facciola <i>et al.</i> 2002) Involved in allelopathy (Cutillo <i>et al.</i> 2003; Thaler <i>et al.</i> 2003)
	<b>Tyramine hydroxylase</b>	Octopamine (Oct)	Inhibits growth in plant cell culture Accumulates in <i>Bromus</i> plants subject to stress (Matsuda <i>et al.</i> 2005) Precursor of hydroxycinnamoyl compounds (Facciola <i>et al.</i> 2002; Matsuda <i>et al.</i> 2005) and involved in allelopathy (Facciola <i>et al.</i> 2002)
	<b>°) Tyrosine hydroxylation and decarboxylation of DOPA, or °) tyramine hydroxylation</b>	Dopamine (Dop)	Anti-oxidant and promotes growth (Matsuda <i>et al.</i> 2005) High levels correlate with fast growth (Matsuda <i>et al.</i> 2005) Accumulates in <i>P. antiscorbutica</i> under stress (Matsuda <i>et al.</i> 2005) Regulates carbohydrate metabolism (Matsuda <i>et al.</i> 2005) Precursor of hydroxycinnamoyl compounds (Facciola <i>et al.</i> 2007) and involved in allelopathy (Facciola <i>et al.</i> 2007)
<b>Tryptamines</b>	<b>Tryptophan decarboxylase</b>	Tryptamine (Try)	Precursor of indol-acetic acid growth hormone (Facciola <i>et al.</i> 2007) Involved in defense against pathogens (Facciola <i>et al.</i> 2012)
	<b>Tryptamine hydroxylase</b>	Serotonin (Ser)	Involved in several developmental processes (Pelagio-Flores <i>et al.</i> 2003) branching (Pelagio-Flores <i>et al.</i> 2003) Increases in response to senescence (Pelagio-Flores <i>et al.</i> 2003) Involved in defense against fungal pathogens (Pelagio-Flores <i>et al.</i> 2003)

Table 2. Source neighbourhoods affect means (a) and standard deviations (b) of biochemical phenotypes of clones of *Dactylis glomerata* in a common garden. We study total amines, individual amines and C/N-ratios. Models include population-level responses to neighbourhood, i.e. changes in densities of *Dactylis glomerata* (biomass ratios); and presence of species exerting a significant univariate effect on the corresponding dependent variable (given as footnote). Analyses of standard deviations also account for the corresponding mean levels, and do not study NAcPut as it was absent in many individuals

resulting in numerous zero variances. Independent variables excluded by model selection: “excl”, variables included but at  $p>0.1$ : “ns”, direction of variation + or -, variables included at  $P<0.1, 0.05, 0.01, 0.001$ : (\*), \*, \*\*, \*\*\*. Df = error degrees of freedom. Appendix S3 gives the results for all analyses, including those without co-variables. Fig. 1 and 2 illustrate the observed relationships for the amines whose means or standard deviations respond most strongly to source neighbourhoods. Amine abbreviations as in Table 1.

(a) Means

	diversity and distinctness of source neighbourhood				
	phylogenetic distance	number of Poaceae species	number of leguminous species	total species number	number of functional groups
<b>total amines</b>	ns	ns	- *	+ *	- *
<b>Agm</b>	excl	- **	- **	+ **	- *
<b>Put</b>	excl	ns	+ *	excl	excl
<b>Spd</b>	excl	excl	+ (*)	excl	excl
<b>Spm</b>	excl	excl	+ *	excl	excl
<b>DAP</b>	ns	ns	ns	excl	ns
<b>Cad</b>	excl	excl	ns	excl	excl
<b>N<sup>1</sup>AcPut</b>	ns	- (*)	- *	+ *	- *
<b>N<sup>1</sup>AcSpm</b>	ns	excl	excl	excl	excl
<b>Phe</b>	ns	excl	ns	+ (*)	- (*)
<b>Tyr</b>	- *	- *	excl	excl	excl
<b>Oct</b>	excl	- ***	- ***	+ ***	- **
<b>Dop</b>	+ (*)	excl	ns	ns	ns
<b>3M4OHPhe</b>	excl	excl	excl	excl	ns
<b>Try</b>	excl	excl	ns	excl	excl
<b>Ser</b>	- (*)	excl	excl	+ (*)	excl
<b>C/N</b>	ns	excl	excl	- *	ns

Species in source neighbourhood exerting a significant univariate effect and included in the present model: *Anthriscus sylvestris* (positive univariate effect on Agm), *Poa trivialis* (positive univariate effect on Put, Spd, N<sup>1</sup>AcSpm and Oct, negative effect on C/N), *Phleum pratense* (negative univariate effect on N<sup>1</sup>AcSpm and Oct) and *Alopecurus pratensis* (positive univariate effect on Oct).

## (b) Standard deviations

	diversity and distinctness of source neighbourhood				
	phylogenetic distance	number of Poaceae species	number of leguminous species	total species number	number of functional groups
<b>total amines</b>	excl	excl	excl	excl	- (*)
<b>Agm</b>	excl	excl	- *	excl	excl
<b>Put</b>	excl	excl	ns	excl	excl
<b>Spd</b>	excl	excl	ns	excl	excl
<b>Spm</b>	excl	ns	- (*)	+ (*)	- (*)
<b>DAP</b>	excl	excl	excl	- (*)	excl
<b>Cad</b>	+ *	excl	excl	+ *	- **
<b>N<sup>1</sup>AcSpm</b>	excl	+ (*)	ns	excl	excl
<b>Phe</b>	excl	- **	excl	+ **	- **
<b>Tyr</b>	excl	ns	excl	excl	excl
<b>Oct</b>	+*	excl	ns	+ *	ns
<b>Dop</b>	excl	excl	ns	+ **	-**
<b>3M4OHPhe</b>	excl	excl	excl	excl	excl
<b>Try</b>	excl	excl	excl	excl	excl
<b>Ser</b>	excl	excl	excl	excl	excl
<b>C/N</b>	+ **	+ **	ns	excl	- *

Species in source neighbourhood exerting a significant univariate effect and included in the present model: *Phleum pratense* (positive univariate effect on Spd), *Poa trivialis* (positive univariate effect on Spm and N1AcSpm and DAP), *Alopecurus pratensis*, *Geranium pratense* and *Anthriscus sylvestris* (positive univariate effects on Oct, the latter two also on Agm)

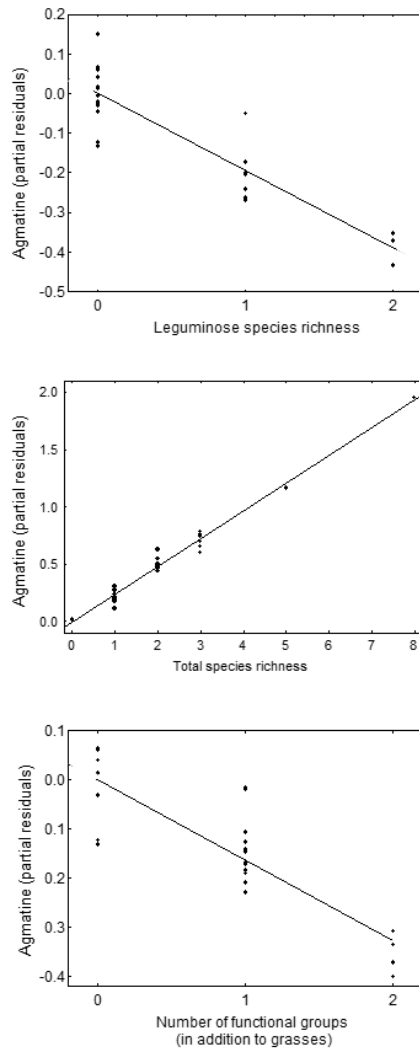


Figure 1. Relationship between diversity and distinctness of the source neighbourhood and Agm level, accounting for biomass ratio as a co-variable, illustrating the corresponding analysis in Table 2a. The y-axis gives partial residuals of Agm, i.e. Agm levels accounting simultaneously for the other independent variables entered into the multiple regression model.



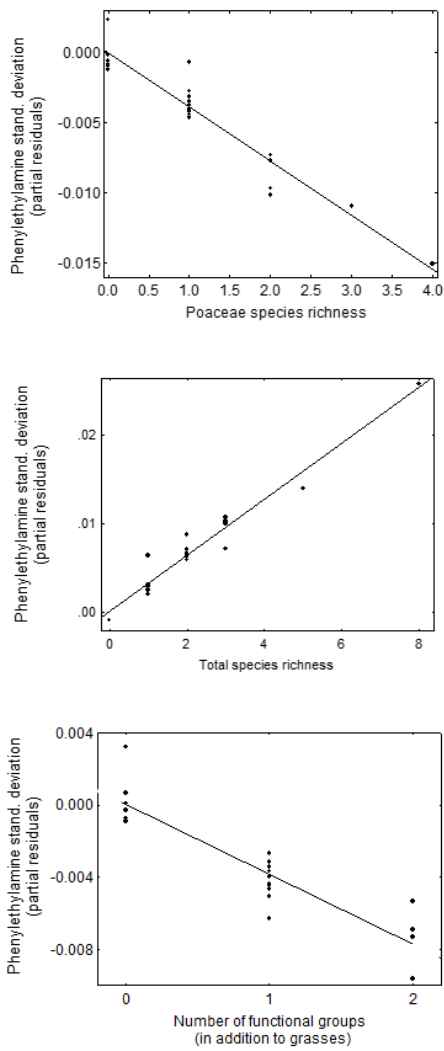


Figure 2. Relationship between diversity and distinctness of the source neighbourhood and the standard deviations of Phe levels, accounting for biomass ratio as a co-variable, illustrating the corresponding analysis in Table 2b. The y-axis gives partial residuals of Phe standard deviations, i.e. Phe standard deviations accounting simultaneously for the other independent variables entered into the multiple regression model.